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mRNA Decay

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Preface

Every transcript synthesized in a cell will ultimately be degraded, and messenger (m)RNAs are no exception to this rule. More than just the elimination of "old" transcripts, mRNA decay is a complex and highly regulated process that plays a pivotal role in maintaining the transcriptome and cellular homeostasis. Not only do RNA degradation factors contribute to precisely regulating the cellular abundance of each transcript, allowing the cell to adapt to environmental conditions, but specialized RNA surveillance pathways also ensure that aberrant mRNAs are efficiently eliminated.

General mRNA decay takes place in the cytoplasm where it is commonly initiated by the enzymatic shortening of the 3′ poly(A) tail (deadenylation) and the removal of the 5′ cap structure (decapping). Besides this general pathway, years of research have uncovered diverse and specialized mechanisms for mRNA degradation, both in the cytoplasm and the nucleus. All these pathways often involve multiple steps, which are tightly regulated.

Though it may seem like a simple, specific topic, mRNA degradation covers a large field of research. In *mRNA Decay: Methods and Protocols*, I aimed to provide both new and experienced RNA researchers with an inspiring collection of protocols developed by world-leading experts, ranging from classical methods for studying RNA degradation at the single RNA level to the latest transcriptome-wide approaches involving long-read sequencing and metabolic labeling.

This volume's theme is so broad that it was not possible to cover everything. In particular, I apologize to the colleagues working on RNA degradation in bacteria and archaea, as this book mainly covers methods for eukaryotic models. For those seeking more protocols for the study of deadenylation, there are other resources available, such as the recent book edited by Dr. Eugene Valkov and Prof. Aaron C. Goldstrohm in the Methods in Molecular Biology series (Volume 2723). Another book edited by Prof. Kazuharu Arakawa focuses on the potential of nanopore sequencing technology (Volume 2632).

As I near the completion of this first experience editing such a book, I'd like to thank all the contributing authors for their enthusiasm and professionalism in sharing their knowledge and expertise. It was a privilege to work with all of you on this project. I am also grateful to series editor Dr. John Walker for the opportunity to edit this volume and for his guidance and advice. Finally, I'd like to thank Charlotte, Marilyn, and Charlie for their support throughout the preparation of this book.

Paris, France — *Maxime Wery*

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Part I

Classical Methods

Chapter 1

Minimal Perturbation Analysis of mRNA Degradation Rates with Tet-Off and RT-qPCR

Cosmin Saveanu

Abstract

Messenger RNA stability is an important variable in gene expression and its dynamics. High stability ensures a constant level of synthesized protein, whereas mRNA instability can be critical for regulatory processes in which protein production needs to be stopped, such as development, inflammation, or adaptation to stress. Accurate measurements of RNA degradation rates are important for understanding how RNA features and RNA binding proteins affect the posttranscriptional life of an mRNA. As an alternative to global transcriptional inhibition methods, the use of a Tet-off repressible promoter has the advantage that cells are minimally perturbed by the addition of doxycyclin during the assay. We illustrate the use of a reporter mRNA expressed from a plasmid in *Saccharomyces cerevisiae* cells, but similar methods can be applied to other regulated promoters, on plasmids or by genome editing, and in other organisms. RNA levels are measured by reverse transcription followed by quantitative PCR. An exponential decay law is then used to estimate how well the measurements follow this expected trend for the simplest possible mechanism of RNA degradation, where the decay is proportional to the amount of RNA present at any given time.

Key words RNA degradation, Yeast, *Saccharomyces cerevisiae*, Repressible promoter, RT-qPCR, Exponential decay

1 Introduction

The stability of an mRNA has a profound impact on its steady-state level at a given transcription rate. Therefore, methods for estimating how long an mRNA is present in a cell and identifying the mechanisms responsible for both stability and degradation of mRNAs are important for understanding gene expression. Many methods can be used to estimate mRNA degradation rates, but the one presented here requires relatively simple equipment and is also inexpensive. Its main disadvantages are the fact that it can only estimate bulk mRNA degradation, the 5′ untranslated region of the mRNA of interest is not the native one, and it can only be applied to individual reporters. By comparison, other methods, such as high-resolution microscopy can track individual mRNA

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molecules in specific subcellular compartments. For example, labeling of the nascent protein and of the 3′ untranslated region of a reporter mRNA can estimate the dynamics of RNA degradation during nonsense-mediated mRNA decay [1]. Single-molecule tracking is not without caveats, as it requires specific RNA elements that modify the mRNA of interest and may also affect its stability or translation. In addition, these elements may also be resistant to degradation of the mRNA fragments generated from the reporters, leading to erroneous estimates of half-life values. Recently, technical solutions have been proposed to prevent such artifacts [2, 3].

To avoid altering the mRNA sequence, metabolic labeling methods have the advantage of estimating mRNA stability for thousands of transcripts in parallel. The level of incorporation of such nucleotides indicates the balance between mRNA synthesis and degradation. Initially based on a relatively challenging and error-prone purification of modified RNA molecules [4, 5], variants of pulse-chase labeling of mRNA now use chemical modification of incorporated nucleotides that can be detected by short reads sequencing [6].

Between microscopy and metabolic labeling, the most widely used method for estimating mRNA degradation rates involves the inhibition of its synthesis and monitoring mRNA levels at different time points. Global inhibition of transcription, which is achieved in mutants that affect the function of RNA polymerase II, or chemical inhibition of transcription with drugs such as ortho-phenanthrolin or thiolutin, can have various secondary effects because a stress response can be induced by such treatments [e.g. 7].

Inhibiting transcription of a single gene by using a specific promoter inserted upstream of the coding region minimally disrupts the cellular metabolism, including general translation and RNA degradation factors. A widely used method for rapid repression of gene expression uses a chimeric transcription factor consisting of the bacterial *tet* repressor fused to the transcription activating domain of the herpes simplex virus [8]. However, the promoter region and the corresponding 5′ untranslated part of mRNA can affect the translation and degradation of the mRNA under investigation. This can be partially avoided by careful analysis of the promoter region to insert the *tet* operator sequences upstream of the potential TATA box, for example [9]. The decrease in mRNA levels can also depend on its nuclear to cytoplasmic distribution, especially if export from the nucleus is slow. Another caveat of using reverse transcription followed by qPCR, as described here, is that the signal observed may not correspond to full length molecules but, for example, to abnormally stable mRNA fragments. An alternative way of looking at mRNA degradation that does not have this problem is Northern blotting, as described elsewhere in this volume. For a comprehensive evaluation of methods allowing the

evaluation of mRNA stability, see the review by Wada and Becskei [10].

Here, I present the protocol that we use for Tet-off-based mRNA degradation rate estimation using a low copy (centromeric) plasmid expressing the mRNA of interest from the pCM189 backbone [11] in yeast. The levels of the target mRNA are measured over time using reverse transcription and quantitative PCR and the results are fitted to an exponential decay model to calculate the mRNA degradation rate and its associated half-life.

2 Materials

2.1 Yeast Growth Media

1. YPD medium: 2% D-glucose, 1% yeast extract, 1% Bactopeptone. Autoclave at 110 °C for 20 min.
2. SD-URA medium: 2% D-glucose, 6.7 g/L yeast nitrogen base containing ammonium sulfate, without amino acids, 1.92 g/L drop-out mix without uracil. Autoclave at 110 °C for 20 min. Alternatively, filter sterilize.

2.2 Cell Lysis and RNA Extraction

1. Lysis buffer: 10 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.5% SDS. Store at room temperature.
2. Phenol-chloroform-isoamyl alcohol (25:24:1) mix, pH 5.2. Store at 4 °C.
3. Ethanol/ammonium acetate mix: 6 volumes of ethanol for 1 volume of 7.5 M ammonium acetate. Store at −20 °C.
4. 70% ethanol. Store at room temperature.
5. Thermomixer of dry bath that can hold 65 °C for 1.5 mL Eppendorf tubes.
6. Optimized DNase I and its buffer containing Mg^{2+} and Ca^{2+} ions (e.g. Turbo DNase).
7. Spectrophotometer for small volumes working at 260 nm (e.g. Nanodrop).
8. Cell culture incubator with shaking (30 ° C).
9. Doxycyclin 1000× stock solution: 10 mg/mL in ethanol. Store at −20 °C.
10. Refrigerated centrifuge for 50 mL and 1.5 mL tubes.

2.3 Reverse Transcription and qPCR

1. Thermal cycler.
2. qPCR system for microplates and its associated software.
3. qPCR premix, containing SYBR Green intercalating fluorophore, thermostable DNA polymerase, dNTPs, and the manufacturer buffer. Usually these premixed solutions are provided as 2× stock. Store at −20 °C.

4. Oligonucleotides specific for the transcript of interest (*see* **Note 1**): 100 μM in water. Store at −20 °C.
5. Reverse transcriptase derived from murine leukemia virus enzyme, with decreased RNase H and DNA endonuclease activities and increased thermal resistance (e.g. SuperScript III or SuperScript IV).
6. 5× reverse transcriptase buffer: 250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$. Store at −20 °C.
7. Actinomycin D: 10 mg/mL. Store at −20 °C.
8. Dithiothreitol (DTT): 0.1 M stock. Store at −20 °C.
9. dNTP mix: 25 mM dATP, 25 mM dGTP, 25 mM dCTP, 25 mM dTTP. Store at −20 °C.
10. Linear polyacrylamide: 5 mg/mL. Store at −20 ° C.

2.4 Software for Analysis of Results and Half-Life Estimation.

1. Computer system with R.
2. RStudio package.
3. *MASS* package.
4. *Reshape2* (optional).

3 Methods

3.1 Cell Culture and Recovery

1. Grow cells in synthetic SD-URA medium, if the reporter mRNA is expressed from a *URA3* marker containing plasmid. Dilute cells grown in selective medium overnight in 300 mL YPD medium at 600 nm to an initial absorbance of 0.15.
2. Incubate the cells with agitation at 30 °C for about 4 h, until the 600 nm absorbance reaches 0.5.
3. Split the culture in 5 flasks marked for incubation with doxycyclin for 0, 5, 10, 15, 20, and 30 min. Continue incubation with agitation at 30 °C.
4. Add 50 μL of doxycycline stock to the flask labeled "30 min." Add doxycyclin 10 min later to the flask "20 min." Follow the indications of Fig. 1a for the addition of doxycyclin in the other flasks. Adapt the duration for more stable transcripts.
5. At the end of the incubation period, move all the flasks to an ice-water mix for rapid chilling of the media and cells. Transfer cells to refrigerated 50 mL Falcon tubes.
6. Recover cells by centrifugation for 10 min at 2000 × *g*, at 4 °C.
7. Transfer the cells using 1 mL of ice-cold water in an Eppendorf tube. Centrifuge for 30 s at 10,000 × *g*. Remove the supernatant and store the tubes at −80 °C or process immediately.

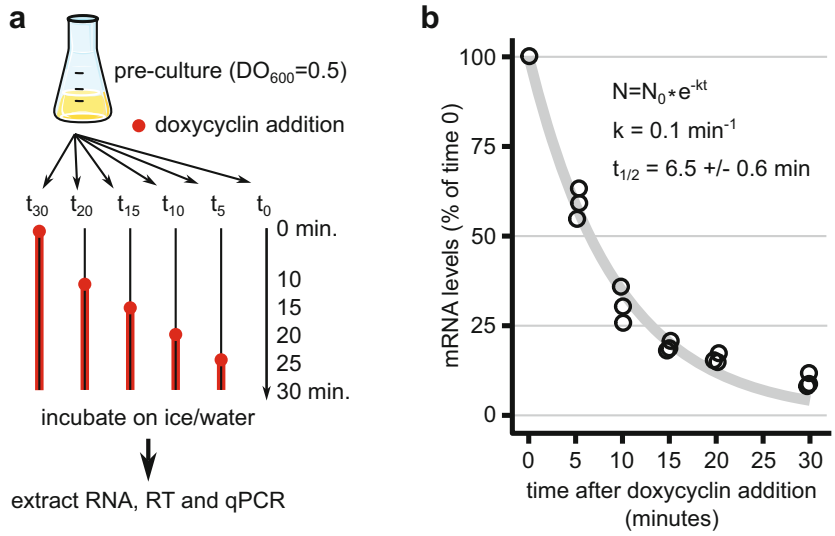

Fig. 1 Typical workflow and result for the half-life estimation using RT-qPCR with a Tet-off reporter. (**a**) Schematics of the culture conditions and a way to synchronize the recovered samples and ensure equal treatment for the different time points. All the time points are recovered together, but with different periods of doxycyclin treatment. The flask image was generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license. (**b**) Example of obtained results [14], with a relatively unstable mRNA. The experiment was done three times independently and the results were fitted to an exponential decay function (indicated). "*k*" is a kinetic first order constant for the degradation of the mRNA

3.2 3.2. Total RNA Extract Preparation with Hot Phenol

1. Resuspend the pellet by pipetting up and down with 400 µL of lysis buffer. Add 400 µL Phenol-chloroform-isoamyl alcohol. Vortex for 10 s.
2. Transfer the tubes to a dry block and incubate for 30 min at 65 °C with occasional mixing.
3. Move the tubes on ice and incubate for 5 min.
4. Separate phases by centrifugation for 5 min at 14,000 × *g*, at 4 °C.
5. Recover the aqueous supernatant and perform two additional phenol-chloroform extractions.
6. Final aqueous RNA extract is mixed in a 1 to 3.5 volumetric ratio with chilled ethanol/ammonium acetate mix. Incubate at 4 °C for 10 min.
7. Recover precipitated RNA by centrifugation for 15 min at 14,000 × *g*, at 4 ° C.
8. Wash the pellet with 800 µL of 70% ethanol twice, using 5 min of centrifugation to recover the pellet at each wash.
9. Remove all the ethanol and leave the pellet to air-dry until their appearance becomes translucent.

10. Add 50 µL of water and resuspend the pellet by vigorous pipetting. Optionally, incubate the tubes at 65 °C for 5 min to ensure that all the RNA has been solubilized.
11. Measure absorbance at 260 nm to estimate the amount of recovered RNA. Adjust the concentration of all tubes to 2 µg/µL. Store the RNA samples at −80 °C.

3.3 Reverse Transcription and qPCR

1. Use 10 µg of total RNA for DNase treatment in a total volume of 15 µL, together with DNase buffer and modified DNase I (2 units). Incubate for 30 min at 37 °C.
2. Add 85 µL of water to increase the working volume and perform a phenol-chloroform extraction.
3. To the recovered aqueous phase, add 8 µL of linear polyacrylamide solution (40 µg) and 3.5 volumes of ethanol-ammonium acetate mix. Incubate on ice for 10 min.
4. Recover the DNase-treated RNA by centrifugation for 20 min at 14,000 × *g*, at 4 °C.
5. Wash once with 0.8 mL of 70% ethanol. After drying the pellet, resuspend in 10 µL of cold water.
6. Use 2 µL of DNase-treated RNA to set up the annealing mix for the reverse transcription reaction. The 5 µL total reaction volume, in a 0.2 mL PCR tube, should contain 1 µL of 5× RT buffer and specific oligonucleotides for the reaction, 0.5 µL of a 5 µM solution for each (*see* **Note 2**). A control reaction, without oligonucleotides, is optional but recommended. An additional control, without RT, can also be performed.
7. Prepare a 2× mix containing the reverse transcriptase. For 5 µL of mix, use 1 µL of 5× RT buffer, 1 µL of 0.1 M DTT, 0.3 µL of dNTP mix, 0.05 µL of actinomycin D, 2.3 µL of water, 0.35 µL of reverse transcriptase. Keep on ice.
8. Use a thermocycler to incubate the annealing mix for 5 min at 85 °C, followed by 20 min at 65 °C and 10 min at 42 °C.
9. While the tubes are in the thermocycler at 42 °C, add 5 µL of reverse transcriptase mix and mix by pipetting up and down.
10. Incubate for 30 min at 42 °C and then inactivate the enzyme for 10 min at 70 °C. Recover the tubes and place them on ice. The RT reaction can be stored at −20 °C.
11. Quantitative PCR reactions are done on serial dilutions of the cDNA obtained in the previous step. An example of serial dilution starts with 5 µL of cDNA in 75 µL of water, followed by dilutions of 10 µL of this initial solution with 70 µL of water, three times. The obtained dilutions differ by a factor of 8, leading to differences in the number of cycles required to get to a threshold fluorescence of about 3.

12. Mix 5 μL of cDNA dilution with 20 μL of SYBR Green mix, which contains 12.5 μL of 2× commercial 2× dNTP, enzyme and SYBR Green, 1.25 μL of 10 μM solution for each of the two PCR primers and 5 μL of water.
13. Perform the qPCR reaction. Typical conditions are 95 °C for 5 min, followed by 40 cycles of 20 s at 95 °C and 1 min at 60 °C, with a fluorescence reading at the end of each cycle. An optional melting curve can be also programmed.

3.4 Data Analysis and RNA Half-Life Estimation

1. Use the qPCR software associated with the qPCR thermocycler to measure cycle threshold values and verify that the differences in cycle values correspond to the dilutions (*see* **Note 3**).
2. Once normalized, express each of the amounts of the transcript of interest as a fraction of the amount of that transcript in the sample that received no treatment with doxycyclin. It is expected that these fractions are lower than 1.
3. Load the results in R in a data frame called *mydf*. The simplest version of such a data frame will have two columns: *time* and *fraction*. If several replicate experiments have been performed, use a three-column data frame, with one of the columns corresponding to a description of which replicate experiment it represents (*see* **Note 4**).
4. Use the *nls* function included in R to estimate the first order kinetic constant for an exponential decay model (*see* **Note 5**). This function uses a named vector for the initial estimates of the estimated parameter, which can be expressed using the following commands: `initialestimate <- c(initial_k); names(initialestimate) <- c("k")`. Starting with an *initial_k* of 0.1 is reasonable for relatively fast RNA degradation experiments (`initial_k <- 0.1` before setting the *initialestimate* variable).
5. To enter the *nls* formula, it is useful to assign *x* to the time values and *y* to the fraction of RNA remaining at each time point: `x <- mydf$time; y <- mydf$fraction`. The formula is: `m <- nls(formula = y ~ exp-(k*x), start=initialestimate)`. The result noted "*m*" contains the fitted parameter. The constant "*k*" can be recovered from the fitted model through `k_estimated <- coef(summary (m)) 1`.
6. For a visual estimation of how well the results fit the observations, compute the theoretical expectation for the fraction of remaining RNA and overlay the curve on the data points, as illustrated in Fig. 1b. A *times* variable, from 1 to 30, is generated with: `times <- seq(from=0, to=30, by=1)`, and is combined with computed fraction of remaining RNA: `estfraction <- exp(-times*k_estimated)`.

`predicteddf <- data.frame(time=times, predicted=estfraction)`. The results are overlaid on a plot of the experimental observations, either as fraction or percentage of value at time 0.

7. Especially if several replicates have been done, but even for a single experiment, obtain an idea of how precise the estimate for *k* is, by using the *confint* function of the *MASS* package: `confm<-confint(m,level=0.95)`. The two values stored in *confm* correspond to the lower and upper estimate for *k*, with a 95% confidence.
8. Finally, computing an estimated half-life from the decay constant is straightforward, as they are linked through the formula: `half-life=ln(2)/k` (*see* **Note 6**).

4 Notes

1. The design of the oligonucleotides for qPCR can be done using specialized tools, such as the one provided by the Potsdam University [12]: https://quantprime.mpimp-golm.mpg.de. For spliced transcripts, oligonucleotides that cover an exon–exon junction might be preferred, as they will not amplify genomic DNA contamination. The PCR fragments amplified for qPCR are generally short, in the 60 to 100 nucleotides range.
2. While random hexamers of heptameric oligonucleotides can be used for reverse transcription, specific oligonucleotides help for a better sensitivity. If random hexamers are used (1 μL of a 100 μM solution for each reaction), the annealing reaction conditions should be adjusted, by incubating the annealing mix for 5 min at 65 ° C and switching to ice for 2 min before the addition of the reverse transcriptase.
3. Quantitative PCR results can be analyzed using an efficiency and threshold cycle difference method described by Michael Pfaffl [13], although I prefer to transform dilutions using base 2 logarithm, rather than base 10, as it is closer to the exponential nature of the PCR reaction. Messenger RNA used for adjusting to the amount of RNA in the qPCR reaction are, for yeast two of the following: *PGK1*, *ACT1* or *RIM1*.
4. For users of spreadsheet software, such as Microsoft Excel or LibreOffice Calc, the results are most often displayed in the so-called wide format. For multiple replicate experiments, it is usual to list their results in separate columns labeled, "exp1," "exp2," and so on. In R, it is often easier to use a "long" format for data analysis. In such a format, each column corresponds to a type of variable. For example, there will be only one column

for the type of experiment and each entry will be one of "exp1," "exp2," etc. The advantage of this format is that all values of interest are found in specific columns. Moreover, graphics packages such as *ggplot2* can use the specific information from a column to assign colors to points in a graph as a function of the discrete values present in that column ("exp1," "exp2," etc.). Switching from a wide to long format for a data frame in R is accomplished with the *melt* function of the *reshape2* package.

5. An exponential decay function might not always correspond to the observations. For example, there might be a lag between the addition of doxycyclin and the transcription inhibition. In this case, one can use time intervals from which a "lag" period has been subtracted. To take into account the possibility that some basal transcription still occurs even in the presence of the drug, leading to an apparent stabilization of the mRNA levels, one can use an additional constant factor in the equation [9]. However, please keep in mind that, in general, the least number of factors we need to guess by nonlinear least squares fitting, the more likely it is that these factors can be given a biological meaning.
6. To establish a confidence interval for the estimated half-life, a possibility is to take the minimal and maximal estimates of the decay constant k and compute the corresponding half-life values. While not formally correct, such computations give a rough idea about how good the fit was and how confident one can be about the reported half-life value.

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Chapter 2

Northern Blotting: Protocols for Radioactive and Nonradioactive Detection of RNA

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Abstract

Northern blotting is a common technique in RNA biology, allowing to detect and quantify RNAs of interest following separation by gel electrophoresis, transfer to a membrane, and hybridization of specific anti-complementary labelled probes. In this chapter, we describe our protocol for efficient RNA extraction from yeast, separation on agarose gel, and capillary transfer to a membrane. We provide two different methods for strand-specific detection of several types of RNAs using oligonucleotide probes, the first using radioactive ^{32}P-labelled probes, the second based on nonradioactive digoxigenin-labelled probes.

Key words Total RNA extraction, Yeast, Northern blotting, Long noncoding RNA, mRNA decay, ^{32}P-labelled oligonucleotide, Digoxigenin, Nonradioactive Northern blotting, Xrn1-sensitive lncRNA

1 Introduction

Northern blotting consists in separating RNA molecules by electrophoresis in a gel, transferring them to a membrane, and then detecting transcripts of interest using specific labelled probes [1]. It has been inspired from the technique developed by Edwin Southern to detect specific DNA sequences from DNA fragments separated on a gel [2].

Northern blotting has become a classical approach in RNA biology, including for the study of ribosome biogenesis [3–5] and nucleolar surveillance [6–8], the regulation of messenger (m)RNA translation and decay [9–14], the biogenesis of transfer (t)RNAs [15–17] and small nucle(ol)ar RNAs [3, 17–19], as well as the characterization of unstable long noncoding (lnc) RNA species [20–23].

Despite other methods developed subsequently are less time-consuming, more sensitive, and easier to implement (e.g., reverse transcription followed by quantitative PCR), the power of

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Northern blotting is that it offers the possibility to visualize the transcript of interest, check its integrity, and estimate its size. This is sometimes critical when studying mRNA decay intermediates with different boundaries [12] or overlapping RNA isoforms produced from the same locus and that are difficult to discriminate by other techniques [24, 25].

Traditionally, Northern blotting involves radioactive labelling of probes using ^{32}P and the detection of the radioactive signal by autoradiography following hybridization of the labelled probe. However, handling radioactive isotopes raises several issues, especially in terms of safety. Moreover, the relatively short half-life of ^{32}P (14.3 days) implies that the labelled probes cannot be stored for long periods as they rapidly lose their activity.

An alternative, nonradioactive method is based on digoxigenin (DIG) labelling [26]. DIG is a steroid produced by *Digitalis* plants, which are the only natural sources of DIG. Thus, anti-DIG antibodies are highly specific as they do not bind to other biological material.

In this chapter, we describe a robust method for total RNA extraction and radioactive Northern blotting that we have successfully used to detect RNAs from the baker's yeast *Saccharomyces cerevisiae* [25, 27], the fission yeast *Schizosaccharomyces pombe* [28, 29] and also less conventional yeast models such as *Naumovozyma castellii* [30]. We also provide a protocol for nonradioactive detection of RNA using DIG-labelled oligonucleotides. Both methods of detection have been validated for the detection of mRNAs and less abundant transcripts, including Xrn1-sensitive lncRNAs. While DIG-labelled probes are more convenient to manipulate, RNA detection using radioactive probes appears to be more sensitive and should therefore be preferably envisaged when working with lowly abundant RNAs.

2 Materials

Prepare all solutions with ultrapure, deionized, sterile water and store them at room temperature, unless specified. For the resuspension and dilution of RNA samples, only use water which is guaranteed nuclease-free. When manipulating RNA, clean working surfaces, pipettes, and electrophoresis equipment with RNase removal solution. Wear gloves to avoid contaminating RNA samples with RNase.

2.1 Preparation of the Cells and Total RNA Extraction

1. 50 mL centrifugation tubes.
2. Safe-lock, RNase-free 1.5 mL tubes.
3. Refrigerated centrifuge and microcentrifuge.
4. Heating block.

5. Ice-cold sterile water.
6. Liquid nitrogen.
7. TES buffer: 10 mM Tris-HCl pH 7.5, 5 mM EDTA pH 8.0, 1% SDS.
8. Phenol solution: phenol saturated with 0.1 M citrate buffer, pH 4.3.
9. Phenol:chloroform:iso-amyl alcohol (125:24:1) solution (pH 4.3–4.7).
10. Ethanol absolute.
11. Nuclease-free water.
12. Spectrophotometer.

2.2 Electrophoresis and Transfer

1. FA buffer (10×): 200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA. Adjust the pH to 7.0 with HCl. Do not autoclave. Store in the dark as MOPS is sensitive to light (the bottle containing the buffer can be wrapped in an aluminum foil). Trash the buffer whenever it turns to yellow.
2. Agarose.
3. Ethidium bromide: stock solution at 10 mg/mL.
4. RNA loading buffer (2×): 95% formamide, 0.02% SDS, 0.02 bromophenol blue, 0.01% xylene cyanol, 1 mM EDTA. Store in aliquots at −20 °C.
5. UV transilluminator.
6. 50 mM NaOH: freshly prepared from a 1 M (40 g/L) stock solution, stored at room temperature.
7. 1 M Tris pH 7.5: for 1 L, weight 127 g of Tris hydrochloride and 23.6 g of Tris base, and dissolve in water.
8. Neutralizing buffer: 0.5 M Tris pH 7.5, 1.5 M NaCl.
9. Saline-Sodium Citrate buffer (SSC, 20×): 3 M NaCl, 0.3 M sodium citrate, pH 7.0.
10. Positively charged nylon membrane (e.g., Hybond XL).
11. Whatman 3MM CHR chromatography paper.
12. Parafilm M.
13. Ultraviolet cross-linker.

2.3 Detection Using ^{32}P-Labelled Probes

1. Safe-lock 1.5 mL tubes.
2. Screw cap 2 mL microcentrifuge tubes.
3. Plexiglas protective screen.
4. Heating block.
5. Microcentrifuge.
6. Hybridization tubes.

Table 1
Oligonucleotides

Name	Sequence 5′-3’	Target
AMO606	CCAGAAGGAAAGGCCCCGTTGGA	18S rRNA
AMO1482	ATCCCGGCCGCCTCCATCAC	*scR1*
AMO1595	GGGAAAAGTTTGTTTGGCTTATTCTGGTGGTTTAG	*XUT1678* & *SUT768*
AMO1788	TGAGCGGTACCGAAGGCATC	*PGK1* mRNA
AMO3661	GATGGAATCCACTAGTTCGCTGTGAAAGCG	*RPL22B* pre-mRNA

7. Hybridization incubator.
8. Oligonucleotides at a concentration of 10 μM, in nuclease-free water (*see* Table 1).
9. ATP, [γ-^{32}P]- 3000 Ci/mmol 10 mCi/mL. Store the source at 4 °C.
10. T4 polynucleotide kinase (10 U/μL). Store the enzyme and its buffer at −20 °C. *See* **Note 1**.
11. Size exclusion chromatography columns (1 per probe). Store at 4 °C. *See* **Note 2**.
12. ULTRAhyb™-Oligo Hybridization Buffer (Ambion). Store at 4 °C.
13. Washing buffer 1: 2× SSC, 0.1% SDS.
14. Washing buffer 2: 0.1× SSC, 0.1% SDS.
15. Storage phosphor screen.
16. Autoradiography cassette.
17. Phosphorimager.
18. Storage phosphor screen image eraser.
19. Stripping solution: 0.1% SDS.

2.4 Nonradioactive Detection Using DIG-Labelled Probes

1. DIG-labelled oligonucleotides (*see* **Note 3** and Table 1).
2. Heating block.
3. Maleic buffer: 0.1 M maleic acid, 0.15 M NaCl, pH 7.5 (adjusted with NaOH).
4. 10% blocking reagent: prepare a 10× concentrated stock solution by dissolving blocking reagent powder (Roche) at 10% (w: v) in maleic acid buffer. Heat if necessary. Aliquots can be stored at −20 °C.
5. Hybridization tubes.
6. Hybridization incubator.

7. ULTRAhyb™-Oligo Hybridization Buffer (Ambion). Store at 4 °C.
8. Washing buffer 1: 2× SSC, 0.1% SDS.
9. 1× blocking solution: dilute the 10× blocking reagent stock solution in maleic acid buffer. Always use a freshly prepared batch.
10. Maleic washing buffer (MWB): 0.1 M maleic acid, 0.15 M NaCl, pH 7.5 (adjusted with NaOH), 0.3% Tween 20.
11. Antibody solution: anti-Digoxigenin-AP Fab fragments (Roche) diluted at 1:10,000 (75 mU/mL) in 1× blocking solution. Centrifuge the antibody stock for 5 min at 10,000 rpm prior to each use, and then pipette the necessary amount carefully from the surface.
12. Detection buffer: 0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5 (20 °C).
13. CDP-Star solution: CDP-Star (Roche) diluted at 1:100 in detection buffer.
14. Parafilm M.
15. Hybridization bags or plastic sleeves.
16. Imaging System (Chemidoc imaging system or equivalent).

3 Methods

3.1 Preparation of the Cells and Total RNA Extraction

1. Harvest 25 mL of exponentially growing cells by centrifugation for 3 min at 4 °C (*see* **Note 4**). As an indication, we use a speed of 3000 rpm with an A-4-81 rotor in a 5810 R Eppendorf centrifuge.
2. Discard the supernatant, and then wash the pellet in 10 mL of ice-cold sterile water.
3. Centrifuge as above.
4. Discard the supernatant, resuspend the pellet in 1 mL of ice-cold sterile water and transfer the cells into a 1.5 mL microcentrifuge tube.
5. Centrifuge for 1 min at 4 °C at 10,000 rpm.
6. Completely discard the supernatant and then flash-freeze the cell pellet in liquid nitrogen.
7. Store the pellet at −80 °C or directly proceed to total RNA extraction.
8. Gently thaw the cells on ice.
9. Resuspend the cells in 350 μL of TES buffer.

10. Under a fume hood, add 350 µL of phenol solution (*see* **Note 5**).
11. Incubate the tube for 20 min at 65 °C in a thermal mixer, under vigorous agitation (1400 rpm). If using a non-mixing heating block, vortex the tubes every 2 min.
12. Flash-freeze the tubes in liquid nitrogen (ensure that the caps are well locked).
13. Carefully remove the tubes from the liquid nitrogen and then centrifuge them for 15 min at room temperature at maximum speed (*see* **Note 6**).
14. Transfer the upper (aqueous) phase into a new 1.5 mL microcentrifuge tube containing 350 µL of phenol:chloroform:isoamyl alcohol solution.
15. Vortex at full speed for 15 s.
16. Centrifuge for 5 min at room temperature at maximum speed.
17. Transfer 250 µL of the upper (aqueous) phase into a new 1.5 mL microcentrifuge tube containing 750 µL (3 volumes) of 100% ethanol. Mix by vortexing.
18. Incubate at −80 °C for at least 1 h (usually overnight).
19. Centrifuge for 30 min at 4 °C at maximum speed.
20. Completely remove the ethanol using a micropipette, and then dry the RNA pellet keeping the tubes open on the bench or under hood for 5 min.
21. Resuspend the RNA pellet in nuclease-free water.
22. Determine the RNA concentration by measuring the absorbance at 260 nm and 280 nm in a spectrophotometer (*see* **Note 7**).

3.2 Denaturing Gel Electrophoresis and Transfer

1. Prepare a 1.2% agarose gel by adding 1.2 g of ultrapure agarose in 100 mL of 1× FA buffer and heat until the agarose has completely melted. Let the mixture cool down to 55 °C, then add 1.8 mL of 37% formaldehyde and 2 µL of ethidium bromide (stock solution at 10 mg/mL). Mix thoroughly for 1–2 min and then pour the gel into the tray. Allow the gel to polymerize at room temperature.
2. Fill the electrophoresis cell with 1× FA buffer.
3. Carefully remove the polymerized gel from the tray and place it into the electrophoresis cell.
4. In a clean, RNase-free tube, mix 10 µg of RNA extract with an equivalent volume of 2× RNA loading buffer (*see* **Note 8**).
5. Denature the RNA samples by heating at 65 °C for 5 min, then chill on ice. Quickly spin the tubes at 4 °C to bring all samples down.

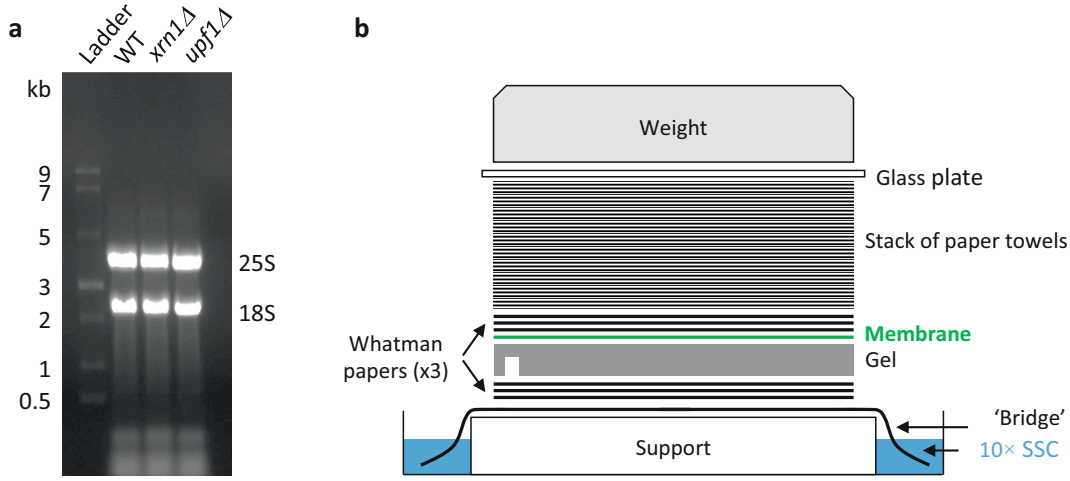

Fig. 1 (**a**) Typical profile of yeast total RNA observed upon staining of the agarose gel with ethidium bromide. Total RNA was extracted from WT cells and from mutant cells, lacking the cytoplasmic 5′-3′ exoribonuclease Xrn1 (*xrn1Δ*) or the NMD core factor Upf1 (*upf1Δ*). The bands corresponding to the 25S and 18S rRNAs are indicated. (**b**) Schematic representation of the transfer system setup

6. Load the RNA samples in the gel.
7. Run the gel at 80 V (*see* **Note 9**).
8. Control the migration by visualization on a UV transilluminator. The profile expected for total RNA extracted from exponentially growing wild-type yeast cells is shown in Fig. 1a (*see* **Note 10**).
9. When the migration is complete, remove the gel from the electrophoresis cell and place it in a clean tray.
10. Soak the gel in 250 mL of 50 mM NaOH for 15 min, at room temperature and under agitation (*see* **Note 11**).
11. Rinse the gel in sterile water.
12. Wash the gel in 250 mL of neutralizing buffer for 15 min, at room temperature and under agitation.
13. Repeat **step 12** (i.e., second wash in neutralizing buffer).
14. Rinse the gel in sterile water.
15. Rinse the gel in 1× SSC buffer.
16. Cut a Hybond XL membrane and six pieces of Whatman 3MM CHR chromatography paper at the dimensions of the gel. Cut an additional large piece of Whatman 3MM CHR chromatography paper.
17. Fill a second glass or plastic tray with 1 L of 10× SSC.
18. Prewet the membrane and the pieces of Whatman paper in 1× SSC.

19. Arrange a glass support into (or over) the second tray. Place the large piece of Whatman paper (bridge) over it, with the edges immersed in the 10× SSC buffer (*see* Fig. 1b). Place two of the six pieces of prewet Whatman paper on the bridge.
20. Take the gel out of the 1× SSC buffer using a glass plate carefully slid under it. Place one of the four remaining pieces of prewet Whatman papers on the gel; then holding the Whatman on the gel with one hand, turn over the glass plate and dissociate it from the gel (*see* **Note 12**). Place the Whatman paper and the gel on the two pieces of Whatman previously placed on the bridge (*see* **step 19**). The gel is now placed upside down on the three pieces of Whatman paper.
21. Place the prewet membrane on the gel. Remove all bubbles by rolling a plastic pipette over the surface.
22. Place the last three pieces of prewet Whatman paper on the membrane.
23. Cut four strips of Parafilm M and place them on the bridge on each side of the gel (*see* **Note 13**).
24. Place a stack of paper towels (≈10 cm high) on the top of the assembly.
25. Place a plastic or glass plate and a weight on the paper towels stack.
26. Allow the capillary transfer to proceed overnight.
27. After the transfer, carefully remove the membrane and place it in a UV cross-linker (with RNAs facing the UV).
28. Cross-link the membrane twice at 1200 × 100 μJ/cm^2.
29. Optional: take a picture of the cross-linked membrane and of the gel using a UV transilluminator to control transfer efficiency.
30. Store the membrane at room temperature if you will proceed to RNA detection using radioactive probes (*see* **Note 14**). For nonradioactive detection, store the membrane at 4 °C or − 20 °C.

3.3 Radioactive Labelling, Hybridization, Washes, and Exposure

1. In a 1.5 mL microcentrifuge tube, mix 20 pmol of oligonucleotide (2 μL of a 10 μM dilution), 2 μL of 10× PNK buffer, 13 μL of sterile water, and 1 μL of T4 polynucleotide kinase (10 U). Keep on ice.
2. Add 2 μL of γ-^{32}P ATP and homogenize by pipetting up and down (*see* **Note 15**).
3. Incubate for 1 h at 37 °C in a heating block.
4. During the labelling, place the membrane in a hybridization tube, add 10–15 mL of preheated Ultrahyb-Oligo Hybridization buffer and pre-hybridize for at least 30 min at 42 °C, with rotation.

5. Prepare a Micro Bio-Spin 6 Chromatography column: invert the column several times to resuspend the gel and remove bubbles, snap off the bottom tip, place the column on a 2 mL collection tube, remove the cap, and then centrifuge for 2 min at room temperature at 1000 × *g* to remove the buffer. Trash the collection tube and place the column in a clean 2 mL microcentrifuge tube, with screw cap (*see* **Note 16**).
6. After labelling of the probe, add 30 μL of sterile water. Homogenize by pipetting up and down and then load on the chromatography column. Centrifuge for 4 min at room temperature at 1000 × *g*. Discard the column (it contains the unincorporated radioactivity).
7. Denature the probe for 5 min at 95 °C.
8. Hybridize the probe at 42 °C with rotation for at least 2 h, up to overnight (see **Note 17**).
9. After hybridization, remove the unbound probe and hybridization buffer. Rinse the membrane with washing buffer 1.
10. Wash the membrane with washing buffer 1 at 25 °C, for 15 min.
11. Repeat **step 10** (i.e., the membrane should be washed twice with washing buffer 1).
12. Wash the membrane with washing buffer 2 at 25 °C, for 15 min.
13. Dry the membrane, place it in a plastic pocket, then in a cassette. Place a screen and expose (*see* **Note 18**).
14. Scan the membrane using a Phosphorimager. Process and analyze the image file.
15. The membrane can be stripped to allow detection of another RNA. For stripping, boil 100 mL of 0.1% SDS and then directly pour on the membrane. Incubate for 10 min at room temperature, under agitation. Repeat up to two times (*see* **Note 19**).
16. To hybridize a new probe on the stripped membrane, go back to **step 1** (if labelling of the new probe is required) or directly to **step 4** (if the probe has been previously labelled). Figure 2a shows the results of the detection of several RNA species using radioactive probes.

3.4 Nonradioactive Detection Using DIG-Labelled Probes

1. Place the membrane in a hybridization tube, add 10–20 mL of preheated Ultrahyb-Oligo Hybridization buffer and pre-hybridize for at least 30 min at 42 °C, with rotation.
2. Determine the quantity of DIG-labelled probe required to get the recommended concentration of 0.9 pmol/mL (i.e., 18 μL of a 1 μM dilution for 20 mL).

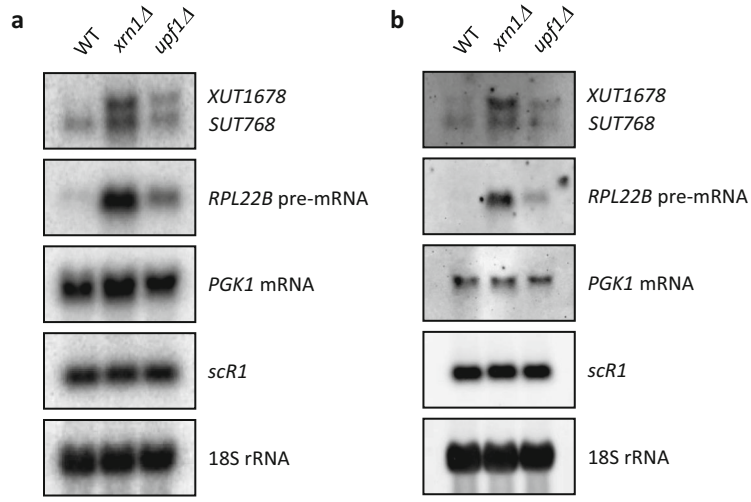

Fig. 2 (**a**) RNA detection using radioactive probes. *XUT1678* (NMD-sensitive lncRNA) and its overlapping isoform *SUT768* [25], pre-*RPL22B* (the unspliced precursor of *RPL22B* mRNA, which is also targeted by NMD [31]), *PGK1* mRNA, *scR1* (the highly abundant ncRNA subunit of the Signal Recognition Particle [32]), and the mature 18S rRNA were detected using ^{32}P-labelled oligonucleotides AM01595, AM03661, AM01788, AM01482, and AM0606 (*see* Table 1). (**b**) RNA detection using nonradioactive DIG-labelled probes. The same RNAs were detected from the same extracts using the same oligonucleotides with DIG added in their 3′ extremity

3. Denature the probe for 8 min at 68 °C.
4. Hybridize the probe overnight at 42 °C (depending on the probe).
5. When hybridization is complete, pour off the hybridization buffer with the probe into a new tube (*see* **Note 20**).
6. Wash the membrane with washing buffer 1 at room temperature, for 10 min.
7. Repeat **step 6** three times (i.e., the membrane should be washed four times with washing buffer 1).
8. Rinse the membrane with MWB for 2 min at room temperature.
9. Add 20 mL of 1× blocking solution on the membrane. Incubate for 30 min at room temperature, under agitation.
10. Discard the blocking solution. Add 20 mL of antibody solution on the membrane. Incubate for 30–60 min at room temperature, under agitation.
11. Discard the antibody solution.
12. Wash the membrane with 20 mL of MWB for 15 min at room temperature, under agitation.

13. Repeat **step 12** (i.e., the membrane is washed twice with MWB).
14. Equilibrate the membrane for 2–5 min in 20 mL of detection buffer, at room temperature, under agitation.
15. For a 100 cm^2 membrane, prepare at least 2 mL of CDP star solution (*see* **Note 21**).
16. Place the membrane with RNA facing up on a piece of Parafilm M (*see* **Note 22**). Apply the CDP star solution on the membrane, ensuring to spread the solution on the whole surface of the membrane, avoiding air bubbles.
17. Incubate the membrane with the CDP star solution for 5 min at room temperature.
18. Eliminate the excess liquid. Place the membrane into a transparent plastic sleeve. Seal the sleeve to avoid the membrane to dry during exposure as it would result in dark background.
19. Acquire the chemiluminescent signal using an imaging system. *See* **Note 23**.
20. The membrane can be stripped to allow detection of another RNA. For stripping, rinse the membrane with distilled water, then pour 100 mL of boiling 0.1% SDS on the membrane. Incubate for 5–10 min at room temperature, under agitation. Repeat two times. Rinse the membrane in 2× SSC for 5 min at room temperature.
21. To hybridize a new probe, go back to **step 1** (*see* **Note 24**). Figure 2b shows the results of the detection of the same RNAs as in Fig. 2a, but using nonradioactive DIG-labelled probes (*see* **Note 25**).

4 Notes

1. We usually work with T4 polynucleotide kinase from New England Biolabs but it can be replaced by an equivalent from another manufacturer.
2. We use Micro Bio-Spin 6 columns (Bio-Rad), but equivalent columns from other manufacturers can be used.
3. DIG-labelled probes can be directly purchased from many oligonucleotide synthesis companies. Alternatively, the user can label their favorite oligonucleotide in their own lab, using DIG oligonucleotide labelling kits produced by different manufacturers.
4. The typical amount of total RNA extracted using this protocol is 200–250 μg for a culture of 25 mL of WT cells of *S. cerevisiae* harvested at OD_{600} 0.5. However, this value may vary from an experiment to another.

5. Phenol is corrosive and highly toxic. It should therefore be manipulated with great caution. Wear gloves and work under a fume hood. Use safe-lock tubes and ensure that they are perfectly locked before incubating them at 65 °C and then plunging them in liquid nitrogen. When the extraction is completed, discard residual phenol and all contaminated materials according to local regulation.
6. It is not necessary to centrifuge at 4 °C at this stage. Centrifuging at room temperature allows the samples to thaw during the centrifugation and does not impact RNA integrity.
7. An absorbance of 1 unit at 260 nm corresponds to 40 μg of RNA per mL. However, the spectrophotometer measures the absorbance of all molecules present in the sample that absorb at the selected wavelength, including nucleic acids, residual proteins, and other potential contaminants. RNA and DNA have absorbance maxima at 260 nm, while proteins display an absorbance peak in the UV spectrum at 280 nm due to the aromatic residues. The A_{260}/A_{280} ratio for pure RNA should be ≥ 2.0. Absorbance at 230 nm can also be measured as it can be indicative of a contamination by residual phenol in the sample. An A_{260}/A_{230} ratio ≥ 2.0 is expected for pure RNA; a lower ratio is likely to be due to the presence of residual traces of phenol. If this is the case, add nuclease-free water to the RNA sample to reach a volume of 350 μL, and then repeat the extraction with phenol:chloroform:iso-amyl alcohol and the precipitation (*see* **steps 14–22** of Subheading 3.1).
8. We usually load 10 μg of total RNA per lane, but it is possible to load less or more RNA. To reduce the difference of loading between lanes (due to pipetting error), we recommend to dilute the RNA extracts at the same concentration (≈1.5 μg/μL) before adding the loading buffer and work with volumes that are within the range of maximal accuracy of your micropipette.
9. Usually, we run 10 × 15 cm gels at 80 V. We use constant voltage to keep the migration rate relatively constant during the run. It is possible to use a different voltage. Reducing it will extend the running time. Conversely, increasing the voltage will accelerate the run but it will also generate more heat. After 1 h of migration, we use to change the buffer.
10. Two sharp bands should appear at 3.4 and 1.8 kb, corresponding to the mature 25S and 18S rRNAs, respectively. Smearing of these bands may be indicative of RNA degradation. The bands observed at the low molecular weights correspond to the 5.8S and 5S rRNAs, sn(o)RNAs and tRNAs.

11. It is not necessary to work under RNase-free condition after the electrophoresis. The incubation in 50 mM NaOH induces alkaline partial hydrolysis of RNA.
12. There are alternative ways to set up the gel-transfer system. The gel should be handled carefully to avoid breaking it. Using a glass plate to get it out from the 1× SSC buffer and placing a piece of Whatman paper over it reduces the risk of gel break during the manipulation.
13. The pieces of Parafilm M on the side of the gel/membrane/Whatman assembly prevent the stack of paper towels to be in contact with the bridge and directly absorb the transfer buffer.
14. RNA is covalently immobilized on the membrane after UV cross-linking. The membrane can be stored and reused for a long time.
15. Radioactivity should be handled carefully, following rigorous training and in strict compliance with the local regulations. Always wear gloves and appropriate protection. Always work under a protective Plexiglas screen. Control the working environment with a Geiger counter.
16. For safety, we recommend to recover the labelled probe into a tube with screw cap, which can prevent sudden opening and accidental projection of radioactive material during probe denaturation at 95 °C.
17. Probes are usually hybridized overnight at 42 °C. However, for very abundant RNAs (such as *scR1* or the 18S and 25S rRNAs), the time of hybridization can be reduced to 2 h.
18. The exposure time depends on the abundance of the transcript of interest, the amount of RNA loaded on the gel, the probe and the activity of the source. We recommend to check the signal of the washed membrane using a Geiger counter before exposure. As an indication, the typical signals and exposure times are as follows: 18S or 25S rRNAs (>200 cps, 5 min), *scR1* (100 cps, 15 min), *PGK1* mRNA (20 cps, 4–5 h), lncRNAs (< 1 cps, overnight).
19. Beware of projection when boiling the 0.1% SDS solution (especially when using a microwave). When successively hybridizing different probes, we recommend to start from the less abundant transcript and proceed to the most abundant. If a lowly abundant RNA should be detected after a highly abundant one and if the membrane cannot be fully stripped, just wait until the radioactive signal corresponding to the abundant RNA gets back to the background (this can be controlled using a Geiger counter).
20. The hybridization buffer with the DIG-labelled probe can be stored at −20 °C for reuse within 1 year.

21. The manufacturer recommends 1 mL of CDP star solution for a 100 cm^2 membrane. However, we prefer to prepare a larger volume to ensure the whole membrane to be covered by the solution.
22. Hybridization bags can be used as an alternative. In that case, place the membrane with the RNA facing up in a hybridization bag, apply the CDP star solution on the membrane and immediately cover it with the second sheet of the bag to spread the solution on the whole surface of the membrane, avoiding air bubbles. After the 5 min incubation, eliminate the excess liquid and seal the bag.
23. The chemiluminescent signal continues for at least 2 days, allowing multiple images to be acquired as exposure of a few minutes is usually sufficient. Classical X-ray films can be used as an alternative.
24. The membrane should not be allowed to dry at any time in case it has to be reprobed.
25. As shown in Fig. 2, in our hands, radioactive probes are more sensitive than their DIG-labelled counterparts for the detection of the NMD-sensitive pre-*RPL22B* mRNA and the lncRNAs (*XUT1678* and *SUT768*). We therefore recommend the use of ^{32}P-labelled probes to detect the less abundant RNAs.

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Chapter 3

RNA Blotting by Electrotransfer and RNA Detection

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Abstract

Northern blot, or RNA blot, is a widely used technique in molecular biology to detect and semi-quantify specific RNAs. The advantage of this method is its ability to simultaneously estimate the sizes and quantities of degraded or processed RNA products. Northern blotting involves the use of electrophoresis to separate RNA samples by size. These RNAs are then transferred and immobilized on a membrane, and the RNAs of interest are detected by hybridization using a sequence-specific labeled DNA probe. Agarose gels are typically used in routine procedures to allow for the separation and specific detection of high molecular weights (ranging from tRNA size to several kb RNAs). However, acrylamide gels are preferred when the separation of lower weight RNAs (ranging from 20 to 200 nucleotides) is required. Both approaches for RNA separation are presented here. The term "northern blot" originally referred to the capillary transfer of RNA from the electrophoresis gel to the blotting membrane. However, RNA transfer to the membrane by electrotransfer has also been proven to be a suitable and fast method for transferring RNAs from acrylamide and agarose gels to the membrane.

Key words RNA separation, Electrophoresis, Northern, Blot, Electrotransfer, Agarose gel, Acrylamide gel, Hybridization, Probe labelling

1 Introduction

Northern blotting, a traditional method in RNA biology, has been widely utilized for various purposes such as investigating messenger (m)RNA processing and decay [1, 2], as well as characterizing the existence and fate of many other noncoding RNA species [3–6]. Despite the availability of alternative methods, northern blotting remains highly valuable for its ability to directly visualize the transcript of interest and simultaneously estimate the sizes and quantities of all degraded or processed RNA products that share the targeted RNA sequence.

A typical blotting procedure generally begins with the extraction of total RNA from biological materials. The RNA samples are then separated by size on an agarose or acrylamide gel using

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electrophoresis. Agarose gels allow for the separation and specific detection of high molecular weights (ranging from transfer RNA size to several kb RNAs), while acrylamide gels are preferred when the separation of lower weight RNAs (ranging from 20 to 200 nucleotides) is necessary. These two approaches, for instance, were very useful in estimating the amount of full-length mRNA targeted by the No Go decay pathway (using agarose gel) and in parallel providing information about the production of small RNA species by endonucleases attacking this full-length mRNA (using acrylamide gel) [7].

Due to the fragility of gels and the limited ability of probes to penetrate the gel matrix effectively, the separated RNAs are transferred to a membrane and fixed using UV irradiation. The term "northern blot"originally referred to the capillary transfer of RNA from the gel to the blotting membrane. Capillary transfer is typically performed overnight. Here, we describe an electrotransfer method that facilitates transfer within 90 minutes. This allows for probing of the membrane on the same day as electrophoresis, representing a significant time-saving advantage and facilitating the detection of several RNAs of interest and RNA standardization within a week.

RNAs are detected using a DNA probe designed to be complementary to the target sequence. The labeled probe is added and allowed to hybridize with the immobilized RNA. After successive washes to remove unbound probes, the membrane is exposed to a radioactivity-sensitive screen. The screen is then scanned, providing an estimation of the size and quantity of the RNAs of interest.

2 Materials

Ribonucleases are widely distributed in biological sources, and their environmental contamination easily occurs during the analysis steps, mainly through fingers. It is crucial to wear talc-free latex or nitrile gloves in all steps to limit RNase contamination (*see* **Note 1**).

2.1 Preparation of RNA Sample for Loading Gel

1. Safe lock RNAse-free tubes of 1.5 mL (*see* **Note 2**).
2. 2× Gel Loading Buffer II: 95% formamide, 18 mM EDTA, 0.025% SDS, 0.025% bromophenol blue, 0.025% xylene cyanol (*see* **Note 3**).
3. RNA ladders (*see* **Note 3**).
4. RNase-free water.
5. Heating block.
6. Microcentrifuge.
7. Fume Hood.

2.2 Agarose Gel Preparation and Apparatus for Electrophoresis

1. Agarose ultrapure.
2. 10× TBE: 0.9 M Tris, 0.9 M Borate, 0.02 M EDTA, pH 8.3.
3. 250 mL Erlenmeyer flask.
4. RNase-free water.
5. Ethidium Bromide: 10 mg/mL. Another nucleic acid dye can be used.
6. Submerged horizontal agarose gel electrophoresis apparatus (*see* **Note 4**).
7. Generator.

2.3 Acrylamide Gel Preparation and Apparatus for Electrophoresis

1. Acrylamide bis-acrylamide (19/1) for nucleic acids.
2. 10× TBE: 0.9 M Tris, 0.9 M Borate, 0.02 M EDTA, pH 8.3.
3. Urea.
4. RNase-free water.
5. Mini-PROTEAN system for casting acrylamide gels (Bio-Rad). Many equivalent systems exist.
6. 10% ammonium persulfate (APS).
7. N, N, N′, N′-Tetramethylethylenediamine (TEMED).
8. RNase cleaning solution (*see* **Note 5**).
9. 50 mL tubes.
10. Mini-PROTEAN electrophoresis system (Bio-Rad).
11. Generator.

2.4 Checking the Quality of Migration (For Agarose Gel)

1. UV transilluminator (*see* **Note 6**).

2.5 RNA Transfer from Agarose Gel to Membrane

1. GENIE BLOTTER or equivalent apparatus.
2. 10× TBE: Tris 0.9 M, Borate 0.9 M, EDTA 0.02 M, pH 8.3.
3. Positively charged nylon membrane (s*ee* **Note 6**).
4. Whatman 3MM paper.

2.6 RNA Transfer from Acrylamide Gel to Membrane

1. Mini-PROTEAN Protein transfer system (Bio-Rad) or equivalent.
2. 0.5× TBE pre-chilled at 4 °C.
3. Positively charged nylon membrane (*see* **Note 6**).
4. Whatman 3MM paper.

2.7 UV Cross-Linking of RNA and Methylene Blue Staining

1. Ultraviolet cross-linker (*see* **Note 7**).
2. Methylene blue solution: 0.2% methylene blue in 0.3 M Na-Acetate.

2.8 DNA Probe Preparation and Labelling

See **Note 8.**

1. DNA oligonucleotide specific and complementary to the RNA of interest (*see* **Note 9**).
2. T4 PolyNucleotide Kinase (PNK) (*see* **Note 10**).
3. ATP gamma ^{32}P, 3000 Ci/mmol 10 mCi/mL.
4. Distilled water.
5. Water bath.
6. Sephadex Micro-column (*see* **Note 11**).
7. Radiation shield.
8. Plexiglass box and rack for radiation shielding.
9. Geiger counter.

2.9 DNA Probes Hybridization and Revealing

1. Hybridization buffer (*see* **Note 12**).
2. 20× SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0 (*see* **Note 13**).
3. Washing solution I: 5× SSC, 0.01% SDS. Pre-warm at 42 °C.
4. Washing solution II: 1× SSC, 0.01% SDS. Pre-warm at 42 °C.
5. Hybridization bottle.
6. Hybridization oven.
7. Water bath.
8. Storage phosphor screen and cassette.
9. Phosphorimager.
10. Phosphor screen eraser (*see* **Note 14**).
11. Stripping solution: 0.1% SDS.

3 Methods

3.1 Preparation of Agarose Gel and Electrophoresis

1. For a 1% agarose gel (15 cm wide and 10 cm long), add 1 g of agarose to 100 mL of 1× TBE buffer in an Erlenmeyer flask (*see* **Note 15**).
2. Begin dissolving the agarose in the microwave at 800 W for 1 min. Heat for an extra minute and/or stop the microwave as soon as the first bubbles appear. Mix slowly while shaking the Erlenmeyer flask.
3. When the agarose solution has cooled (approximatively around 60 °C), add 4 μL of ethidium bromide (or another nucleic acid dye) under a fume hood.
4. In a fume hood, pour the agarose solution in the gel caster, add the comb, and allow it to set (approximatively 30 min).

5. The gel caster can now be handled outside of the hood. Remove the comb and place the gel in the electrophoresis cell. Add 1× TBE buffer in the tank to cover the gel with at least 1–2 mm of buffer.
6. Aliquot 5 µg of total RNA and mix it with the same volume of 2× Gel Loading Buffer. Note that depending on the combs used, a total volume of 15–25 µL can usually be loaded (*see* **Note 16**).
7. Denature the RNA samples for 3 min (heating block set at 85 °C). Put the samples on ice immediately, briefly centrifuge (5–10 s) to collect the liquid, and load on the gel.
8. Migrate at 100 V in 1× TBE buffer until the bromophenol blue reaches 1 cm from the end of the gel (*see* **Note 17**).
9. Examine the gel on a UV transilluminator to visualize the RNA and take a picture (*see* **Note 18**). *See* Fig. 1a.

3.2 Preparation of Acrylamide Gel and Electrophoresis

1. Mount plates on the gel casting system.
2. For a total volume of 15 mL (required to cast two 8% acrylamide gels), solubilize 6.3 g of urea in 5.85 mL of distilled water in a 50 mL plastic tube. This will create an endothermic reaction. Warm the tube by stirring it in a beaker containing hot water to accelerate urea solubilization.
3. Add 3 mL of 40% acrylamide-Bis acrylamide solution.
4. Add 1.5 mL of 10× TBE.
5. For ethidium bromide staining of the gel (optional), add 0.5 µL of ethidium bromide stock solution.
6. Finally, add 150 µL of 10% APS and 15 µL of TEMED.
7. Homogenize by turning the 50 mL tube upside down.
8. Pour the content between the two mounted glass plates until the mixture overflows slightly, then place the comb. Allow the gel to polymerize for at least 1 h (*see* **Note 19**).
9. Place both gels on the migration system. If only one gel is used, replace the second gel with a plastic plate (mandatory for the Bio-Rad system). The unused gel should be appropriately stored for future use (no longer than a week).
10. Fill the tank with 1× TBE solution.
11. Remove the comb. Then preheat the gel for 30 min by running at 80 V.
12. After 30 min, carefully clean the wells of urea and acrylamide by ebb and flux using a long pipet tip or a syringe filled with buffer.
13. Aliquot 2–5 µg of total RNA and mix it with the same volume of 2× Gel Loading Buffer.

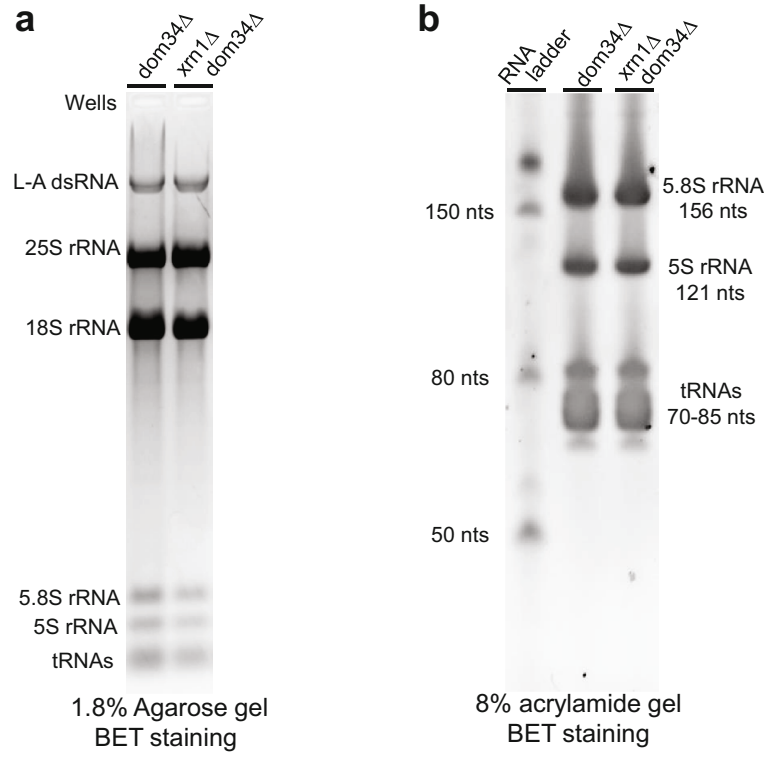

Fig. 1 BET gel staining. (**a**) A 1.8% agarose gel stained with ethidium bromide. 5 μg of total RNA from *dom34* and *xrn1 dom34* mutant strains has been loaded. Abundant RNAs can be observed, such as 25S, 18S, 5.8S, and 5S ribosomal (r)RNAs. Transfer (t)RNAs can also be easily observed. L-A virus dsRNA (a 4.6 kbp size double-stranded RNA) can also be observed in the total RNA extract of many *S. cerevisiae* laboratory strains. (**b**) An 8% acrylamide gel stained with ethidium bromide. Samples identical to those in (**a**) were loaded. A better resolution of small RNA fragments produced in these strains can be observed. A small RNA marker has also been used to facilitate RNA sizing

14. Denature the RNA samples for 3 min (heating block set at 85 °C). Put the samples on ice immediately, microcentrifuge briefly (5–10 s) to collect the liquid, and then load on gel.
15. Run for around 2 h at 80 V. Stop the migration 20–30 min after the bromophenol blue leaves the gel.
16. When migration is complete, empty the tank and discard the buffer in the appropriate disposal tank. Remove the gel-containing plates.
17. Unmold the gel by gently using a flat plastic spatula inserted between the two glass plates to carefully separate the gel from the plates. We use the green lever provided by Bio-Rad.

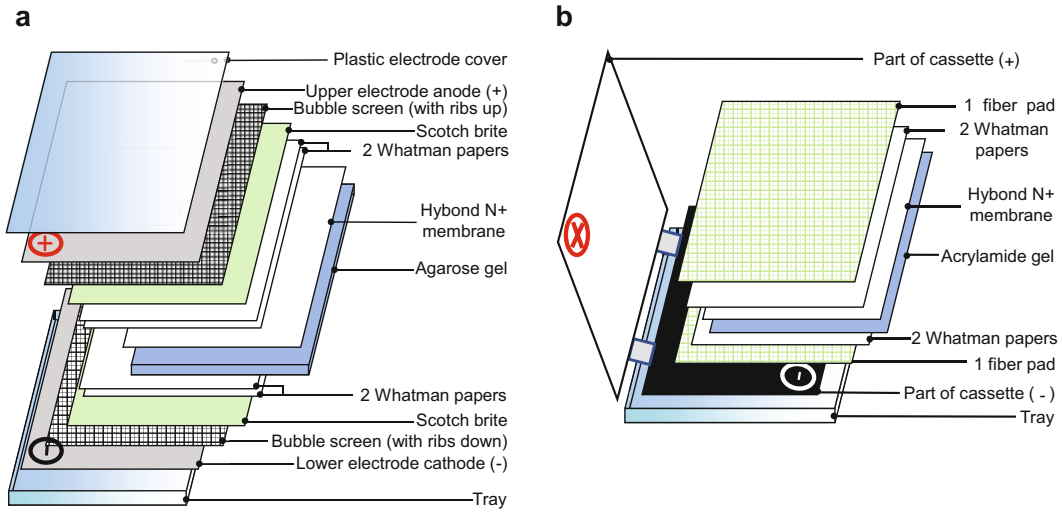

Fig. 2 Assembly for RNA blotting. Diagram (**a**) illustrates the assembly for RNA blotting using an agarose gel, while diagram (**b**) shows the assembly for RNA blotting using an acrylamide gel

18. Examine the gel on a UV transilluminator to visualize the RNA if you chose to add ethidium bromide at **step 5** and take a picture. Figure 1b shows the different RNA species that can be observed using a UV transilluminator.

3.3 RNA Transfer from Agarose Gel (Genie Blotter)

1. Pre-soak the gel in a tank filled with TBE 0.5× for 10 min.
2. Cut four pieces of Whatman 3MM paper to the same size as the gel and a piece of Hybond N+ membrane to the same size of the gel. Pre-soak the pieces of Whatman 3MM paper and the membrane in TBE 0.5×
3. Load the GENIE blotter as described in Fig. 2a, respecting the following order.
4. Put the cathode in the bottom of the tray.
5. Place a plastic bubble screen (ribbed side down) on the cathode.
6. Wet a fiber pad (we use Scotch-brite pads) and place it in the tray.
7. Place, one by one, 2 wet pieces of Whatman 3MM paper on top of the Scotch-brite pad. After each sheet, squeeze out air bubbles by rolling a pipet over the surface (a 10 mL plastic pipet reduced to the inner size of the tray will do and will be reusable).
8. Put the pre-soaked gel above the two first layers of Whatman 3MM paper.
9. Place the pre-wetted membrane on the gel. Again, try to avoid getting air bubbles between the gel and the membrane.

10. Place the remaining two wet sheets of Whatman 3MM paper on top of the membrane. Squeeze out air bubbles by rolling a pipet over the surface, taking care not to crush the gel.
11. Place a wet Scotch-brite pad. Add 0.5× TBE buffer until the tray is almost full.
12. Place a plastic bubble screen, ribbed side up, on the pad and place the anode on it.
13. Put the two-holed plastic anode cover over the anode.
14. Compress the pad/paper/gel/membrane/paper/pad sandwich by approximately 2 mm to slide into the tray support.
15. Slowly tilt the GENIE to an upright position. The buffer should cover the top of the blotting area. Add more buffer if it is not the case, but respect the maximum buffer volume recommended for the apparatus.
16. Connect the GENIE to the generator and begin blotting for 90 min at 12 V.
17. Once the transfer is complete, place the membrane on a Whatman 3MM paper (RNA side up) to absorb the excess of 0.5× TBE.

3.4 Electrotransfer of RNA from Acrylamide Gel to Membrane

1. Open the plastic cassette to prepare a sandwich between the fiber pads, Whatman papers, the gel, and the nylon membrane (*see* Fig. 2b).
2. Pour pre-chilled 0.5× TBE in a tray for assembly.
3. Handle the red or transparent part of the cassette (anode) vertically and lay the black part (cathode) in the tray containing transfer buffer.
4. On the submerged black part, add the fiber pad, and thoroughly soak it.
5. On top of the fiber pad, place the first Whatman paper, and then the second. Use a roller (or broken plastic pipet) to remove any air bubbles.
6. Place the negatively charged membrane, use the roller.
7. Place two more Whatman papers, use the roller.
8. Place the fiber pad and finally finish the assembly by closing the cassette.
9. Place the assembly in the Bio-Rad tank and fill with chilled TBE 0.5× buffer.
10. Perform transfer in a cold chamber at 200 mA for 90 min.
11. After migration, remove carefully the membrane and place it on a Whatman paper.

3.5 Fixing of RNA to the membrane by UV Irradiation

1. Note the date on the "RNA" side (using pencil or pen, but avoid felt-tips) and make a notch to remind the loading direction.
2. Cross-link the RNAs to the membrane by placing it in the Stratalinker ("RNA" side facing the UV lamp). Irradiate at 1200 × 100 μJ/cm^3 (*see* **Note 20**).
3. Place the membrane in a plastic film and store it at 4 °C until hybridization.

3.6 Methylene Blue Staining (Facultative)

To control the quality of transfer, the gel can be exposed to UV to check if there is any RNA remaining on it. Additionally, the membrane can be stained with methylene blue. This allows to check if the transfer was homogeneous or if there was any bubble for instance. Fig. 3a shows a methylene blue staining of a membrane after electrotransfer of RNAs from an agarose gel.

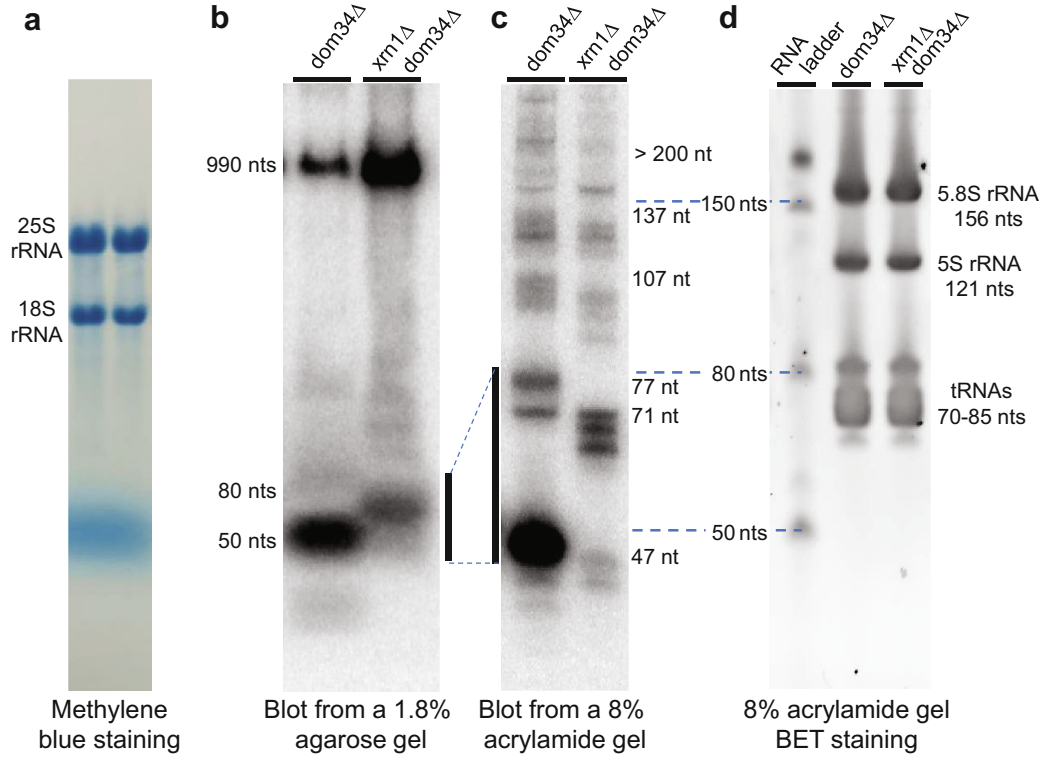

Fig. 3 RNA detection on membrane. (**a**) Methylene blue staining of a membrane after electrotransfer from an agarose gel. (**b**) Detection of a 0.9 kb RNA of interest using a specific radiolabelled DNA probe [7]. This RNA produces small RNA fragments ranging from 50 to 80 nts that can be better resolved using 8% acrylamide gel (**c**). In (**c**), identical RNA amounts were loaded and the same probe has been used. Small RNA sizes indicated have been deduced by primer extension analysis [7]. (**d**) is identical to Fig. 1b. Images from (**c**) and (**d**) can be superimposed and corroborate perfectly small RNA sizing

1. Immerse the membrane (dry or wet) in methylene blue solution and carry out staining for 5–10 min at room temperature.
2. Pour the methylene blue solution back into a bottle for reuse.
3. Wash the membrane three times with water, each time gently shaking the membrane for 5–10 s.
4. RNA bands stain blue against the white to bluish background of the membrane (*see* Fig. 3a). The methylene blue solution will be removed from the membrane during prehybridization in the following steps, and this does not affect the hybridization.

3.7 DNA Probe Preparation and Labelling

1. In a 1.5 mL sterile tube, mix 5 µL of oligonucleotide (10 pmoles/ µL), 1.5 µL of 10× PNK buffer, 1 µL of T4 PNK (10 units), 3 µL of ATP γ-^{32}P, and water up to a total volume of 15 µL. Incubate for 30 min at 37 °C.
2. Purify the probe on a G25 Sephadex micro-column (*see* **Note 11**). Vortex the micro-column to resuspend the sephadex in the buffer. Put the Spin column in a 1.5 mL tube and centrifuge for 2 min at 1000 × *g* in a microcentrifuge to remove the buffer. Use another 1.5 mL tube. Add 35 µL of H_2O to the labelled oligonucleotide and load on the column (in the middle of the column). Centrifuge for 2 min at 1000 × *g*. The eluate volume should be approximatively 40–45 µL.

3.8 DNA Probes Hybridization and Revealing

1. Put the membrane in a hybridization bottle, RNA side facing inward, avoiding contact with the tube wall.
2. Add 12 mL of hybridization buffer for small bottle (size: 3.5 cm × 14 cm, total volume of about 140 mL) or 15 mL for larger ones (size: 3.5 cm × 25 cm, total volume of about 240 mL).
3. Pre-hybridize for 1 h at 42 °C.
4. Dilute the labelled probe in 500 µL of hybridization buffer and add it to the bottle.
5. Hybridize for a minimum of 1–3 h or overnight at 42 °C. *See* **Note 21**.
6. Pre-warmed the washing solution I in a water bath at 42 °C.
7. Discard the hybridization solution containing the probe in the radioactive liquid waste bottle (or store it at −20 °C for reuse).
8. Add 20 mL of the pre-warmed washing solution I in the hybridization bottle and rotate at 42 °C for 15–20 min to efficiently wash the membrane and eliminate a maximum of radioactivity. Ensure that the membrane is submerged in the liquid during rotation.
9. Pre-warmed the washing solution II at 42 °C.

10. Discard the first wash in the radioactive liquid waste bottle. Wash twice with 20 mL of the washing solution II. Rotate at 42 °C for 15–20 min.
11. Discard the last wash in the radioactive liquid waste bottle, and gently remove the membrane using appropriate tweezers.
12. Put the membrane on a 3MM Whatman paper for 1 min and enclose it in a plastic film.
13. Place the membrane in a cassette and expose it to a phosphor screen with the white side of the screen against the membrane and black side on top. Allow exposure for several hours to several days depending on the signal intensity. Scan the screen using a phosphoimager (*see* **Note 22**). Figure 3 shows the results of identical RNA samples run in parallel on agarose and acrylamide gels, transferred to a nylon membrane, and hybridized with an identical probe [7].
14. After exposure, still observe safety protocols, always protecting yourself from radiation, store the membrane at 4 °C in a plastic pocket, ensuring it remains flat. Use a plexiglass box for radiation shielding. *See* **Note 23**.

4 Notes

1. All materials (glassware, solutions, etc.) used are specified RNase-free or sterilized through appropriate autoclaving. Prepare all solutions in nuclease-free water. Autoclaved distilled water is sufficient; some laboratories may prefer to use DEPC-treated water. It is commercially available, but if prepared in the laboratory, ensure to remove all traces of DEPC by autoclaving.
2. We use Eppendorf tubes. We have observed that 1.5 mL tubes are not contaminated by RNases. Take a new tube bag, use it only for RNA preps and always wear gloves. Some may prefer to autoclave all tubes for RNA preps, it is not mandatory.
3. We use commercial 2× Gel Loading Buffer II (Ambion). Different commercial RNA ladders exist, consisting of RNA fragments for sizing single-stranded RNA. They can be very helpful when you need to determine your gel percentage and migration time conditions.
4. We use the Sub-Cell GT Cell and the Powerpac™ HC High-Current Power Supply (Bio-Rad).
5. We use RNAse Zap on all surfaces of the gel caster, tray, comb, and the inner part of the cell. Gently spread it using paper towels on all surfaces that can be in contact with RNA samples. Rinse with distilled water, leave it dry or use paper towels.
6. We use Amersham Hybond-N+.

7. We use a Stratalinker 1800 UV cross-linker.
8. All handling of radioactivity must comply with health and safety rules.
9. We use an oligonucleotide containing at least 10 C or G with a minimum of 18–20 nucleotides in length. We verify the specificity of this sequence for the RNA of interest.
10. We use commercial T4 PolyNucleotide Kinase (New England Biolabs).
11. We use G25 Spin column (Amersham).
12. We use Roti-Quick hybridization buffer (Carl Roth).
13. To prepare washing solutions I and II, begin by preparing 1 L of SCC 20×. Dissolve the following in 800 mL of distilled H_2O: 175.3 g of NaCl, 88.2 g of sodium citrate. Adjust the pH to 7.0 with a few drops of 1 M HCl. Sterilize by autoclaving.
14. We do not use expensive Phosphor screen eraser. A white light illuminator table is largely sufficient. Expose for 30 min.
15. We use a range of agarose concentration up to 1.8% when the size of RNAs of interest is expected between 500 bases and the size of tRNAs. This limits fuzzy signals.
16. We load 5 μg of yeast total RNA in routine. It is possible to load 10 to 20 μg of total RNA from yeast or other species.
17. To set correctly the voltage, calculate a voltage according to this rule: 5–10 V multiplied by the distance between electrodes in cm.
18. Protect the gel from RNases by putting a plastic film on the transilluminator glass. The picture could be taken with a ruler laid alongside the gel, so the band positions can later be identified on the membrane.
19. After polymerization, it is possible to store the gel wrapped in damp paper and in a plastic bag at 4 °C for the following day.
20. If you use a UV light box (254 nm wavelength), leave the membrane for 5 min ("RNA" side facing the UV lamp).
21. Hybridize for 1–3 h if the RNA of interest is known to be abundant, like ribosomal RNAs or like *ScR1* noncoding RNA (used as a routine loading standard for *S. cerevisiae*).
22. When handled properly, membranes can easily be reused for 3–4 additional hybridizations with different probes (and even more depending on the blots). Membranes can be stripped if the radioactive signal can mask the appropriate detection of another RNA of interest. It is always more convenient to detect the less abundant RNAs first. For this reason, all RNA standards (usually abundant noncoding or coding RNAs) are

probed at the very end. For stripping, 100 mL of boiling SDS (0.1%) can be added directly to the membrane in a hybridization bottle. Rotate for 15 min at ambient temperature and discard the SDS solution properly. The membrane is then ready for a new step of pre-hybridization. It is time-consuming, but very important, to expose the membrane to ensure that all previous signals have disappeared or have been reduced before any new hybridization.

23. Membranes can be stored for months at −20 °C. Handle them with care during a couple of minutes after removing from the freezer, and subsequent hybridizations will also be successful.

Acknowledgements

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Part II

Methods for the Study of Deadenylation

Chapter 4

Dynamic Evolution of Poly-A Tail Lengths Visualized by RNAse H Assay and Northern Blot Using Nonradioactive Probes in Yeast

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Abstract

Poly-A tail length dynamics have been extensively studied from yeast to human, mostly using reporter transcripts. Recent studies have been carried out genome-wide to determine the status of poly-A tails at steady state. However, poly-A tail measurement at equilibrium gives an overall length that reflects a mixture of the different poly-A tail sizes for a single transcript. New genome-scale techniques are emerging to estimate dynamic of poly-A tails lengths, but they are not yet routine and individual validation experiments are useful. In this chapter we describe a protocol for visualizing poly-A tail lengths following transcription inhibition for a reporter mRNA using denaturing poly-acrylamide gel electrophoresis and northern blot assay. This protocol is quick to set up, requires the purchase of only a few specific reagents, does not rely on radioactivity for RNA monitoring, and can be easily implemented in any molecular biology laboratory.

Key words Poly-A tail, Tet-OFF, mRNA, Yeast, *Saccharomyces cerevisiae*, Northern blot, RNAse H, Deadenylation, Digoxygenin, DIG

1 Introduction

Poly-A tail addition is a ubiquitous and non-templated RNA modification that first occurs co-transcriptionally. From their early life in the nucleus to their export to the cytoplasm, poly-A tails are bound by poly-A binding proteins that are associated with mRNA stability and protein translation. Poly-A tail shortening is mediated through the action of two major conserved deadenylase complexes in yeast: the Pan2/Pan3 complex that starts the poly-A tail shortening [1] and the Ccr4-Not complex, a master regulator of gene expression, which shortens the remaining of the poly-A tail through its dual deadenylase activity [2, 3].

Several methods have been developed to evaluate poly-A tail lengths genome-wide such as PAL-Seq [4] or Tail-Seq [5], and the arrival of Nanopore direct sequencing was a new step forward in

Maxime Wery (ed.), *mRNA Decay: Methods and Protocols*, Methods in Molecular Biology, vol. 2863,
https://doi.org/10.1007/978-1-0716-4176-7_4,

determining poly-A tail sizes [6]. However, Nanopore direct sequencing can miss very short poly-A tail measurements, and most established protocols using Illumina sequencing rely on PCR for cDNA amplification that can generate biases for poly-A tail estimations [7]. Furthermore, steady-state poly-A tail measurements provide an average poly-A tail size for a transcript at a given time and might lack information on the proportion of long and short poly-A tails. Using time-course mRNA labelling and poly-A tail measurements with a modified PAL-Seq protocol, a recent study was able to measure dynamic poly-A tail changes, but such a method takes time to set up and may not be suitable for rapid estimation of the poly-A tail size dynamics for a few transcripts of interest [8].

Here we describe a fast and easy way to visualize the dynamics or steady-state poly-A tail sizes in yeast using RNase H/oligonucleotide cleavage and northern blot analysis. This method was one of the first described to study poly-A tail length but using probes labelled with radioactive isotopes for hybridization and detection [9, 10]. We propose as an alternative to radioactive labelling, the use of probes labelled with digoxigenin, thus avoiding specific equipment for handling and disposal of radioactive waste. This method is free from PCR amplification steps and minimally perturbs the cell metabolism, through the use of a tetracycline-dependent promoter for the gene of interest. Such an assay can be used to screen for factors or conditions that lead to poly-A tail shortening or extension over time. Using the RNAse H enzyme that specifically hydrolyses RNA/DNA hybrids and a specific primer in the 3′ extremity of the RNA of interest, poly-A tail fragments are generated and compared to a fragment without a poly-A tail (*see* Fig. 1). These fragments are then analyzed on a urea-containing poly-acrylamide gel that allows nucleic acid denaturation and separation. The obtained images are analyzed using appropriate software, typically ImageJ.

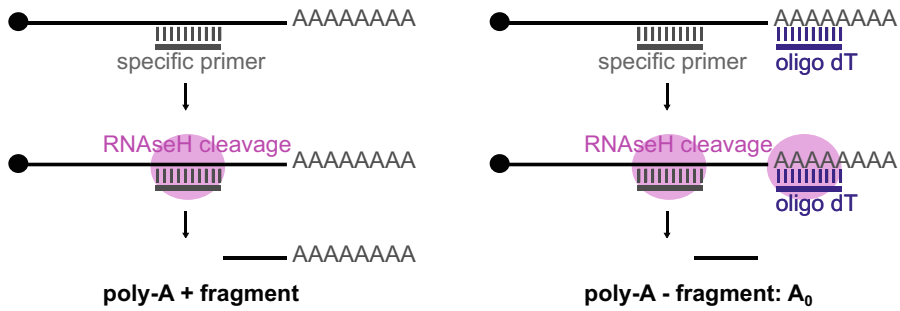

Fig. 1 Poly-A fragments are generated using a specific primer in the 3′ region of the targeted mRNA and the RNAse H action. Addition of an oligo-dT allows analysis of the cleaved fragment without a poly-A tail that corresponds to the "A_0" reference point

2 Materials

Prepare all solutions using RNase-free water. Clean material with RNase away surface decontaminant. Keep an RNAse-free area, use filter tips, handle all material destined for RNAs with gloves. The equipment and reagents from a standard molecular biology laboratory are required including the following.

2.1 Yeast Strains, Media, and Plasmids

1. *Saccharomyces cerevisiae* strains of interest.
2. YPD (Yeast Extract–Peptone–Dextrose) or synthetic selective media for plasmid maintenance.
3. Plasmid with the gene of interest under a tetracycline-dependent promoter such as pCM189 or pCM190 (*see* **Note 1**) [11].
4. Doxycycline stock solution: 10 mg/mL (in RNAse-free water).

2.2 RNA Extraction

1. TES buffer: 10 mM Tris pH 7.5, 10 mM EDTA, 0.5% SDS.
2. Molecular-grade acid phenol.
3. Molecular-grade chloroform.
4. 3 M sodium acetate, pH 5.2.
5. Absolute ethanol.
6. 70% ethanol.
7. 10 mg/mL glycogen (molecular grade).
8. ThermoMixer or equivalent.
9. Nanodrop ND1000 Spectrophotometer or equivalent.

2.3 RNAse H Cleavage Assay

1. Specific primer for cleavage (*see* Subheading 3.4).
2. Oligo dT (12–18) primer (0.5 μg/μL).
3. 10X annealing buffer: 0.1 M Tris pH 7.5, 10 mM EDTA, 0.5 M NaCl.
4. RNAse H (5,000 units/mL) and RNAse H buffer.
5. Stabilized formamide (FORMAzol, MRC) containing 0.01% of bromophenol blue and 0.01% of xylene cyanol.

2.4 Northern Blot

2.4.1 Gel Preparation

1. Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad) or equivalent.
2. 10X TBE buffer: 890 mM Tris-borate, 890 mM boric acid, 20 mM EDTA.
3. Acrylamide/Bis-acrylamide 19:1, 40% (w/v) solution.
4. Urea powder.
5. 10% freshly prepared ammonium persulfate (APS)

6. Tetramethylethylenediamine (TEMED).
7. Ethidium bromide, SYBR Safe or equivalent.

2.4.2 RNA Blotting and Probe Hybridization

1. Semi-dry transfer apparatus.
2. Positively charged nylon membrane (e.g. Hybond N+).
3. Cellulose filter paper.
4. UV cross-linker.
5. Hybridization oven and tubes.
6. 20X SSC: 3 M sodium chloride, 0.3 M sodium citrate, pH 7.0.
7. 10% SDS.
8. Methylene blue solution: 0.02% (w/v) methylene blue in 0.3 M NaOAc (pH 5.5).

2.4.3 DNA Probe Preparation

1. DIG DNA labelling mix (Roche). This mix contains dUTP-DIG.
2. Forward and reverse primers for the detection of mRNA of interest (*see* Subheading 3.6.1).
3. DNA polymerase enzyme with its buffer.
4. dNTP stock at 10 mM.
5. Molecular-grade agarose.
6. Agarose gel electrophoresis system.
7. PCR purification kit.

2.4.4 RNA Probe Preparation

1. DIG RNA labelling Kit (Roche). The kit contains the T7 RNA polymerase, UTP-DIG, and an RNase inhibitor.
2. T7 promoter primer (5′-taatacgactcactatagggaga -3′) and primer for mRNA of interest with reverse T7 promoter sequence (*see* Subheading 3.6.2).
3. 0.2 M EDTA, pH 8.0.

2.4.5 RNA Detection and Visualization

1. ULTRAhyb™ Ultrasensitive buffer (ThermoFisher).
2. Blocking buffer and washing buffer: DIG Wash and Block Buffer Set (Roche).
3. Anti-Digoxigenin-POD, Fab fragments (Roche).
4. ECL reagent and chemiluminescence detection system.

3 Methods

This protocol describes how to perform dynamic poly-A tail visualization on yeast mRNAs. However, it can be applied to other organisms using the same RNA starting amount. Probe preparation (*see* Subheading 3.6) can be done in parallel for time saving.

3.1 Cell Culture and Transcriptional Arrest

For steady-state analysis, skip **steps 2** and **3**.

1. Grow yeast cells with constant shaking—250 rotations-per-minute (rpm)—at 30 °C, in YPD or in selective media for plasmid maintenance until exponential phase, typically, to $OD_{600} = 0.6$ (*see* **Notes 2** and **3**).
2. When cells reach the exponential growth phase, divide the culture in the appropriate number of flasks.
3. For the transcriptional shut-off, add doxycycline to the culture at a final concentration of 10 μg/mL and return the flask at 250 rpm at 30 °C (*see* **Note 4**).
4. After relevant time, transfer cells into appropriate tubes and spin for 5 min at room temperature at 3000× *g*. Remove and discard supernatant. Transfer the tubes to liquid nitrogen or place them on dry ice (*see* **Note 5**).
5. Store the cell pellets at −20 °C or −80 °C or directly proceed to Subheading 3.2.

3.2 Total RNA Extraction

Here we describe the classical hot acid phenol RNA extraction protocol [12] (*see* **Note 6**). Total RNA extraction using commercial kits can be an alternative.

1. Add 400 μL of acid phenol to the cell pellet and 400 μL of TES buffer (s*ee* **Note 7**).
2. Place the samples in a ThermoMixer for 30 min at 65 °C with shaking at 1400 rpm.
3. Spin the samples for 5 min at 20,000× *g* at room temperature.
4. Recover the top aqueous layer and transfer it in new tubes containing 400 μL of acid phenol.
5. Vigorously vortex the tubes for 30 s and spin the samples for 5 min at 20,000× *g* at room temperature.
6. Recover the top aqueous layer in new tubes containing 400 μL of chloroform.
7. Vigorously vortex the tubes for 30 s and spin the samples for 5 min at 20,000× *g* at 4 °C.
8. Recover the top aqueous layer in new tubes containing 1 mL of ice-cold 100% ethanol and 40 μL of 3 M NaOAc. Invert the tubes several times to mix.
9. At this stage, tubes can be kept at −20 °C or −80 °C or proceed directly to **step 10**.
10. Spin the samples for 20 min at 20,000× *g* at 4 °C.
11. Carefully discard the supernatant. A viscous and transparent pellet can be visible.

12. Wash the pellet with 500 µL of 70% ethanol and spin for 5 min at 20,000× *g* at 4 °C.
13. Discard the supernatant without disturbing the pellet. Air-dry the samples at room temperature for 30 min.
14. Resuspend the pellet in RNase-free water (*see* **Note 8**). To ensure complete dissolution, the samples can be placed at 65 °C for 5 min.
15. Check RNA concentration using a Nanodrop or another nucleic acid quantification instrument.
16. Store RNAs at −20 °C or −80 °C or directly proceed to Subheading 3.3 (*see* **Note 9**).

3.3 RNA Quality Assessment

RNA quality can be assessed by loading total RNA on an agarose gel. Ribosomal RNA presence as sharp bands reflect overall RNA quality. Alternatively, RNA quality can be assessed using the Agilent RNA 6000 Nano Kit on the Agilent Bioanalyzer 2100 instrument.

1. Prepare a 1% agarose gel in 1X TBE containing an intercalating agent (ethidium bromide or alternatively SYBR safe) (*see* **Note 10**).
2. Resuspend 5 µg of RNAs in FORMAzol containing 0.01% of bromophenol blue and 0.01% of xylene cyanol (*see* **Note 11**).
3. Denature the samples for 5 min at 85 °C then keep them on ice.
4. Before loading, rinse the gel wells by up and down pipetting with 1X TBE buffer. Load the samples and run the gel in 1X TBE buffer for 1 h at 100 V (30 cm distance between electrodes).
5. Visualize the RNA on a UV transilluminator. For *Saccharomyces cerevisiae*, 25S and 18S should be visible (*see* Fig. 2).
6. *Optional:* the gel can be used to do a northern blot (*see* Subheading 3.9).

3.4 RNAse H Cleavage

If comparing several time points, the same quantity of total RNA should be used for each point.

1. Prepare tubes containing 20 µg of total RNA, 1 µL of specific primer (stock at 50 µM), 2 µL of 10X annealing buffer, and add RNase-free water to reach a final volume of 20 µL (*see* Fig. 1 and **Note 12**.)
2. Prepare a tube for the non-poly-A point ("A_0") containing 20 µg of total RNA, 1 µL of specific primer (stock at 50 µM), 2 µL of 10X annealing buffer, 1.5 µL of oligo dT (12–18) (stock at 0.5 µg/µL), and add RNase-free water to reach a final volume of 20 µL (*see* Fig. 1).

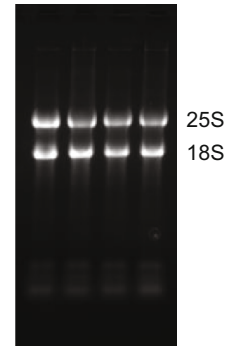

Fig. 2 Image of a 1% agarose 1X TBE gel for *Saccharomyces cerevisiae* total RNA. The upper band corresponds to the 25S rRNA and the lower band correspond to the 18S rRNA. Bands at the bottom of the gel correspond to the 5.8S and 5S rRNAs and to tRNAs

3. Denature the samples for 5 min at 85 °C using a Thermomixer and slowly cool down the tubes to 37 °C to allow primers annealing. This can be done by turning off the machine and waiting for it to reach the correct temperature.
4. Add directly to the prepared tubes 5 μL of 10X RNAse H buffer, 1 μL of RNAse H (stock at 5000 U/mL), and 24 μL of RNase-free water to reach a final volume of 30 μL and incubate the reaction for 1 h at 37 °C.
5. To stop the reaction and purify the RNAs, add 200 μL of acid phenol to the tubes and 150 μL of RNase-free water, vigorously vortex for 30 s at room temperature.
6. Spin the samples for 5 min at 20,000× *g* at room temperature.
7. Collect the top aqueous layer in new tubes containing 1 mL of ice-cold 100% ethanol, 40 μL of 3 M sodium acetate, and 15 μg of glycogen. Invert the tubes several times to mix.
8. At this stage, the tubes can be kept at −20 °C or −80 °C or directly proceed to **step 9**.
9. Spin the samples for 20 min at 20,000× *g* at 4 °C.
10. Carefully discard the supernatant.
11. Wash the pellet with 500 μL of 70% ethanol and spin for 5 min at 20,000× *g* at 4 °C.
12. Discard the supernatant without disturbing the pellet. Air-dry the samples at room temperature for 30 min.
13. Resuspend the pellet in 20 μL of FORMAzol containing 0.01% of bromophenol blue and 0.01% of xylene cyanol.
14. The tubes can be kept at −20 °C or proceed directly to Subheading 3.5.

3.5 Migration and Transfer

This part describes how to prepare a gel (8.4 × 8.4 cm – 1.0 mm thickness) that fits into our vertical electrophoresis apparatus. Quantity should be adjusted depending on the size of the gel (*see* **Notes 13** and **14**).

1. Prepare a denaturing 6–8 M urea poly-acrylamide gel (*see* **Notes 15** and **16**). Example for 8 M urea 6% poly-acrylamide-gel: 1.2 mL of 10X TBE, 1.8 mL of 40% (w/v) acrylamide/bis solution (19:1), 5.8 g of urea, 50 µL of 10% APS, 18 µL of TEMED, and add RNase-free water to reach a final volume of 12 mL.
2. Denature the RNAs at 85 °C for 5 min and then keep them on ice.
3. Before loading the samples, carefully rinse the wells by up and down pipetting with 1X TBE after having pre-warmed the gel by running for 15 min at 180 V.
4. Load the samples and run the gel for 1 h at 180 V in 1X TBE (*see* **Notes 17** and **18**).
5. *Optional:* after migration, RNAs can be detected by incubating the gel in 1X TBE containing SYBR gold.
6. Cut four cellulose filter paper sheets and the membrane at the size of the gel.
7. Wet the four cellulose filter paper sheets and the membrane in 0.5X TBE (*see* **Note 19**).
8. From the anode to the cathode using a semi-dry transfer blot apparatus: place the two cellulose filter paper sheets, the membrane, the gel and two more cellulose filter paper sheets, chase the bubbles (*see* Fig. 3).
9. Transfer at 10 V for 1 h.
10. Crosslink the RNAs to the membrane using a UV cross-linker with a setting of 0.120 Joules.
11. To control RNA transfer, immerse the membrane in a methylene blue solution for 5 min at room temperature and then rinse three times for 5 min with RNase-free water.
12. Proceed to Subheading 3.6 or store the membrane at −20 °C (*see* **Note 20**).

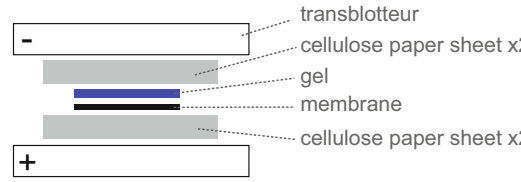

Fig. 3 Schematic drawing of a semi-dry nucleic acid transfer stack

3.6 Probe Preparation

Two possibilities are described for probe preparation, either a single-stranded DNA probe or an RNA probe. The choice should be done depending on the available material and probe specificity considerations (*see* **Notes 21–23** and Fig. 4).

3.6.1 DNA Probe

This part describes how to make a single-stranded DNA probe labelled with DIG-dUTP. DNA template should be the region of interest (*see* Fig. 4). If interested in antisense RNA, add a forward primer in **step 3**.

1. Run a PCR reaction (30–35 cycles), with 100 ng of DNA template, 1 μL of forward primer (stock at 20 μM), 1 μL of reverse primer (stock at 20 μM), 5 μL of 10X Taq PCR buffer, 2 μL of dNTP (stock at 10 mM), 0.5 μL of Taq DNA polymerase (stock at 5000 U/mL), and add RNase-free water to reach a final volume of 50 μL.
2. Purify the DNA fragment away from the free nucleotides (using a PCR purification kit) and quantify the DNA fragment using a Nanodrop or another nucleic acid quantification instrument.
3. Run the PCR for digoxigenin (DIG)-dUTP incorporation with 10 to 50 ng of the DNA purified fragment, 2 μL of the reverse primer (stock at 20 μM), 10 μL of the PCR DIG labelling mix (contains DIG-dUTP), 10 μL of 10X Taq PCR buffer, 1 μL of Taq DNA polymerase (stock at 5000 U/mL), and add RNase-free water to reach a final volume of 100 μL (*see* **Notes 24** and **25**).
4. Store the probe at −20 °C (*see* **Note 26**).

3.6.2 RNA Probe

This part describes how to make a single-stranded RNA probe labelled with UTP-DIG. The T7 RNA polymerase can perform RNA transcription from a T7 DNA duplex promoter. Transcription

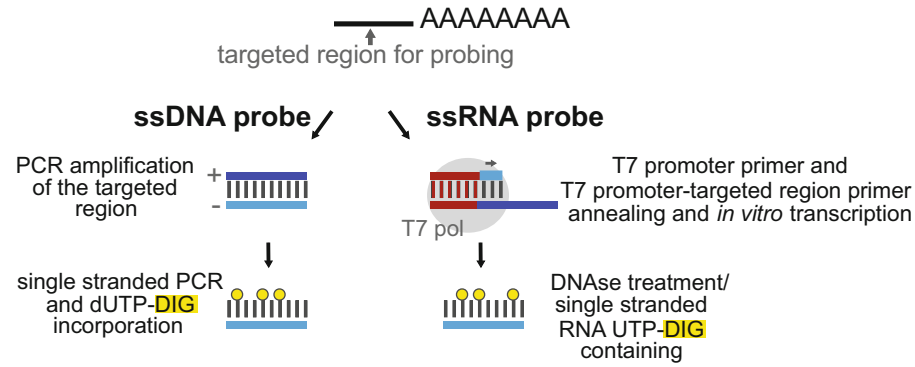

Fig. 4 Differences between ssDNA and ssRNA cold probes generation

is performed on the single-stranded overhang that should correspond to the reverse sequence of the targeted RNA (*see* Fig. 4).

1. Mix the T7 promoter primer with the designed primer (T7 promoter—targeted region) at a final concentration of 10 μM.
2. For primers annealing, denature mixed primers at 95 °C for 5 min and slowly cool down to room temperature.
3. Store the annealed primers at −20 °C or directly proceed to **step 4**.
4. Prepare the following reaction: 2 μL of annealed primers (stock at 10 μM), 2 μL of 10X NTP labelling mixture (contains DIG-UTP), 2 μL of 10X transcription buffer, 1 μL of RNase inhibitor (stock at 20 U/μL), 2 μL of T7 RNA polymerase (stock at 20 U/μL), and add RNase-free water to reach a final volume of 20 μL.
5. Incubate for 1 to 2 h at 37 °C.
6. Add 2 μL of DNase I (stock at 10 U/μL) to eliminate the template DNA.
7. Stop the reaction by adding 2 μL of 0.2 M EDTA (pH 8.0) and store the probe at −20 °C (*see* **Notes 26** and **27**).

3.7 RNA Hybridization

1. Warm the hybridization buffer at 50 °C until it becomes liquid and transparent.
2. Put the membrane in a roller tube, RNA side facing towards the inside.
3. Block the membrane in the hybridization buffer for 1 h under rotation at 50–70 °C. Depending on the size of the membrane, 5 mL to 15 mL should be sufficient (*see* **Notes 28–30**).
4. Denature the DNA probe (*see* Subheading 3.6.1) at 95 °C for 5 min or at 65 °C for 5 min for the RNA probe (*see* Subheading 3.6.2).
5. Add the probe to the membrane and incubate for 1 h to overnight at 50–70 °C (*see* **Notes 30** and **31**).
6. Remove the probe and wash the membrane with 2X SSC, 0.5% SDS buffer at 50–70 °C for 10 min (*see* **Notes 32**). Repeat this step once.
7. Wash the membrane one time with 1X SSC, 0.5% SDS buffer at 50–70 °C for 10 min.
8. Transfer the membrane in a plastic box and incubate it in 1X washing buffer for 5 min at room temperature (*see* **Note 33**).
9. Block the membrane in the 1X blocking buffer (prepared fresh: 1X blocking solution diluted in 1X maleic acid buffer) at room temperature for 30 min (*see* **Note 34**).

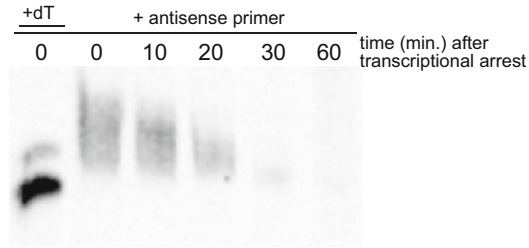

Fig. 5 Evolution of poly-A tail length after transcription shut off using a reporter transcript under a tetracycline-dependent promoter. Lane 1 (+dT) corresponds to the A_0 fragment, without a poly-A tail

10. Incubate the membrane with the Anti-Digoxigenin-POD (poly) conjugated with horse-radish peroxidase, diluted in 1X blocking buffer (1:2000) for 1 h at room temperature.
11. Wash the membrane with 1X washing buffer at room temperature three times for 10 min.
12. Reveal the membrane using a ECL detection reagent (*see* **Note 35**). Typical results are shown in Fig. 5.

3.8 Poly-A Tail Analysis Using Fiji

Fiji can be downloaded at this address: https://imagej.net/software/fiji/downloads

ImageJ2 version 2-9.0/1.53 t was used.

1. Open the image with Fiji (Fiji can notably support TIFF or JPEG formats, but can also open proprietary files, such as ".scn" from Bio-Rad).
2. Go to Edit > Invert colour, to have the background in black and the signal in white.
3. Go to Image > Transform > Rotate 90° Right.
4. Select the rectangle on the Fiji panel and draw a rectangle on the first lane (*see* Fig. 6a, yellow rectangle).
5. Go to Analyse > Plot profile. The profile of the signal intensity should appear with "Distance (pixels)" in *x* and "Gray values" in *y*.
6. Go to "Data" on the profile window and select "Save data" in .csv format.
7. Repeat these steps for all your points by moving the rectangle from **step 4** to the other points (*see* **Note 36**).
8. Plot the profiles for each point using a spreadsheet software (*see* Fig. 6b).

3.9 Optional: Total RNA Northern Blot

This protocol describes the capillarity blotting that is simple to set up. Blotting using a vacuum pump is a faster alternative.

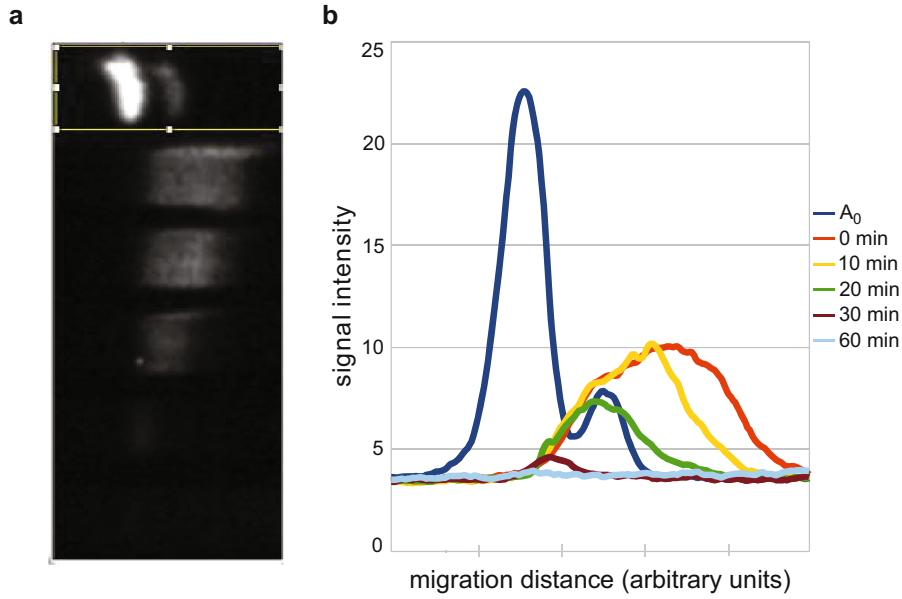

Fig. 6 Poly-A tail images (**a**) can be analyzed to compare poly-A tail signal intensity and length (migration distance) (**b**)

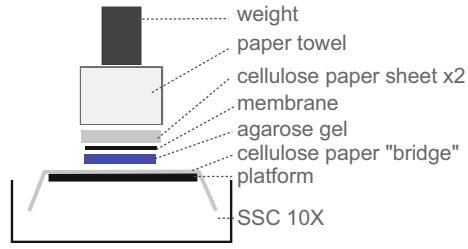

Fig. 7 Schematic drawing of capillarity transfer for northern blot

1. Prepare a tank and a glass/platform that will be placed across the tank (*see* Fig. 7).
2. Cut two cellulose filter paper sheets and the membrane at the size of the gel. Cut another cellulose filter paper sheet at the widest of the membrane but with a longer size that will form the "bridge" (*see* Fig. 7).
3. Place the elements with the following order: the "bridge" cellulose filter paper sheet that should be in contact with 10X SSC solution from both sides, the gel, the membrane and two cellulose filter paper sheets. Chase the bubbles (*see* Fig. 7).
4. Put tissues on top and a weight and let transferring overnight.
5. The next day, tissues should be wet. Remove all elements. Proceed from **step 10** from Subheading 3.5.

4 Notes

1. A Galactose-inducible (GAL) expression system can be an alternative and appropriate media containing galactose should be prepared, whereby addition of glucose then shuts down transcription.
2. Select the total volume of growing cells according to the total number of points for the kinetic study. 15 to 30 mL per point gives a good RNA yield.
3. RNA half-life is dependent on temperature, adjustment of the optimal working temperature for yeast growth should be done to determine the best conditions for your mRNA of interest [13].
4. Proceed in the same way for each time point.
5. It is important to quickly freeze the pellets to stop the kinetic.
6. Acid phenol and chloroform are toxic, wear appropriate protections and proceed in a chemical fume hood.
7. There is no need to wait for the pellets to be thawed if they were kept at −20 °C or −80 °C.
8. When resuspended in water, RNA is highly sensitive to degradation: RNA-containing tubes should be kept on ice.
9. RNAs can be safely stored at −80 °C for several months but several cycles of freezing and thawing can alter RNA integrity.
10. Ethidium bromide is classified as a potent mutagen, take appropriate protection measures.
11. A volume of diluted RNAs in a volume of FORMAzol dye works well. Alternatively, a commercial RNA loading dye can be used.
12. The position of the specific primer determines the size of the targeted fragment and the possibilities for the position of the probe. Choose the specific primer with a size of about 20 to 25 nucleotides and with about 50% GC content.
13. Non-polymerized acrylamide is an irritant and potential neurotoxic compound. Wear appropriate protections and proceed in a chemical fume hood.
14. Poly-acrylamide gel concentration should be chosen depending on your expected poly-A tail sizes. Longer poly-acrylamide gels will increase the resolution.
15. Urea solubilization is endothermic and can be sped up by short microwave treatment or heating in a water bath.
16. Poly-acrylamide urea gels can be kept in wet atmosphere (with wet 1X TBE cellulose paper sheets around) at room

temperature overnight. Cold temperature (4 °C) will result in urea crystallization.

17. Control the migration by visualization of the bromophenol blue and the xylene cyanol that should respectively migrate at positions corresponding to 26 and 106 nucleotides in a 6% urea denaturing poly-acrylamide gel [14].
18. A low range single-stranded RNA ladder can be loaded to determine RNA sizes.
19. 6% poly-acrylamide—urea gel can be difficult to manipulate for the blotting: place a dry cellulose filter paper sheet on the gel that is still on the glass and carefully remove the cellulose filter paper sheet that will have the gel attached to it.
20. The membrane can be stored at −20 °C (with wet 1X TBE cellulose paper sheets around) for several months and probed later. Thaw the membrane at room temperature until it is completely unfrozen before use.
21. Ensure that your probe is specific to your mRNA of interest. If several similar genes are present in the genome with closely related sequences, it can be relevant to design a probe in the 5′ or the 3′UTR of the transcript, which are more likely to be divergent. An alignment software can be used to check for off target sequences, https://www.yeastgenome.org/blast-sgd for *Saccharomyces cerevisiae* or https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome for other organisms.
22. RNA:RNA hybrids are more stable than DNA:RNA hybrids, but DNA probes can be longer than RNA probes.
23. U-rich probes enhance the signal.
24. Adapt the volume if the probe quantity should be increased for better signal detection.
25. As there is only the reverse primer in the PCR reaction, the reaction is not exponential. 40 cycles of linear amplification are recommended.
26. A dot blot assay can be useful to determine probe efficiency and required dilution for effective signal.
27. Avoid repeated freezing and thawing of RNA probes.
28. Other hybridization buffers can be used, and signal efficiency should be tested accordingly. An alternative of the ULTRAhyb™ Ultrasensitive (ThermoFisher) that is formamide-free is the DIG Easy Hyb™ (Sigma). Homemade buffers containing nonspecific nucleic acid can also be used.
29. Hybridization buffer can be reused several times.
30. Hybridization temperature depends on the melting temperature of the probe.

31. Probes in hybridization buffer can be stored at −20 °C and reused several times.
32. Reducing the SSC percentage will result in increased stringency and should be adjusted accordingly to the probe specificity.
33. Washing buffer contains maleic acid buffer with 0.3% tween 20.
34. Other blocking solutions can be used, such as a homemade one with maleic acid buffer containing 0.3% tween 20 and 5% fat dry milk.
35. The membrane can be stripped with 0.5% SDS 2 mM EDTA at 80 °C for 1 h, washed three times for 15 min in RNAse-free water and probed again.
36. Be careful to move the rectangle only horizontally to not modify the *x* axis values as the different profiles are compared based on the same *x* axis values.

Acknowledgments

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Chapter 5

Assessing Deadenylation Activity by Polyacrylamide Gel Electrophoresis Using a Fluorescent RNA Substrate

Rafailia A. A. Beta and Nikolaos A. A. Balatsos

Abstract

The poly(A) tail is a dynamic structure at the 3′- end of the majority of RNA polymerase II transcripts. It is a critical feature, particularly for mRNAs, as the length of the poly(A) tail regulates their translational efficiency and lifespan. The shortening of the tail is catalyzed by deadenylases that trim and finally remove it, triggering mRNA degradation. Conventional assays to evaluate and measure deadenylase activity rely on radiolabeling of the substrate while colorimetric alternatives lack sensitivity. In this chapter, we describe a poly(A)-shortening assay using a 5′-Cy3-labeled RNA substrate bearing a 3′-oligo(A) sequence. The reaction products representing substrates with shortened tails are analyzed by denaturing acrylamide electrophoresis and are visualized using a gel documentation system. This assay is efficient, it requires standard lab equipment, including a fluorescence-detecting gel documentation system, and presents a quantifiable and safer alternative to radioactivity-based methods for evaluating deadenylase activity.

Key words Deadenylase, Deadenylation, Fluorescence assay, Poly(A) tail, Polyacrylamide gel electrophoresis

1 Introduction

The addition of the poly(A) tail to the newly synthesized RNAs is important for their stability as well as export from the nucleus and translation [1]. The length of the poly(A) tail determines translational efficiency [2] and mRNA life span, since shortening of the tail beyond a critical point, triggers degradation of the mRNA and translational silencing. Shortening of the tail, also known as deadenylation, is a regulated process that is catalyzed by deadenylases [1, 3]. Deadenylases are a family of exoribonucleases that hydrolyze poly(A) tails and are classified into two groups: the DEDD and the exonuclease–endonuclease–phosphatase nucleases (EEP) based on the residues that comprise their active sites [1, 4].

The standard assay used to assess deadenylase activity is by visualizing a radiolabeled oligonucleotide substrate and reaction products on a gel, which are laborious and difficult to quantify

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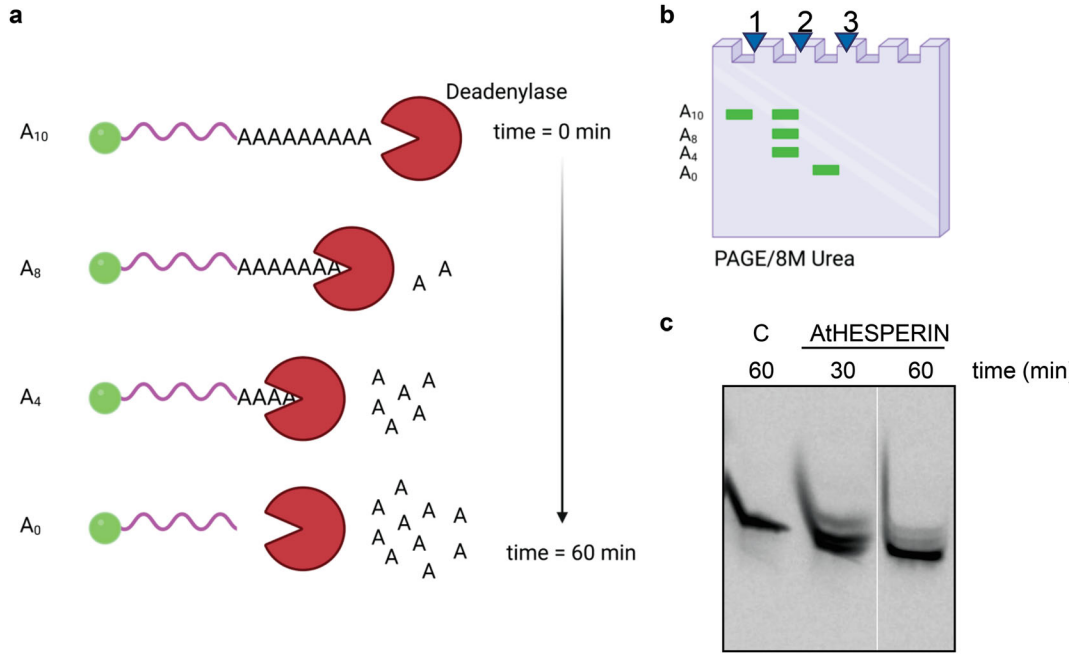

Fig. 1 (**a**) Schematic representation of the deadenylation reaction catalyzed by AtHESPERIN. As reaction time progresses, the deadenylase trims the A_{10} tail of the synthetic RNA, stopping when it reaches non-adenonsine residues of the RNA (A_0). (**b**) Depiction of the expected reaction products run in a denaturing gel. Lane 1, unreacted fluorescent substrate (A_{10}); lane 2, reaction progress (A_{10}-A_4); lane 3, a completely reacted RNA (A_0). (**c**) AtHESPERIN reactions following the same pattern as described in (**b**); C: control reaction with no enzyme

[5] as well as pose an occupational risk of radiation exposure. Nonradioactive alternatives have been reported, namely a colorimetric assay where methylene blue dye intercalates to the RNA—which is a mixture of poly(A) stretches—and enzyme activity is monitored by observing a decrease in the absorption that correlates with the release of methylene blue from the poly(A) molecules and therefore deadenylase activity [6, 7]. It is worth noting that this kind of assay is not very sensitive and requires large reaction volumes, and therefore large amounts of enzyme and substrate.

Here, we describe a poly(A)-shortening assay to evaluate potential deadenylase activity, using a short RNA substrate with a 3′ oligoadenosine tail labeled at the 5′ end with a Cy3 fluorescent moiety. The activity is assessed by denaturing acrylamide gel electrophoresis. The substrate is shortened due to deadenylase activity in a 3′ to 5′ direction; the shortened RNAs migrate faster through the gel and are directly visualized due to the 5′-end fluorescent label (Fig. 1). This assay requires standard laboratory equipment, polyacrylamide gel electrophoresis capabilities (casting and running the gel) as well as a gel documentation system that detects fluorescence.

2 Materials

All solutions must be RNase-free and prepared with DEPC-treated ultrapure deionized water (resistance of 18 MΩ × cm at 25 °C). Take care to follow good RNA practices throughout the procedure by decontaminating working areas with RNase Zap and using RNase-free bottles and RNase-free filtered pipette tips.

2.1 Preparation of Electrophoresis Solutions

1. DEPC-treated water: add 1 mL of DEPC in 1 L of ultrapure water (*see* **Notes 1** and **2**) to achieve a concentration of 0.1% DEPC and autoclave.
2. 10× TBE: 0.89 M Tris, 0.89 M boric acid, 0.025 M EDTA. Dissolve 108.8 g of Tris, 55.02 g of boric acid, and 7.3 g of EDTA in a final volume of 1 L of the DEPC-treated water, and mix thoroughly. Filter through a 0.2 μm filter in an RNase-free bottle (such as the Millipore Stericup Vacuum Filtration System).
3. 40% acrylamide mix, 5% C (*see* **Note 3**). Dissolve 38 g of acrylamide and 2 g of bis-acrylamide in 100 mL of DEPC-treated water, and mix thoroughly (*see* **Note 4**). Filter through a 0.2 μm filter in a RNase-free bottle. Protect from light and keep at 4 °C.
4. 20% acrylamide, 8 M urea mix. Dissolve 24.02 g of urea in 25 mL of the 40% acrylamide mix and 5 mL of 10× TBE (*see* **Note 5**). After the urea is dissolved, adjust the volume to 50 mL with DEPC-treated water and pass through a 0.2 μm filter in a RNase-free bottle.
5. 10% ammonium persulfate (APS): 1 gr of ammonium persulfate powder in 10 mL of DEPC- treated water. The solution can be aliquoted and stored in the freezer.
6. 2× RNA sample buffer: 95% formamide, 5 mM EDTA, and 0.025% SDS. In 9.5 mL of formamide, dissolve 0.015 g of EDTA and 0.0025 g of SDS. Mix thoroughly and adjust the volume to 10 mL with DEPC-treated water and filter through a 0.2 μm filter in an RNase-free bottle (*see* **Note 6**).

2.2 Consumables and Equipment

1. 15 mL and 50 mL conical tubes (RNase- free).
2. 1.5 mL snap-cap tubes.
3. 0.2 μm filters.
4. 21-gauge hypodermic needles.
5. 5 or 10 mL syringes.
6. Circulating water bath.
7. Heating block.
8. Volume adjustable pipettes and appropriate pipette tips.

9. Gel casting system (e.g. Mini-Protean® Tetra Handcast system).
10. Vertical electrophoresis system and power supply (e.g. Mini-Protean® Tetra Cell).
11. Gel documentation system with specifications that align with detection requirements. More specifically, epi- or transillumination at the appropriate wavelength for the fluorophore used, filters that detect the fluorescence emitted by the fluorophore, and a sensitive camera to capture the signal, preferably a cooled Charge Coupled Device (CCD) camera.

2.3 Preparation of Reaction-Specific Solutions

1. RNase inhibitor (10 U/μL).
2. Deadenylation reaction buffer: 50 mM Na-Citrate (pH 6), 0.1 M NaCl, 2 mM $MgCl_2$, 10% Glycerol (*see* **Note 7**).
3. Reaction buffer Master Mix (MM): 0.1 U/μL RNase Inhibitor, 1 mM DTT, diluted in reaction buffer. This buffer must be freshly prepared before use.
4. 17-mer oligonucleotide RNA substrate. The RNA substrate can be synthesized by several companies. It should have the following sequence 5′-CCUUUCCAAAAAAAAAA-3′ (*see* **Note 8**), and it should be labeled at its 5′ end with Cy3. It should be resuspended in DEPC-treated water at a final concentration of 100 μM according to the manufacturer's instructions. The substrate is then diluted at 20 μM in DEPC-treated water for the reactions.
5. Deadenylase (*see* **Note 9**). The enzyme should be diluted in the appropriate reaction buffer at a recommended final concentration of 1.6 μM and kept on ice (*see* **Note 10**).

3 Methods

3.1 Reaction Setup

The reaction volume is usually 10–20 μL and the substrate concentration is fixed at 8 μM (*see* **Notes 10** and **11**).

1. Prepare reaction tubes corresponding to the number of reaction conditions to be tested (*see* **Note 12**) and prepare reactions as follows. Control reaction: 12 μL Master Mix, 8 μL substrate (5′-Cy3-RNA). Deadenylase reaction: 8 μL substrate (5′-Cy3-RNA), the calculated volume for 1.6 μM final concentration and bring to final volume (20 μL) with Master Mix.
2. Incubate the reactions at the appropriate conditions for the enzyme tested, as different enzymes have slightly different optimal conditions. For instance, for the AtHESPERIN deadenylase, the optimal reaction conditions are 25 °C for 1 h [4].

3. Stop the reaction by adding 20 μL of 2× RNA sample buffer and heat at 85 °C for 3 min.

3.2 Electrophoresis

1. Cast the gel by mixing 10 mL of the 20% acrylamide / 8 M urea mix with 10 μL of TEMED and 50 μL of 10% APS. Mix thoroughly and avoid the formation of bubbles. Pour in the glass plates adding the comb for the required number of wells (*see* **Notes 13** and **14**), taking care to not introduce air bubbles between the gel and the comb.
2. Once the gel is set, carefully remove it from the caster and place it in the electrophoresis chamber. Fill the inner and outer chambers of the electrophoresis tank with 1× TBE buffer (*see* **Note 15**).
3. Carefully flush each well of the gel with 1× TBE buffer to remove any urea that will prevent the samples from running smoothly in the gel (*see* **Note 16**).
4. Pre-run the gel at 200 V for 30 min.
5. After the pre-run is done, repeat the flushing of the wells with 1× TBE buffer and then load your samples.
6. Run the gel at 200 V for 30–40 min or until the dye front has reached the bottom of the gel.

3.3 Image Analysis

1. After the gel run is finished, remove the gel from the glass plates and place it in a container with 1× TBE to avoid the gel drying out.
2. Take a picture of the gel using the appropriate settings of a gel documentation system.
3. Analyze the images using the gel documentation system software or the ImageJ software [8] by standard densitometric analysis (*see* **Note 17**).

4 Notes

1. DEPC inactivates RNases by covalently modifying histidine, lysine, cysteine, and tyrosine residues [9].
2. Mix thoroughly and leave overnight before autoclaving.
3. %C refers to the percentage of crosslinker (bis-acrylamide) present in the acrylamide mix and it is equal to [grams crosslinker/(grams crosslinker + grams acrylamide)] × 100. %C determines the diameter of the pores in the gel and is required for better electrophoretic separation. This percentage does not affect pore size; the resulting gel using the composition described here is 20% and leads to a gel with very small pores that can resolve smaller RNA fragments. Keep in mind to

handle the gel gently, as a gel with high %C is inflexible and therefore easy to break [10, 11].

4. Acrylamide is a neurotoxin and a possible carcinogen and can be unsafe to users if not handled properly [12].
5. Protect from light while dissolving. The large amount of urea may take a considerable time to dissolve. A heated water bath with intermittent agitation or mixing can greatly decrease the required time.
6. Depending on the range of products to be detected (17 nucleotides and shorter, in this case) and the sensitivity of the gel documentation system, it is optional but recommended to add dyes to the sample buffer. Typical amounts are 0.025% of xylene cyanol and 0.025% of bromophenol blue. If the dyes interfere with detection, do not include them in the sample buffer. Rather, load an adjacent well with only the dye-containing sample buffer so that electrophoresis can be monitored using the dye front.
7. Different enzymes require specific buffers for optimal activity, so special care should be taken to identify the optimal reaction conditions for each deadenylase to be studied. Here, the reaction buffer for AtHESPERIN is described.
8. The substrate sequence design was based on the substrate described by Maryati et al. [5]. The choice of fluorophore was based on the capabilities of the gel documentation system available when the assay was designed. Therefore, Cy3 can be substituted with another fluorophore, as long as it is placed at the 5′ end of the RNA molecule so that detection is not compromised.
9. The deadenylase described here is AtHESPERIN and was produced in-house from recombinant bacteria. In the first report of AtHESPERIN [13] the effect of different reaction buffer compositions was tested, and later [4] optimized purification conditions and reaction buffer were reported for this enzyme. The reader is encouraged to search for reports regarding the deadenylase they're studying for purification methods and reaction conditions, as to expand here would be out of the scope of this protocol.
10. To assess enzymatic activity, all active sites of the enzyme should be saturated by the substrate [14]. Therefore, the substrate is typically in molecular excess by at least fivefold.
11. The amount of substrate was determined based on the detection sensitivity of the gel documentation system used. It is useful to run a range of concentrations of substrate to determine the minimum amount required for reliable and reproducible detection.

12. Reactions can be set up so that different aspects of enzyme activity can be tested; either a number of reaction tubes reflecting the number of different time points to be assayed or reflecting the presence of some modulator of enzymatic activity. Here, only the basic set up is described. For a set up including modulators of activity, refer to [4].
13. The polymerization of the gel is an exothermic reaction. Therefore, the temperature of the solution increases as the gel polymerizes, which increases the speed of the polymerization reaction [10, 11].
14. Do not perform the gel polymerization step in a fridge or cold room, as it may affect the polymerization of acrylamide chains and negatively affect the reproducibility of electrophoresis. Allow the gel temperature to reach room temperature after polymerization before running [10, 11].
15. Prepare the 1× TBE buffer by adding 100 mL of 10× TBE buffer in 900 mL DEPC-treated water in an RNase-free bottle and mix thoroughly.
16. A 5 or 10 mL syringe and a 21-gauge hypodermic needle can be used to flush the wells, taking care not to prick through the wells.
17. The intensity analysis performed depends on user preference. Additionally, there are resources on use of ImageJ for analysis (for example, see [15, 16]).

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Part III

Methods for the Study of Decapping and Exoribonuclease Activity

Chapter 6

Studying Exoribonuclease Activity Using Fluorescence Anisotropy Assay

Krzysztof Kuś and Lidia Vasiljeva

Abstract

Fluorescence anisotropy is a powerful technique, widely used for investigating ligand-macromolecule binding and high-throughput screens for drugs. Here, we employ fluorescence anisotropy to quantitatively study the activity of exoribonucleases exemplified by the Xrn2 enzyme. Recording changes in the fluorescence anisotropy over time allows real-time detection of enzymatic activity and provides a framework that can be tailored to particular questions. We discuss the experimental setup, the potential substrate RNAs and highlight data analysis. We envision that this assay can be applied to study other nucleic acid-degrading enzymes and further expanded to include competition and inhibitor screens.

Key words Fluorescence anisotropy assay, FA assay, Fluorescence polarization, Exoribonuclease, Xrn2

1 Introduction

Exoribonucleases belong to a class of enzymes involved in the degradation and processing of RNA molecules and can be classified as 5′→3′ or 3′→5′ enzymes [1]. Xrn2 (Rat1 and Dhp1 in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe, respectively*) is a highly conserved, processive 5′→3′-exoribonuclease involved in many aspects of RNA metabolism including termination of RNA polymerase II (Pol II) transcription and rRNA processing [2–4]. Many different assays have been developed to study exoribonuclease activity in vitro. Denaturing gel electrophoresis (UREA-PAGE) is frequently used to separate degradation products, and the extent of degradation can be visualized either using fluorescent, radioactive probes on RNA or dyes interacting with RNA. It is possible to perform time-course experiments, but the quantification of gels is rather cumbersome. Other techniques include real-time fluorescence detection, which takes advantage of fluorescence resonance energy transfer (FRET) to monitor the enzymatic activity. A fluorescent RNA substrate is matched with a DNA quencher

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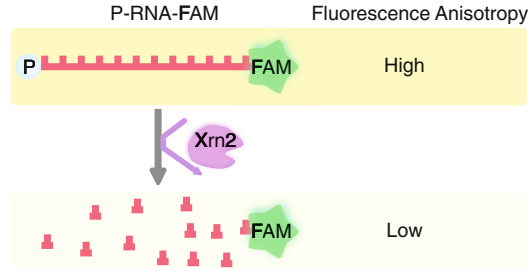

Fig. 1 Schematic principle of fluorescence anisotropy (FA) degradation assay. An RNA molecule with a 3′-FAM fluorophore exhibits high fluorescence anisotropy. When 5′-monophosphate RNA is degraded by 5′→3′-exoribonuclease (Xrn2), FAM is released leading to low FA signal

and an increase of the fluorescence upon RNA degradation is measured [5]. The changes in the signal can be captured in real-time although this setup requires two fluorescent probes and might not be suitable for enzymes that cannot process DNA–RNA hybrids. Here, we report a simple, fluorescence-based approach to study exoribonucleolytic activity by fluorescence anisotropy (FA). FA is widely employed as a biophysical approach allowing in-solution characterization of binding affinities, small molecule screening and studies of enzyme kinetics [6–9]. FA is based on a physical phenomenon in which fluorophore is excited with linearly polarized light and emitted fluorescence is measured at two perpendicular polarization angles. Small fluorescent molecules tumble quickly, losing preferential polarization, which results in low anisotropy. This is in contrast when a small molecule is bound to a macromolecule and impacts the ability to rotate leading to anisotropy increase [10, 11]. Additional applications of fluorescence anisotropy include enzymatic assays, with this technique previously employed in investigating proteasomal protein degradation, amyloid beta-protein cleavage, and ribonuclease P activity [12–14]. In this setup, a fluorescently labelled substrate is incubated with an enzyme that cleaves it into smaller fragments. Therefore, intact substrates have high polarization values, which decrease over time upon incubation with the enzyme (Fig. 1). Here, we describe the protocol that highlights the utilization of FA assay to study real-time RNA degradation kinetics of 5′-monophosphate, 3′--fluorescently labelled substrate catalyzed by Xrn2 enzyme. The description of the method, protein purification, its potential modifications, and data analysis are discussed.

2 Materials

All solutions are prepared with Milli-Q water (ultrapure water with resistivity >18.2 MΩ·cm) and analytical-grade chemicals.

2.1 Protein Preparation

This assay requires purified 5′→3′-exoribonuclease. In this chapter, Xrn2 involved in the termination of Pol II transcription is evaluated. Xrn2 is a processive exonuclease that degrades single-stranded 5′ monophosphorylated RNA.

1. Plasmid containing Xrn2-Strep in pRSFDuet plasmid (kanamycin resistance). C-terminally truncated *S. pombe* Xrn2 construct encompasses residues 1-885 and is C-terminally fused with twin Strep-tag (*see* **Note 1**).
2. 2× TY broth: Tryptone 16 g/L, Yeast extract 10 g/L, NaCl 5 g/L (autoclaved, pH 7 ± 0.2).
3. *E. coli* expression strain (i.e., Rosetta—chloramphenicol resistant).
4. Antibiotics: 40 mg/mL kanamycin and 25 mg/mL chloramphenicol (1000× concentrate).
5. Shaking incubator with temperature control.
6. 1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG).
7. French press (cell disruptor).
8. EDTA-free protease inhibitors cocktail.
9. 200 mM Phenylmethylsulfonyl fluoride (PMSF in ethanol).
10. SuperNuclease (50 U/μL) or Benzonase.
11. Fast protein liquid chromatography (FPLC) system (i.e., AKTA).
12. High-speed refrigerated and tabletop refrigerated centrifuges.
13. Bottle-top filtration system and 0.22 μm filters.
14. SB buffer: 25 mM HEPES-NaOH pH 7.9, 500 mM NaCl, 1 mM $MgCl_2$ and 1 mM β-mercaptoethanol. Keep at 4 °C.
15. Desthiobiotin.
16. Centrifugal concentration filter devices with 50 kDa cut-off.
17. StrepTrap HP or XT prepacked chromatography column.
18. Optional: QA buffer: 50 mM Tris-HCl pH 7.5, 30 mM NaCl, 10% glycerol, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol. Keep at 4 °C.
19. Optional: QB buffer: 50 mM Tris-HCl pH 7.5, 1000 mM NaCl, 10% glycerol, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol. Keep at 4 °C.
20. Optional: HiTrap Q High Performance 5 mL or equivalent ion exchange column.
21. Superdex 200 HiLoad 16/600 or Superdex 200 10/300 GL or equivalent gel filtration column.
22. Liquid nitrogen.
23. SDS-PAGE gels, running buffer, protein ladder, loading dye.

2.2 RNA Substrate

Xrn2 is a 5′→3′ exoribonuclease and therefore a fluorescent probe is attached to the 3′-end of the RNA substrate.

1. Design your RNA substrate including 5′-phosphorylation and 3′-end fluorescent label (*see* **Note 2**). RNA with 5′--monophosphate group and fluorescein amidite (FAM) attached to the 3′-end (P-RNA-FAM): 5′P- UGAAU CUAUUUCUUUUAUCGAGAGGU -3′-FAM is commercially synthesized with standard desalting.
2. A tube with a pellet containing P-RNA-FAM is briefly centrifuged in a tabletop centrifuge to collect all the material.
3. Resuspend the RNA in ultrapure water (500 μM). Aliquot and store at −20 °C.

2.3 Fluorescence Anisotropy

1. An instrument with the capacity to measure fluorescence anisotropy (i.e., PHERAstar FS plate reader, BMG Labtech).
2. The fluorescence anisotropy instrument compatible measurement container. Here, black 96-well half-area microplates (Corning, *see* **Note 3**).
3. Assay buffer (AB buffer): 10 mM HEPES-NaOH pH 7.9, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol.

3 Methods

Procedures are conducted at room temperature except for protein purification, which is performed at ~4 °C (with ice-cold buffers). Solutions used for FPLC are filtered using a 0.22 μm filter bottle-top device.

3.1 Protein Purification

1. Transform 50 μL of chemically competent Rosetta *E. coli* with 100 ng of Xrn2-Strep containing plasmid following standard protocol. After recovery, inoculate 50 mL of 2× TY broth supplemented with appropriate antibiotics (40 μg/mL kanamycin, 25 μg/mL chloramphenicol) with 1/5 of the transformation mixture. Grow overnight at 37 °C with 200 rpm shaking. The next day, the transformed bacteria are inoculated into 2–4 L of 2× TY broth with antibiotics (5 mL of culture per 1 L of fresh media). Grow cells at 37 °C with shaking (200 rpm) until OD_{600} reaches 0.8–1. Move the cells to a 20 °C incubator and induce with 0.3 mM IPTG. Continue the growth at 20 °C, shaking for 16–18 h. Harvest the cells by centrifugation (5000× *g* for 10 min at 4 °C). The cell pellet can be frozen and stored at −80 °C before purification.
2. Resuspend the cell pellet in a beaker with ice-cold SB buffer supplemented with protease inhibitors and 50 U/mL

SuperNuclease (5 mL of buffer per 1 g of wet *E. coli* mass). Place the beaker with a stirrer in a cold room on a magnetic plate mixer and allow the cell pellet to fully dissolve (~20 min).

3. Precool the centrifuge and the French press (cell disruptor). To equilibrate the French press, pass 100 mL of cold SB buffer.
4. Lyse the cells using the French press at 20 kpsi (one pass is sufficient). After the cells are disrupted, add drop-by-drop PMSF, mixing with the lysate (to reach a final concentration of 1 mM) (*see* **Note 4**).
5. Transfer the lysate to centrifuge tubes and spin-down at 40,000× *g* for 20 min at 4 °C.
6. Filter the pre-cleared lysate through a 0.22 μm filter (bottle-top filtration system)—use a prechilled bottle.
7. Apply the filtrate to a StrepTrap HP column equilibrated with cold SB buffer using FPLC at 4 °C (*see* **Note 5**).
8. Wash the column with 50 mL of SB buffer and elute the protein with 50 mL of SB containing 5 mM desthiobiotin.
9. An optional step includes purification using ion exchange chromatography. The fractions containing the protein are diluted with a QA buffer (to reach under 100 mM NaCl—1:10 dilution is sufficient). Apply this sample to the ion exchange column (HiTrap Q or equivalent equilibrated with QA buffer), and wash with at least 4 column volumes of QA buffer. Elute with a gradient from 0–50% of QB using 16 column volumes (*see* **Note 6**).
10. Pool the fractions containing the protein and concentrate to 500–1000 μL using a 50 kDa centrifugal concentrator. After concentration, transfer the sample to the microcentrifuge tube and spin at 10,000× *g*, 4 °C for 10 min to remove any potential aggregates. Subject this sample to gel filtration (Superdex 200 HiLoad 16/600). Analyze protein fractions on SDS-PAGE. The protein is expected to elute with a peak at ~71 mL and should migrate above 100 kDa on SDS-PAGE gel (Fig. 2).
11. Measure the concentration of the purified exonuclease, i.e., reading absorbance at 280 nm and applying Beer-Lambert law: $c = \frac{A}{\varepsilon \cdot l}$, where c is the concentration (M), A-absorbance at 280 nm, l-path length (cm), ε-extinction coefficient ($\frac{1}{M \cdot cm}$).
12. Aliquot proteins to microcentrifuge tubes and snap-freeze on liquid nitrogen. Store the aliquots at −80 °C.

3.2 Fluorescence Anisotropy Assay

1. Turn on the fluorescence anisotropy instrument in advance to allow the light source to warm up.

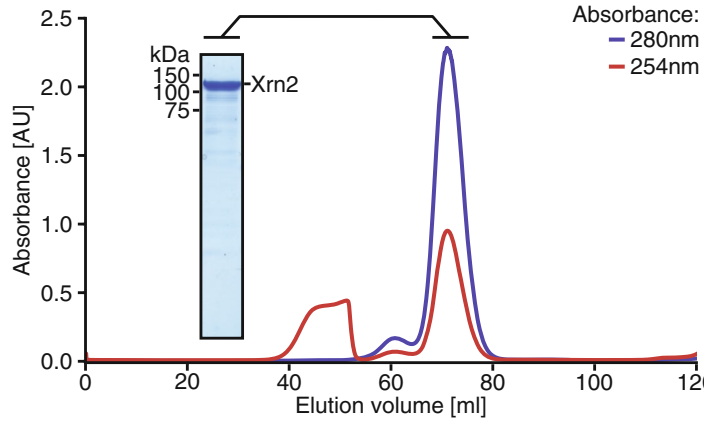

Fig. 2 Xrn2 gel filtration chromatogram. Separation is performed on the Superdex 200 HiLoad 16/600 column. Absorbance at 280 and 254 nm is included (blue and red trace, respectively). The inset depicts Coomassie-stained SDS-PAGE gel for combined fractions of Xrn2

2. Set up a protocol that will allow performing time-course measurements in the software controlling fluorescence anisotropy device. This protocol uses PHERAstar software. The "Test Setup" tab allows the user to define a new protocol ("Test Protocols"). Create "New Protocol for Fluorescence polarisation" with "Reading Mode" set to "Plate mode (slow kinetics)." Choose the appropriate plate from the list and add the name of the protocol. In "Kinetic window 1" specify the number of cycles (i.e., 20) and cycle time (60 s to obtain measurement every minute). In the "Optic" section select "Top optic" and verify that "Optic settings" are appropriate for your fluorophore (for FAM suitable selection is FP 485 520 520) (*see* **Note 7**). In the panel "Concentrations/Volumes/Shaking" adjust shaking to "double orbital" with 700 rpm and select "Before the first cycle" to facilitate better mixing. In the "Layout" section select cells/wells which will be measured (selecting a full plate would not be possible with 60 s cycle time). Before the first experiment, click "Start measurement" and in the next window change the "Gain Adjustment Target mP" to 120.
3. Dilute the RNA to 250 nM with AB buffer (serial dilution would be required from 500 μM P-RNA-FAM stock). The total volume will depend on the number of reactions, and 50 μL is required per well (here three reactions are prepared for degradation and three for negative control/mock—in total 350 μL with 50 μL excess).
4. Dilute Xrn2-Strep (20 μM) 350 times (2 μL of 2.1 mg/mL protein diluted with 698 μL of AB buffer) (*see* **Note 8**).

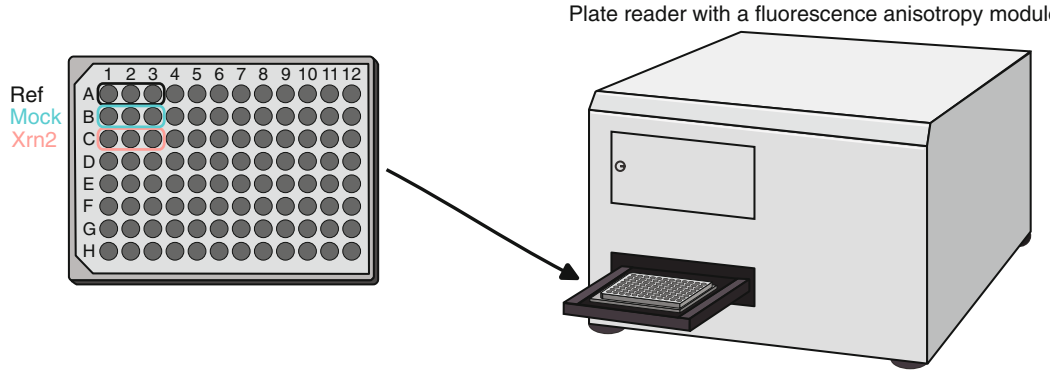

Fig. 3 Half-area 96-well plate with an exemplary setup that will be measured in a plate reader with a fluorescence anisotropy module

5. Pipet 50 μL of AB buffer to three wells—this will serve as a reference (Ref) for the fluorescence signal (Fig. 3, *see* **Note 9**).
6. Pipet 45 μL of diluted RNA to six wells (three will serve as a negative control—mock and three for degradation assay) (Fig. 3).
7. The plate is ready for the experiment. Open the protocol window and verify if the correct wells are selected that are meant to be measured. Eject the tray for the plate (Fig. 3).
8. First, add 5 μL of AB buffer to each of the mock wells and quickly stir with a pipet tip.
9. Now, quickly add 5 μL of diluted Xrn2 to three wells and also mix with a pipet tip.
10. Immediately, insert the plate into the device and click "Start Measurement." On the following window select well with RNA only (mock) and click "Start adjustment" (*see* **Note 10**).
11. Click "Start Measurement" once more (*see* **Note 11**).
12. Wait for the program to finish. Open the analysis software to export fluorescence anisotropy values. We use the PHERAstar MARS software to export values of fluorescence anisotropy and raw data to verify fluorescence intensities.

3.3 Data Analysis of Expected Outcomes and Potential Assay Extensions

1. Exported Excel files can be analyzed in any appropriate fitting/graphing software.
2. Plot the data and verify that fluorescence anisotropy is not changing over time if exoribonuclease is absent. The addition of the exoribonuclease should result in the decay of the signal over time (Fig. 4). FA signal decay can be fitted with a nonlinear single-exponent function: $FA \sim (A - B) \cdot e^{-k \cdot t} + B$—where A and B are constants (related to max and min values of FA), k and t are rate constant and time, respectively. To obtain the

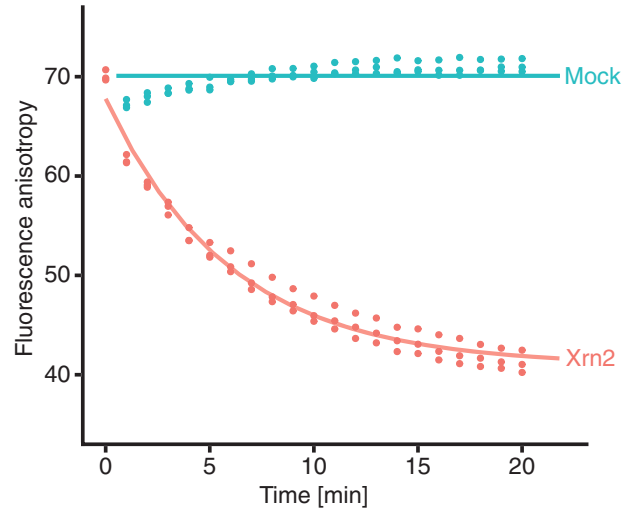

Fig. 4 Anticipated results—addition of Xrn2 results in anisotropy decay. Experimental data can be fitted with a one-phase exponential decay that provides a half-life of the signal (solid line, time point 0 is an average value for signal without Xrn2). Mock (negative control)—refers to wells where the buffer was added instead of Xrn2 and FA is stable over the measurement window

half-life ($\tau_{1/2}$) of the FA signal calculate: $\tau_{1/2} = \frac{\ln(2)}{k}$. In the experiment presented in (Fig. 4), the half-life is approximately 4 min ($\tau_{1/2} \approx 4$ min).

3. Potential extensions of this assay include testing of additional factors/small molecules to study their impact on exoribonucleases. If extra components are evaluated, it would be important to include a control with RNA/factor mix to ascertain that there is no additional external ribonucleolytic activity. We described only one possibility of how to initiate the degradation reaction. Xrn2 requires metal ions for its activity. Xrn2 can be prepared with EDTA, which makes it reversibly inactive. Exoribonuclease activity can be fully restored with the addition of Mg^{2+} ions.

4 Notes

1. Expression of full-length, fission yeast Xrn2 protein in *E. coli* has been challenging with severe proteolysis observed and therefore low protein yield. Xrn2 construct encompassing 1-885 amino acids is expressed at higher yield and affinity purified using C-terminal Strep-tag (described in this protocol) or 8×His-tag [15, 16].

2. Fluorescent labels can be chosen based on the available instrumentation. Additionally, experiments with a substrate that lacks a 5′-phosphate group might be performed to test given exoribonuclease preference for this moiety RNA could contain secondary structure motifs to evaluate its impact on nucleolytic activity. When 3′→5′-exoribonuclease is tested, the fluorescent label would be at the 5′-end of the RNA molecule.
3. Some plates exhibit high autofluorescence or are designed for high protein binding capacity and are not suitable for measurements. Follow instrument recommendations regarding plate/container selection.
4. PMSF is added to the extract immediately after passage through the French press.
5. StrepTrap HP was replaced by StrepTrap XT. Purification can be performed with other compatible resins and might be executed in batch mode.
6. Purification by ion exchange is optional as affinity purification and gel filtration result in satisfactory protein preparation. Nevertheless, this step allows the cleanup of residual degradation products of Xrn2. Protein starts eluting from the column ~20% QB buffer using the HiTrap Q HP column.
7. Additional parameters include: "Focal height" adjusted to 5.7 mm, "Positioning delay": 0.3 s, "No of flashes per well and cycle": 300.
8. Too high concentration of enzyme results in complete RNA degradation and only low fluorescence anisotropy would be observed. Therefore, depending on the enzyme activity, optimization of exoribonuclease concentrations might be required, with different enzyme dilutions assessed with this assay.
9. Reference (Ref) can also have fluorescence anisotropy, but fluorescence intensity values should be only a fraction of fluorescence intensity from P-RNA-FAM wells. This will validate that fluorescence is measured.
10. Manual injection of the enzyme into the well might be a source of variability. On PHERAstar instrument adjustments take ~30 s and shaking 20 s. Consequently, the first time point is typically measured after around 60 s if executed swiftly. Having an instrument equipped with an automated pump to inject Xrn2 or RNA solution would decrease variability concerns.
11. Make a note of the temperature (here experiments were performed at 23 ± 0.5 °C). If your instrument allows temperature control, different temperatures can be tested.

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Chapter 7

Purification of Enzymatically Active Xrn1 for Removal of Non-capped mRNAs from In Vitro Transcription Reactions and Evaluation of mRNA Decapping Status In Vivo

Karolina Drążkowska, Rafał Tomecki, and Agnieszka Tudek

Abstract

The cap is a 7-methylguanosine attached to the first messenger RNA (mRNA) nucleotide with a 5′-5′ triphosphate bridge. This conserved eukaryotic modification confers stability to the transcripts and is essential for translation initiation. The specific mechanisms that govern transcript cytoplasmic longevity and translatability were always of substantial interest. Multiple works aimed at modeling mRNA decay mechanisms, including the onset of decapping, which is the rate-limiting step of mRNA decay. Additionally, with the recent advances in RNA-based vaccines, the importance of efficient synthesis of fully functional mRNAs has increased. Non-capped mRNAs arising during in vitro transcription are highly immunogenic, and multiple approaches were developed to reduce their levels. Efficient and low-cost methods for elimination of non-capped mRNAs in vitro are therefore essential to basic sciences and to pharmaceutical applications. Here, we present a protocol for heterologous expression and purification of catalytically active recombinant Xrn1 from *Thermothelomyces* (*Myceliophthora*) *thermophilus* (Tt_Xrn1). We also describe protocols needed to verify the enzyme quality.

Key words Xrn1, Heterologous expression, Recombinant protein, mRNA cap, Decapping, In vitro degradation of non-capped mRNAs, mRNA vaccines, Direct RNA sequencing

1 Introduction

Xrn1 is a conserved 5′-3′ exonuclease that is essential to cytoplasmic mRNA decay in Eukaryotes [1]. Extremities of protein-coding transcripts emerging from the nucleus are modified co-transcriptionally with a 5′ cap and 3′ polyadenosine tail (pA-tail) to protect the mRNA from immediate exoribonucleolytic decay in both the nuclear and cytoplasmic compartments. In the cytoplasm the cap is bound by cap-binding proteins, which are required for translation initiation. The pA-tail is coated with

Karolina Drążkowska and Rafał Tomecki contributed equally.

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polyA-binding protein (Pab1 in yeast and PABPC1 in human) that is believed to stimulate both translation and gradual pA-tail deadenylation. The widely accepted model postulates that a substantial pA-tail shortening, mediated by Ccr4-Not and Pan2/3 deadenylases, induces mRNA decapping by Dcp1/2 complex. This in turn enables Xrn1 to initiate the transcript degradation from its 5′-end [2]. Consequently, Xrn1 action terminates the life cycle of a functional mRNA, as opposed to other decay mechanisms, that operate from the 3′-end on aberrant mRNAs, which mostly display erroneous translation [3]. Since cap removal is the rate-limiting step of mRNA decay, identification of the onset of decapping in vivo is important for thorough characterization of mRNA decay dynamics.

The mRNA 7-methylguanosine cap is a 5′-end modification specific to Eukaryotes, which occurs in three most common forms: cap-0, cap-1, and cap-2 [4]. The cap-0 consists of an *N*7-methylguanosine attached to the first transcribed nucleotide with a 5′-5′ triphosphate bridge [m^7G(5′)ppp(5′)N]. This form is sufficient to protect the RNA from Xrn1-mediated degradation [5] and is the only cap structure present in yeast. In higher Eukaryotes the mRNA cap is further modified by methylation of the 2′-OH group of the first, or both the first and second, ribose of the 5′-terminal nucleotides. These modifications generate the cap-1 [m^7G(5′)ppp(5′)N1$_m$pN2p] or cap-2 [m^7G(5′)ppp(5′)N1$_m$pN2$_m$p] structures, respectively. Regardless of the species, Xrn1 enzymes are only active towards monophosphorylated RNA 5′-ends [5] that emerge from the decapping reaction mediated by Dcp1/2 [6]. Cap-1 and cap-2 are particularly important to higher Eukaryotes, since they mark endogenous mRNA as self to the innate immune system [7]. Transcripts with other 5′-ends (e.g. triphosphate or cap-0) are in turn sensed as nonself, resembling foreign (mostly viral) RNA molecules, and are thus highly immunogenic. Such exogenous 5′-ends are recognized by IFIT1-5 or RIG-I receptors, which induce signaling cascades exerting an inflammatory response [4, 5].

mRNA vaccines are synthesized by in vitro transcription (IVT), which yields transcripts bearing exclusively 5′-triphosphates if nonmodified nucleotides are used for the reaction setup. Including molar excess of cap analogs for IVT reaction results in the synthesis of capped RNA fraction. Alternatively, in vitro transcribed 5′--triphosphorylated mRNA can be capped enzymatically following IVT, using *Vaccinia* capping enzyme and cap 2′-*O*-methyltransferases [8–10]. The efficiency of mRNA capping depends on the method, protocol, and cap analog selected for the particular reaction. Notably, even trace amounts of RNA bearing 5′-terminal triphosphates, which are synthesized as by-products, irrespective of the capping method employed, are likely to induce undesirable immunological responses and should be thus removed from the vaccine preparation. Some of the methods used to attach the cap to

the mRNA also provide means for removal of non-capped mRNAs [8, 10, 11]. Among procedures to reduce the fraction of uncapped mRNAs in vitro, a routinely utilized approach involves conversion of the 5′-terminal triphosphate to monophosphate by the use of the 5′-polyphosphatase, followed by Xrn1-mediated digestion of the mRNA body [7, 12].

Utilizing commercially available Xrn1 enzyme to deal with large amounts of in vitro produced mRNA is cost-ineffective. In this chapter, we present a protocol for efficient in-house purification of Xrn1 enzyme from *Thermothelomyces thermophilus* (also known as *Myceliophthora thermophila*) filamentous fungus (Tt_Xrn1) and provide simple protocols to evaluate its enzymatic activity on RNA oligonucleotides, on in vitro synthesized mRNAs with variable 5′-end status and on RNA preparations from living organisms. Those enzymatic tests also sketch some of the possible Xrn1 applications. The first aims at eliminating the uncapped transcripts' fraction from the pool of in vitro transcribed mRNAs. The second application relates to transcriptomics performed using Direct RNA Sequencing [13] and utilizes Tt_Xrn1 to evaluate the pA-tail length status of uncapped mRNAs in budding yeast in vivo.

2 Materials

2.1 Expression of Recombinant Tt_Xrn1

1. pET-28-Tt_Xrn1-6xHis (*see* **Note 1**).
2. *Escherichia coli* BL21-CodonPlus(DE3)-RIL chemo-competent cells.
3. Luria-Broth (LB)-agar plates containing 50 μg/mL kanamycin.
4. Liquid LB medium.
5. Auto Induction Medium (AIM) Super Broth Base including Trace elements (Formedium).
6. Glycerol stock.
7. Kanamycin stock solution (1000×): 50 mg/mL, in water.
8. Chloramphenicol stock solution (1000×): 34 mg/mL, in 70% ethanol.
9. Sterile inoculation loop.
10. Orbital incubation shaker.
11. Sorvall RC3-series centrifuge with H6000A/HBB6 swinging-bucket rotor.
12. 1000 mL plastic bottles for centrifugation.
13. 10–12% SDS-PAGE gel for protein analysis (home-made or commercial).

14. Laemmli buffer (3×): 187.5 mM Tris-HCl pH 6.8, 6% (w/v) SDS, 30% (v/v) glycerol, 7.5% (v/v) 2-mercaptoethanol, 0.03% (w/v) bromophenol blue.
15. Coomassie stain: 0.025% Coomassie Brilliant Blue R-250, 7.5% acetic acid, 40% methanol.
16. SDS-PAGE gel destaining solution: 10% ethanol, 4% acetic acid.

2.2 Cell Lysis

1. Cell lysis buffer: 20 mM Tris-HCl pH 7.4, 200 mM NaCl, 10 mM imidazole, 10 mM 2-mercaptoethanol, and protease inhibitors (added freshly: 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.02 μM pepstatinA, 0.02 μg/mL chymostatin, 0.006 μM leupeptin, 20 μM benzamidine hydrochloride).
2. Lysozyme stock solution (1000×): 50 mg/mL in water.
3. EmulsiFlex-C3 High Pressure homogenizer (Avestin).
4. Rotating wheel.
5. Sorvall WX ULTRA SERIES ultracentrifuge with F37L rotor and appropriate tubes.
6. Glassware (bottle/flask).
7. Vortex.

2.3 Chromatography-Based Purification of Recombinant Tt_Xrn1

1. ÄKTAxpress and ÄKTA Purifier FPLC systems (GE Healthcare) (*see* **Note 2**).
2. Ni-NTA column (*see* **Note 3**).
3. Affinity chromatography low-salt (AC-LS) buffer: 20 mM Tris-HCl pH 7.4, 200 mM NaCl, 10 mM imidazole, 10 mM 2-mercaptoethanol.
4. Affinity chromatography high-salt (AC-HS) buffer: 20 mM Tris-HCl pH 7.4, 1 M NaCl, 10 mM imidazole, 10 mM 2-mercaptoethanol).
5. Affinity chromatography buffer E: 20 mM Tris-HCl pH 7.4, 200 mM NaCl, 300 mM imidazole, 10 mM 2-mercaptoethanol.
6. 96-well 2 mL deep-well plates.
7. 15 mL and 50 mL Falcon tubes.
8. 10–12% SDS-PAGE gels for protein analysis (home-made or commercial).
9. Molecular weight marker for protein electrophoresis.
10. Coomassie stain: 0.025% Coomassie Brilliant Blue R-250, 7.5% acetic acid, 40% methanol.
11. SDS-PAGE gel destaining solution: 10% ethanol, 4% acetic acid.

12. Superdex 200 Increase 10/300 GL size-exclusion chromatography (SEC) column (Cytiva; *see* **Note 4**).
13. SEC gel-filtration (GF) buffer: 10 mM Tris-HCl pH 7.4, 150 mM NaCl.
14. Resource Q 1 mL anion exchanger column (GE Healthcare) for ion-exchange chromatography (IEX; *see* **Note 5**).
15. IEX-LS buffer: 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM DTT.
16. IEX-HS buffer: 10 mM Tris-HCl pH 7.4, 1 M NaCl, 1 mM DTT.
17. Sterile molecular biology-grade glycerol.
18. Liquid nitrogen.
19. Commercial yeast Xrn1 (New England Biolabs).

2.4 Enzymatic Xrn1 Quality Check

1. 1.5 mL microcentrifuge tubes.
2. Thermoblock.
3. Three synthetic RNA oligonucleotides: ACUCACUCACU CACCAAAAAAAAAAAAACC (*see* **Note 6**), containing at the 5′-terminus either monophosphate (5′-P), hydroxyl group (5′-OH), or methylated cap-0 (5′-m^7Gppp) and labeled with fluorescein amidite (FAM) at the 3′-end (*see* **Note 7**). Substrates should be purified by RNase-free dual HPLC and polyacrylamide gel electrophoresis (PAGE). Order 3 nmoles of each of these substrates in an RNA oligonucleotide synthesis company of choice and suspend them in 15 μL of sterile RNase-free water, so that the final concentration of the stocks is 200 μM.
4. Sterile RNase-free water.
5. Commercial yeast Xrn1 with 10× NEBuffer 3 (New England Biolabs).
6. RiboLock™ RNase Inhibitor (40 U/μL) or equivalent.
7. F-dye (formamide loading dye): 90% deionized formamide in 1× TBE buffer, 0.03% xylene cyanol, 0.03% bromophenol blue, 20 mM EDTA.
8. Liquid nitrogen.
9. 40% polyacrylamide stock: acrylamide:N,N′-methylene-bisacrylamide 19:1 (w/w), in water.
10. TBE buffer (10×): 890 mM Tris-borate pH 8.3, 10 mM EDTA.
11. 20% denaturing polyacrylamide stock with 7 M urea in 1× TBE (*see* **Note 8**).
12. 10% (w/v) ammonium persulfate (APS).

13. N,N,N′,N′-tetramethylethylenediamine (TEMED).
14. 20% denaturing polyacrylamide gel (*see* **Note 9**).
15. Vertical gel electrophoresis system with power supply.
16. Typhoon™ FLA-9500 biomolecular imager (GE Healthcare Life Sciences) or equivalent suitable for detection of FAM fluorescence.
17. Total RNA isolated from yeast (*Saccharomyces cerevisiae*), human (e.g. HEK293), or plant (*Arabidopsis thaliana*) cells (*see* **Notes 10** and **11**).
18. Agarose.
19. Ethidium bromide solution.
20. Horizontal gel electrophoresis system with power supply.
21. UV transilluminator coupled to the camera.
22. In vitro transcribed mRNA of interest. As controls for specificity of Xrn1 digestion we used 5′-monophosphorylated EGFP mRNA (*see* **Note 12**) or luciferase-encoding mRNAs either co-transcriptionally capped with cap-1 analog (e.g. CleanCap, Trilink; reviewed by [8–10]), or bearing 5′-triphosphate or 5′-OH ends (*see* **Note 13**).

3 Methods

3.1 Heterologous Expression of Recombinant Tt_Xrn1

See **Note 14**.

1. Transform an aliquot of chemo-competent *E. coli* BL21-CodonPlus(DE3)-RIL with 25–50 ng of pET-28-Tt_Xrn1-6xHis plasmid by chemo-transformation. Select transformants on plates with solid LB-agar supplemented with 50 μg/mL kanamycin.
2. Inoculate 50–100 mL of liquid LB medium supplemented with 50 μg/mL kanamycin and 34 μg/mL chloramphenicol with antibiotic-resistant clones scraped from the selection plate using a sterile inoculation loop.
3. Grow the pre-culture overnight at 37 °C with shaking at 120 rpm.
4. The next day, use 30 mL of the pre-culture to inoculate 1 L of liquid AIM medium Super Broth Base Including Trace elements, supplemented with 2% glycerol, 50 μg/mL kanamycin, and 34 μg/mL chloramphenicol.
5. Grow the autoinduction culture in an orbital shaker (at 150 rpm) for 72 h at 18 °C.
6. To evaluate Tt_Xrn1-6xHis expression, collect and spin down 100–150 μL of the culture after induction, lyse the bacterial

pellet in Laemmli buffer, and resolve the sample in a 10–12% SDS-PAGE gel. Stain the gel with Coomassie to visualize the cellular proteins. Tt_Xrn1-6xHis is usually visible as shown in Fig. 1a (*see* **Note 15**).

7. Harvest the bacterial cells by centrifugation at 5000 rpm in a Sorvall H6000A/HBB6 swinging-bucket rotor for 15 min at 4 °C (*see* **Note 16**).

3.2 Bacterial Cell Lysis

1. Resuspend a fresh or frozen bacterial pellet in the centrifuge bottle in 100 mL of ice-cold cell lysis buffer containing protease inhibitors (*see* **Note 17**). Add lysozyme at a final concentration of 50 μg/mL.
2. Incubate the suspension for 30 min in a cold cabinet (or cold-room) with head-over-tail rotation.
3. Disrupt the cells using the EmulsiFlex-C3 High Pressure homogenizer at 1500 Bar according to the manufacturer's instructions (*see* **Note 18**). Usually, 4–5 consecutive passes of the cell suspension through the homogenizer are sufficient to achieve satisfactory lysis.
4. Clear the cell lysate by centrifugation in a Sorvall WX ULTRA SERIES ultracentrifuge, using F37L fixed-angle rotor and dedicated centrifuge tubes (*see* **Note 19**) at 33'000 rpm for 45 min at 4 °C.
5. Transfer the supernatant (native protein extract) into a glass bottle or flask by pipetting (*see* **Note 20**) and keep on ice until purification.

3.3 Chromatography-Based Purification of Recombinant Tt_Xrn1

The Tt_Xrn1 purification protocol is based on three consecutive steps: (a) initial protein enrichment via affinity chromatography on a nickel-based resin (Fig. 1b,c), followed by removal of impurities and/or proteolytic degradation products through (b) size-exclusion (Fig. 1d) and (c) ion-exchange chromatography (Fig. 1e). This three-step approach, employing different purification techniques, yields a protein sample that is comparable in purity to commercial Xrn1 enzyme ('NEB_Xrn1'; Fig. 1f).

3.3.1 Enrichment of Tt_Xrn1-6xHis on the Nickel Resin

We recommend performing semi-automated nickel affinity chromatography using the ÄKTAxpress system (*see* **Note 2**), though a manual approach is also possible (*see* **Note 21**).

1. Equilibrate a 5 mL nickel-resin column compatible with the ÄKTAxpress (*see* **Note 3**) with 25 mL of low-salt (AC-LS) buffer at a flow rate of 2 mL/min.
2. Load the protein extract at a flow rate of 1 mL/min.
3. Wash the column with 40 mL of AC-LS buffer at a flow rate of 2 mL/min.

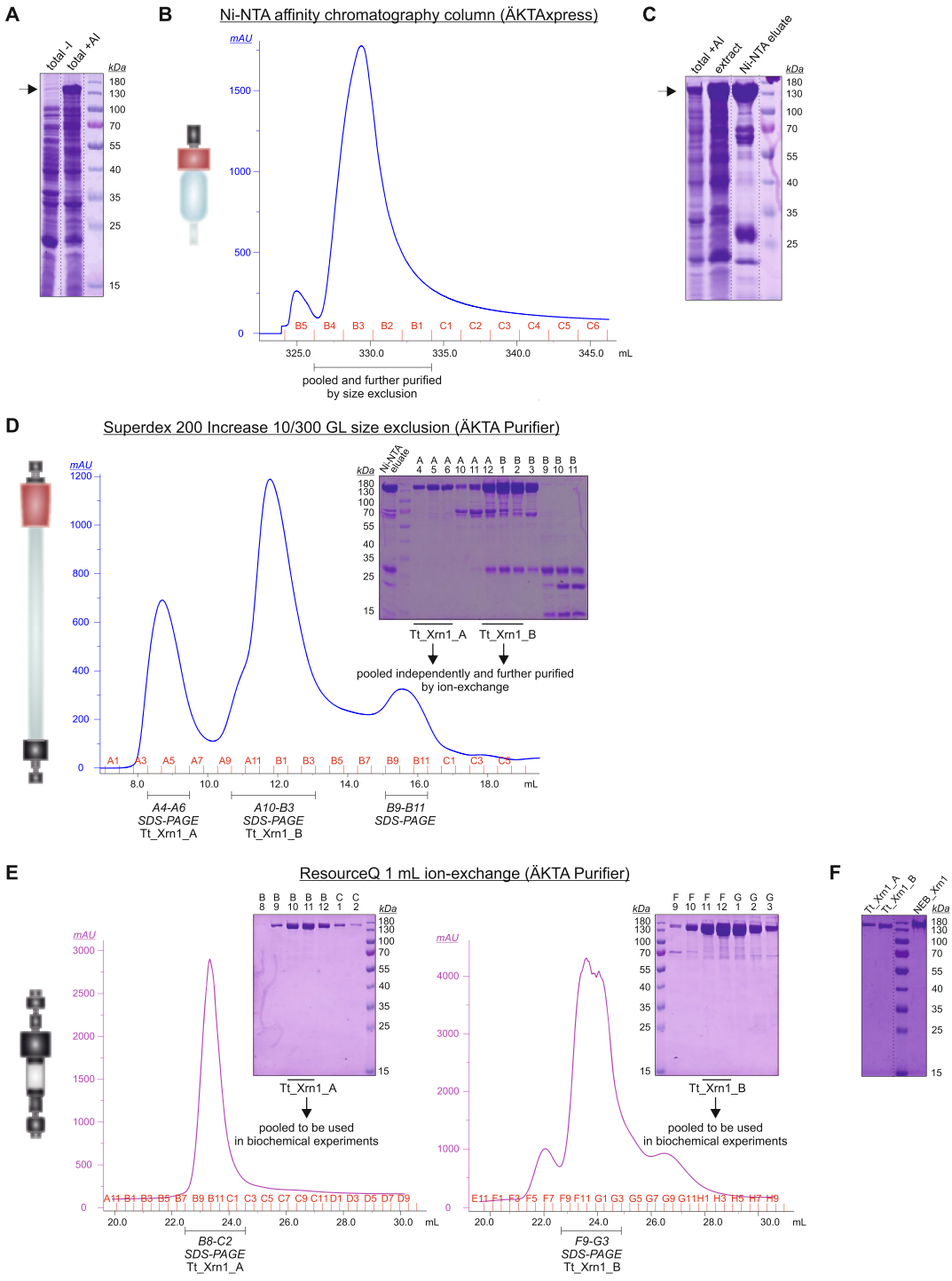

Fig. 1 Tt_Xrn1 heterologous expression and purification. (**a**) Comparison of Tt_Xrn1-6xHis (arrow) expression before (total -I) and after 48 hours (total + AI) of autoinduction. (**b**) Nickel-based affinity chromatography profile ($A_{280\ nm}$) of Tt_Xrn1-6xHis. Fractions subjected to subsequent purification step are indicated. (**c**) SDS-PAGE control of Tt_Xrn1-6xHis (arrow) solubility and enrichment after Ni-NTA chromatography. (**d**) Size exclusion chromatography profile ($A_{280\ nm}$) of Tt_Xrn1-6xHis with SDS-PAGE analysis of relevant fractions.

4. Wash the column with 25 mL of high-salt (AC-HS) buffer at a flow rate of 2 mL/min.
5. Wash the column with 20 mL of AC-LS buffer at a flow rate of 2 mL/min.
6. Elute the proteins bound to the Ni-NTA column by washing the column with 30 mL of buffer E at a flow rate of 1 mL/min.
7. Control the elution progress through constant monitoring of absorbance at UV wavelength 280 nm (Fig. 1b). Collect the eluate fractions (2 mL) into the wells of a 96-well 2 mL deep-well plate.
8. Analyze the eluate fractions by SDS-PAGE and Coomassie staining to evaluate the efficiency of Tt_Xrn1-6xHis purification (Fig. 1c).
9. Pool the fractions containing most of the recombinant protein, transfer them into a single 15 mL tube, and keep on ice until subsequent purification step.

3.3.2 Purification of Tt_Xrn1-6xHis Using Size-Exclusion Chromatography (SEC)

1. To further purify the protein sample obtained following affinity chromatography, load 1 mL of the eluate (*see* **Note 22**) from the nickel resin onto the Superdex 200 Increase 10/300 GL size-exclusion chromatography (SEC) column (*see* **Note 4**) using the ÄKTA Purifier 100 FPLC system with multi-wavelength UV-900 detector. Pre-equilibrate the column with gel-filtration (GF) buffer and perform SEC separation using 1.2 column volume of GF buffer.
2. Monitor the progress of SEC separation by constant measuring of absorbance at UV wavelength 280 nm (Fig. 1d).
3. Collect 0.4 mL fractions into the wells of a 96-well 2 mL deep-well plate.
4. Analyze the fractions corresponding to the absorbance peaks for the presence of Tt_Xrn1-6xHis by running them in 10–12% SDS-PAGE gel. Stain the gel with Coomassie (Fig. 1d) to pinpoint the fractions with the highest yield of Tt_Xrn1-6xHis (*see* **Note 23**).
5. Pool these fractions and keep on ice until subsequent purification step.

Fig. 1 (continued) Tt_Xrn1-6xHis was present in the first two peaks (Tt_Xrn1_A and Tt_Xrn1_B). (**e**) Ion-exchange chromatography profiles ($A_{215\ nm}$ for better protein detection) of the Tt_Xrn1_A (*left*) and Tt_Xrn1_B (*right*) along with SDS-PAGE analyses of relevant fractions. Indicated fractions containing the highest amounts of Tt_Xrn1_A and Tt_Xrn1_B were pooled and used in subsequent biochemical analyses. (**f**) Comparison of the quantity and purity of home-made Xrn1 to the commercial enzyme. 3 μL of Tt_Xrn1_A, 1 μL of Tt_Xrn1_B, and 10 μL of commercial Xrn1 (NEB_Xrn1) were analyzed by SDS-PAGE

3.3.3 Final Polishing of Tt_Xrn1-6xHis Protein Prep Using Ion-Exchange Chromatography (IEX)

1. Subject the Tt_Xrn1-6xHis-enriched pooled SEC fractions to ion-exchange chromatography on an anion exchanger Resource Q 1 mL column (*see* **Note 5**) pre-equilibrated with IEX LS buffer using the ÄKTA Purifier 100 FPLC system. A linear gradient of NaCl within a 150 mM-1 M range in Tris-HCl pH 7.4 and 1 mM DTT, achieved by gradual replacing of IEX-LS with IEX-HS buffer, should be utilized.
2. Monitor the progress of IEX separation by constant measuring of absorbance at UV wavelength of 215 nm (Fig. 1e).
3. Collect 0.3 mL fractions into the wells of a 96-well 2 mL deep-well plate and analyze those corresponding to the absorbance peaks for the presence of Tt_Xrn1-6xHis by running them in 10–12% SDS-PAGE gel (*see* **Note 23**). Stain the gel with Coomassie to identify the fractions with the highest yield of Tt_Xrn1-6xHis (Fig. 1e). Combine these fractions into the single tube and place on ice.

3.3.4 Evaluation of Tt_Xrn1-6xHis Purity and Recommended Long-Term Storage Conditions

1. Analyze the final Tt_Xrn1-6xHis preparation by 10–12% SDS-PAGE and Coomassie staining to evaluate the protein homogeneity. It is recommended to directly compare the purity of the in-house-produced Tt_Xrn1 to that of the commercial Xrn1 run alongside in the same gel (Fig. 1f). The latter serves also as a quantitative reference for the approximate estimation of the protein concentration.
2. The purified protein should be supplemented with glycerol to a final concentration of 30%, divided into aliquots that will require no more than five thawing and refreezing cycles, and flash-frozen in liquid nitrogen for prolonged storage at −80 °C.

3.4 Enzymatic Tt_Xrn1 Quality Control

The purified Tt_Xrn1 should be tested for robustness and specificity of its enzymatic activity, using assays adapted to the needs of the subsequent specific Tt_Xrn1 applications. Below, we present three quality control assays, which are for illustration purposes only. In general, we recommend comparing the efficiency of Tt_Xrn1 digestion to a commercial NEB_Xrn1 enzyme. To this end, three approaches can be used, which consist in digesting either RNA oligonucleotides (*see* **Note 24**), ribosomal RNA predominating in total cellular RNA (*see* **Note 25**), or in vitro transcribed mRNA with various 5′-termini (*see* **Note 26**), as illustrated in Fig. 2a-c.

3.4.1 Digestion of a Synthetic RNA Oligonucleotide Substrate

Figure 2a shows Tt_Xrn1 or commercial Xrn1 activity towards a series of 30-mer RNA substrates labeled with a fluorescent FAM dye at the 3′-end for detection. To control for Xrn1 purity and specificity, the substrates have different 5′-ends. The 5′--monophosphorylated RNA oligonucleotide represents a substrate

suitable for Xrn1, whereas a 5′ cap-0 modification or 5′-OH protect the RNA from Xrn1-mediated degradation.

1. Prepare 20% denaturing polyacrylamide gel (*see* **Notes 8** and **9**).
2. Prepare working solutions of each fluorescently labeled RNA substrate by diluting the 200 μM stock 350-fold with sterile RNase-free water (*see* **Note 27**). The volume of working solution depends on the number of reactions planned. Each reaction requires 5 μL of the RNA oligonucleotide solution.
3. Prepare a working solution of the protein by diluting the Tt_Xrn1 enzyme five-fold in 1× NEBuffer 3 (*see* **Note 27**).
4. Prepare a number of 1.5 mL tubes, each containing 10 μL of the F-dye, equal to the number of samples planned to be collected for analysis.
5. In a 1.5 mL tube, combine 2 μL of 10× NEBuffer 3, 1 μL of the Tt_Xrn1 enzyme solution from **step 3** or an equivalent amount of commercial Xrn1 enzyme as a positive control, 1 μL of RiboLock™ RNase Inhibitor, and sterile RNase-free water up to 15 μL. Place on ice. Remember to include a negative control sample without any enzyme.
6. Add 5 μL of the RNA oligonucleotide solution from **step 2**, mix by pipetting and immediately transfer 10 μL into the first tube with F-dye (t0 sample). Freeze this sample in liquid nitrogen. Place the tube with the remainder of the reaction mixture in a thermoblock set at 37 °C.
7. Add 10 μL of F-dye to the remainder of the reaction mixture at the desired final time point (60 min after the beginning of the reaction). Freeze the sample in liquid nitrogen (*see* **Note 28**).
8. After collecting the last sample, take the tubes one by one out of the liquid nitrogen and open them immediately to avoid any "explosion" due to the sudden temperature change. Leave them opened at room temperature for a few min.
9. Close the lids and denature RNA samples in a thermoblock set at 90 °C for 5 min, and then place on ice.
10. Spin down the samples in a microcentrifuge.
11. Run 5–10 μL of each sample in a 20% denaturing polyacrylamide gel using 1× TBE buffer. For each reaction condition, start by loading the sample for time point 0′ (t0), followed by samples collected at the later time points, to monitor the course of the reaction over time.
12. Conduct the electrophoresis at the constant power of 25 W until bromophenol blue (lower dye) gets approximately 2–3 cm from the bottom edge of the gel.

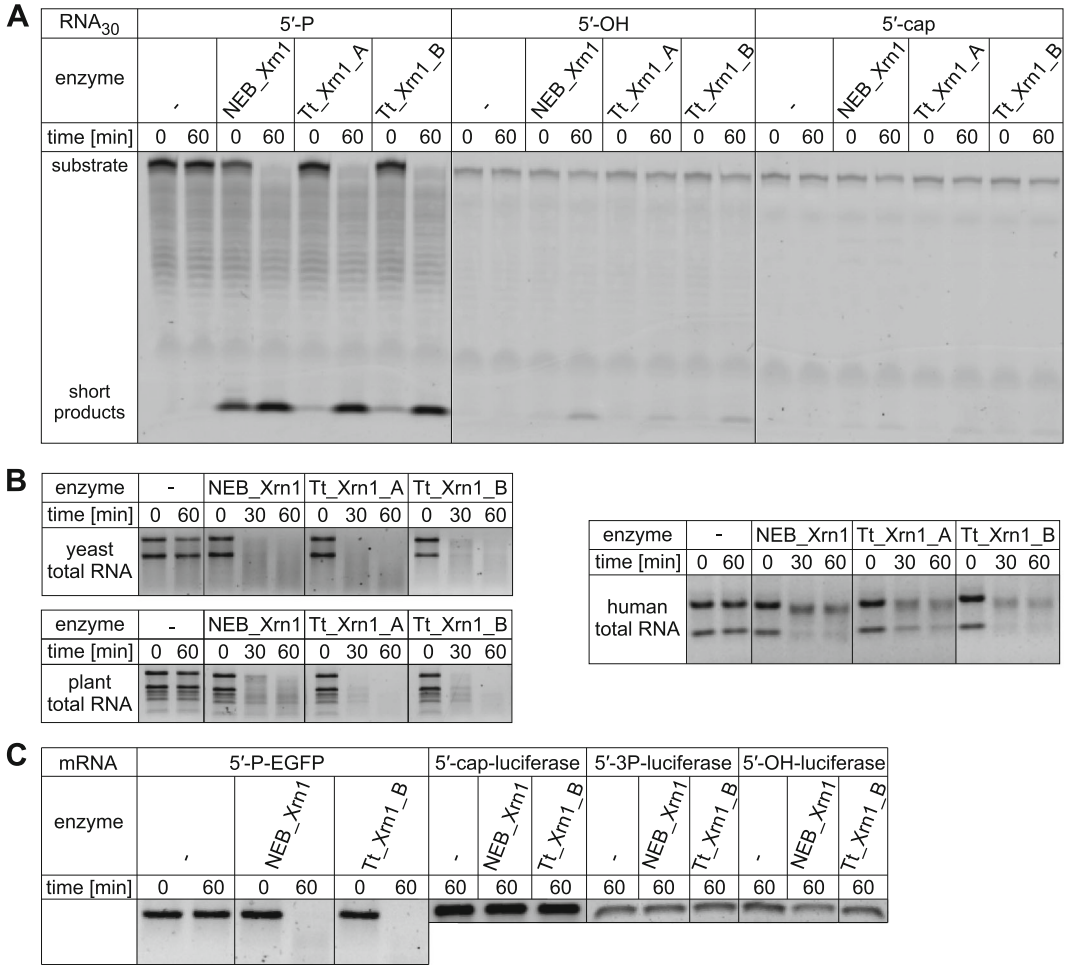

Fig. 2 Quality control of the Tt_Xrn1 enzymatic properties. (**a**) Biochemical assay performed using a synthetic 30-mer oligonucleotide RNA substrate labeled with fluorescent FAM dye at the 3′-end and containing either a 5′-end monophosphate, OH, or cap-0. Equal amounts of each substrate were treated with commercial NEB_Xrn1 or with purified Tt_Xrn1_A or Tt_Xrn1_B. The positions of the substrates and final degradation products are indicated on the left. (**b**) Biochemical assay performed using total RNA isolated from *S. cerevisiae* (top left), *A. thaliana* (bottom left), or HEK293 human embryonic kidney cells (right). (**c**) Biochemical assay performed using in vitro transcribed EGFP or luciferase mRNAs bearing 5′-end monophosphate, cap-1, triphosphate (3P), or hydroxyl group

13. Scan the gel in-between the glass plates (without disassembling the gel cassette) using Typhoon™ FLA-9500 biomolecular imager at settings ensuring that the fluorescent signal is not oversaturated.

3.4.2 Digestion of Total RNA

Figure 2b shows the activity of Tt_Xrn1 or commercial Xrn1 on total RNA samples extracted from budding yeast, human cell line, or plant cells (*see* **Note 10**). Although Xrn1 activity towards total RNA seems robust, the enzyme cannot be used to remove rRNA

prior to transcriptomic analyses (*see* **Note 25**). However, it can be applied to samples enriched in polyadenylated mRNA species with the aim to determine the onset of decapping (*see* **Notes 11** and **29**).

1. Prepare a standard 1% agarose gel with ethidium bromide in 1× TBE buffer.
2. Prepare a working solution of total RNA substrate diluted to 100 ng/μL. Use 10 μL per reaction.
3. Prepare a number of 1.5 mL tubes, each containing 10 μL of the F-dye, equal to the number of samples planned to be collected for analysis.
4. In a 1.5 mL tube, mix 3 μL of 10× NEBuffer 3, 1 μL of the Tt_Xrn1 or equivalent amount of the commercial Xrn1 enzyme as a positive control, 1 μL of RiboLock™ RNase Inhibitor, sterile RNase-free water up to 30 μL, and place the tube on ice. This mix will be sufficient for three time points. Also include a negative control sample without enzyme.
5. Add 10 μL of the total RNA solution from **step 2**, mix thoroughly by pipetting, transfer 10 μL into the first tube containing F-dye (t0 sample) and place it on ice. Place the tube with the remainder of the reaction mixture in a thermoblock set at 37 °C.
6. 30 min after the beginning of the reaction, remove 10 μL reaction aliquots and mix them with F-dye in other preset tubes (**step 3**).
7. Add an extra 0.5 μL of the Tt_Xrn1 enzyme (or an equivalent amount of commercial Xrn1 enzyme) to the sample (except negative controls) to boost RNA degradation efficacy. Place the tubes back in the thermoblock set at 37 °C.
8. Add 10 μL of F-dye to the remainder of the reaction mixture at the desired final time point (60 min after the reaction start).
9. Denature all collected RNA samples at 65 °C for 5 min and then place on ice.
10. Spin down in a microcentrifuge.
11. Run 10 μL of each sample in a 1% agarose-TBE gel with ethidium bromide, using 1× TBE buffer. For each reaction condition, start by loading the sample collected at t0, followed by samples collected at the later time points, to monitor the course of the reaction over time.
12. After electrophoresis for 0.5 h at 7 V/cm, place the gel on a transilluminator to visualize the reaction products and capture the gel image with a digital camera.

3.4.3 Digestion of In Vitro Transcribed mRNA

Figure 2c shows the activity of Tt_Xrn1 or commercial Xrn1 towards in vitro transcribed mRNAs with various 5′-termini. Xrn1 can be used to degrade 5′-monophosphorylated mRNA. In contrast, capped transcripts, as well as molecules with 5′-triphosphate and 5′-OH-ends are not substrates preferred by Xrn1 enzymes. This can be used to assess the purity of the obtained Tt_Xrn1 preparation, since exclusive catalytic activity towards the monophosphorylated substrate will testify against the presence of contaminating bacterial nucleases. This approach also highlights the utility of Tt_Xrn1 for in vitro preparation of mRNA vaccines, as it can be used to remove the uncapped mRNA fraction that is generated as a minority side-product during various protocols for in vitro production of capped mRNAs (*see* **Note 30**).

1. Prepare a standard 1% agarose gel with ethidium bromide in TBE buffer.
2. Prepare a working solution of the mRNA substrate diluted to 100 ng/μL. Use 10 μL per reaction.
3. Prepare a number of 1.5 mL tubes equal to the number of samples planned to be collected for analysis. Add 10 μL of the F-dye in each tube.
4. In a 1.5 mL tube, mix 2 μL of 10× NEBuffer 3, 1 μL of Tt_Xrn1 or equivalent amount of commercial Xrn1 enzyme as a positive control, 0.5 μL of RiboLock™ RNase Inhibitor, sterile RNase-free water up to 10 μL, and then place on ice. Remember to prepare a negative control sample without any enzyme.
5. Add 10 μL of the mRNA solution from **step 2**, mix and transfer 10 μL into the first tube containing F-dye (t0 sample). Place the tube with the remainder of the reaction mixture in a thermoblock set at 37 °C.
6. Add 10 μL of F-dye to the remainder of the reaction mixture at the desired final time point (60 min after the beginning of the reaction).
7. Run 5 or 10 μL of each sample in a 1% agarose-TBE gel with ethidium bromide, using 1× TBE buffer. For each reaction condition, start by loading the sample collected at t0, followed by sample collected at the later time point, to monitor the course of the reaction over time.
8. After electrophoresis for 0.5 h at 7 V/cm, place the gel on a transilluminator to visualize the reaction products and capture the gel image with a digital camera.

4 Notes

1. The pET-28-Tt_Xrn1-6xHis plasmid can be generated by cloning the *XRN1* gene open reading frame from *Thermothelomyces* (*Myceliophthora*) *thermophilus* into pET-28b(+) vector (Novagen). To this end, the full-length open reading frame with codons optimized for expression in *E. coli* (alignment of recoded and native amino-acid and DNA sequences is available at Mendeley: https://doi: 10.17632/343nrfcbp4.1) can be synthesized by a company of choice, or amplified using primer pair Tt_Xrn1For (5′--ttttgtttaactttaagaaggagatataccATGGGCGTCCC-GAAGTTTTTCC-3′)—Tt_Xrn1Rev (5′-atctcagtggtggtggtggtggtgctcgagGCTCTG-CAGTGCTGCGGTCTG-3′). We recommend using the sequence and ligation-independent cloning (SLIC; [14, 15]) method for plasmid construction, using the gel-purified pET-28b(+) vector linearized by digestion with *Nco*I/*Xho*I restriction enzymes as an acceptor. Alternatively, the insert can be excised with the same pair of restriction endonucleases from the plasmid provided by the DNA synthesis company and re-cloned into pET-28b(+) via a standard, T4 DNA ligase-mediated reaction.
2. The overall course of Tt_Xrn1 purification described herein is based on the use of the older generation of FPLC machines still widely utilized in laboratories worldwide, namely ÄKTAxpress and ÄKTA Purifier, which have been discontinued and replaced with newer chromatography systems such as ÄKTA Pure (Cytiva) that can be used as substitutes. Likewise, similar equipment offered by other vendors may be alternatively employed, in conjunction with dedicated chromatography columns, according to the manufacturers' instructions.
3. Any empty 5 mL column compatible with the ÄKTA FPLC systems can be manually filled with Ni-NTA Superflow resin, which is more economical than using commercially available prepacked IMAC columns such as HisTrap (Cytiva). However, the latter are obviously also applicable instead. The column may be used several times for the purification of the same protein. Methods for beads regeneration and recharging with nickel ions using sulfate or chloride salts are usually described by the manufacturer of the particular resin in the accompanying manual.
4. Other SEC columns compatible with ÄKTA Purifier (or equivalent equipment from other vendors) can be used, provided that their working range allows to efficiently fractionate Tt_Xrn1 (theoretical molecular weight of the

Tt_Xrn1 protein with C-terminal 6xHis-tag is approximately 162 kDa). Alternatively, SEC purification can be performed directly after affinity chromatography step on ÄKTAxpress, using e.g. HiLoad 16/600 Superdex 200 pg preparative column.

5. Other anion exchanger columns compatible with ÄKTA Purifier (e.g. Mini Q, Mono Q) or their equivalents working with FPLC systems offered by other vendors can be used as substitutes. Cation exchanger columns (such as Resource S, Mini S, or Mono S) are not recommended, since Tt_Xrn1-6xHis binds poorly to such resins.
6. Any alternative generic oligonucleotide sequence may be chosen, providing that it does not have a tendency to form secondary structures, such as stem-loops (to be checked using RNA secondary structure prediction algorithms available online, e.g. RNAfold).
7. Fluorescent label is recommended, since FAM enables convenient and harmless detection of labeled oligonucleotides in polyacrylamide gels. Moreover, significantly better resolution is observed when fluorescently labeled RNA oligonucleotides are utilized, compared to the substrates labeled with radioisotopes. However, ^{32}P-based labeling of the RNA 3′ -end with T4 RNA ligase I and 5′ [^{32}P]pCp or CutA nucleotidyltransferase and α-^{32}P[CTP] are alternative options [16, 17], enabling visualization of substrates and products generated in the course of Xrn1-mediated digestion by phosphorimaging.
8. Specific recipe is as follows. Add 500 mL of the 40% polyacrylamide stock and 100 mL of 10× TBE buffer in a beaker and mix using a magnetic stirrer with heating plate set at 55 °C. While stirring, add 420.42 g of urea and sterile water to 1 L. When dissolved, filter the solution using a 0.22 μm filter unit attached to the vacuum pump. Store at room temperature, protected from light.
9. Specific recipe to prepare a 20% denaturing polyacrylamide gel is as follows. Assemble the gel cassette using 1 mm-thick spacers; use binding clips to clip and hold the gel plates together and secure the bottom of assembly with adhesive tape. To the volume needed for pouring the particular gel of 20% denaturing polyacrylamide stock, add 1/100 and 1/1000 of gel stock volumes of 10% APS and TEMED, respectively; for example, add 400 μL of APS and 40 μL of TEMED to 40 mL of 20% polyacrylamide stock solution and mix. Immediately pour the solution from the top of the gel cassette avoiding formation of air bubbles and insert a 1 mm-thick comb. Wait for approximately half an hour for complete gel polymerization. Place the gel cassette in the appropriate electrophoresis

tank, pour 1× TBE buffer to the upper and bottom reservoirs, remove the comb and rinse the wells with 1× TBE using a syringe with a needle just prior to loading the samples.

10. Any RNA extraction method best suited for the organism of interest can be used. In case of yeast cells and cell lines alike, the authors have good experience with the hot-acid phenol method. Briefly, resuspend cell pellet in 400 µL of phenol solution saturated with 0.1 M citric acid pH 4.3 and supplemented with 400 µL of TES buffer (10 mM Tris-HCl pH 7.5, 5 mM EDTA, 1% (w/v) SDS). Incubate for 40 min in a thermoblock with shaking at 65 °C. Centrifuge for 10 min at 4 °C and maximum speed. Collect the upper phase and transfer it to a new tube containing 400 µL of phenol solution saturated with 0.1 M citric acid pH 4.3. Incubate for another 20 min in a thermoblock with shaking at 65 °C. Centrifuge for 10 min at 4 °C and maximum speed. Transfer the upper phase to a new tube containing 400 µL chloroform and vortex the tubes several times. Centrifuge for 10 min at 4 °C and maximum speed. Transfer the upper phase to a new tube containing 45 µL of 2 M LiCl and add 1 mL of absolute ethanol. Precipitate RNA for 30 min (or overnight) at −20 °C or −80 °C. Centrifuge for 25 min at 4 °C and maximum speed. Discard the supernatant and add 400 µL of 80% ethanol and mix. Centrifuge for 15 min at 4 °C and maximum speed. Discard the supernatant. Dry the pellet and then resuspend it in nuclease-free water (large pellets can be difficult to dissolve—in such case, water volume can be increased). Measure RNA concentration.

11. If needed, the polyadenylated RNAs, representing mostly mRNAs, can be further purified from total RNA. Many kits for enrichment of polyadenylated RNA from total RNA exist. We have positive experience with the Dynabeads oligo dT(25). Detailed procedures for polyA-enrichment are listed in the manufacturer's information or in ref. [18]. To obtain 10–15 µL of 40–100 ng/µL polyadenylated RNA from total yeast or human RNA, 35 µg of total RNA should be used as an input. The maximum yield of polyadenylated RNA will be therefore equal to around 1–3% (w/w) of the total RNA. For the Dynabeads oligo dT(25), the protocol is as follows. Wash 100 µL of beads slurry per sample three times with 1 mL of Binding Buffer (20 mM Tris-HCl pH 7.5, 1 M LiCl, 2 mM EDTA). Between washes, remove buffer using a magnetic stand. Resuspend the beads in 50 µL of Binding Buffer per sample and set aside. Resuspend 35 µg of the total RNA in 50 µL of nuclease-free water (final concentration: 700 ng/µL; if needed, an excess can be prepared to set aside 10–20 µL of input for further analyses). Add 50 µl of Binding Buffer to 50 µL of RNA. Denature the RNA sample for 2 min at 65 °C.

Place the sample on ice for 30–60 s. Add 50 μL of washed beads in Binding Buffer. Incubate for 20 min at room temperature with occasional mixing. Remove the supernatant. Wash the beads two times with 200 μL of the Wash Buffer (10 mM Tris-HCl pH 7.5, 150 mM LiCl, 1 mM EDTA). Carefully remove all Wash Buffer and resuspend the beads in 10–15 μL of nuclease-free water. Place tube in a thermoblock heated to 80 °C for 2 min and immediately transfer to the magnetic stand to remove the eluate containing the polyadenylated fraction. Measure the concentration of polyadenylated fraction and the input sample to calculate the fraction recovered.

12. 5′-monophosphorylated EGFP mRNA was obtained using in vitro transcription mediated by T7 RNA polymerase. A threefold molar excess of GMP over GTP yielded predominantly 5′-monophosphorylated transcripts, which are preferred substrates for Xrn1.

13. Luciferase mRNAs with various 5′-ends protecting from digestion by Xrn1 were utilized as controls for enzyme specificity. Capped mRNA was obtained during in vitro transcription in the presence of cap-1 analog, which was introduced to mRNA co-transcriptionally. In turn, in vitro transcription performed with just nonmodified NTPs yielded 5′-triphosphorylated mRNA. 5′-OH mRNA was obtained by treating 5′--triphosphorylated mRNA with Calf Intestinal Alkaline Phosphatase (CIAP), which converts 5′-triphosphates to 5′-hydroxyl ends.

14. Here, we provide a protocol for T7 RNA polymerase-driven heterologous overexpression of Tt_Xrn1 in *E. coli* based on the use of autoinduction medium (AIM) [19]. Such medium contains limited amount of glucose and lactose as carbon sources. Glucose is metabolized first, but during logarithmic growth phase bacteria switch to lactose utilization. The latter is converted into allolactose, which in turn elicits the expression of T7 RNA polymerase placed under the control of Lac repressor-regulatable (*lacUV5*) promoter in the derivatives of *E. coli* BL21(DE3) genetic background. This leads to the production of recombinant protein of interest from the plasmid construct introduced by transformation [20, 21]. However, it should be pointed out that induction of protein expression by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a standard LB medium (at the final concentration 0.1–1 M) could be alternatively employed, albeit the duration of induction should be optimized in terms of achieved protein quantity and solubility. Nonetheless, autoinduction has several advantages over IPTG usage. There is no need for monitoring optical density of the bacterial culture and no manual addition of the inducer within a specified time window. In addition, autoinduction cultures

are able to grow to much higher densities, thus yielding considerably more material for protein purification in smaller medium volumes, as compared to the conventional induction procedure.

15. Tt_Xrn1 is usually highly overexpressed and thus easily observable in total protein extracts following SDS-PAGE gel analysis. Briefly, bacterial pellets collected following centrifugation of equal cell numbers of the pre-culture and autoinduction culture can be directly lysed in Laemmli buffer with mild sonication and subsequent incubation at 95 °C. Next, samples should be resolved by electrophoresis in a 10–12% SDS-PAGE gel (home-made or commercial). Following protein separation, the gel should be microwaved briefly at high power, first in deionized water and then in Coomassie stain until the solutions start to boil, and afterwards stained further in Coomassie stain for 1 h followed by at least 3 washes in the destaining solution over approximately 2 h (*see* Fig. 1a), preferably using rocking platform. In the event the result of such examination is not obvious, western-blot using an anti-His tag antibody (e.g. monoclonal Anti-polyHistidine antibody produced in mouse) can be additionally performed. Lower expression level does not necessarily preclude successful protein purification.
16. Following careful decanting of the supernatant, cell lysis can be performed immediately after centrifugation of the autoinduction culture. Alternatively, the bacterial cell pellet may be stored at −20 °C for several weeks before proceeding with cell disruption.
17. Since the recommended protocol for cell lysis presented herein involves the use of high pressure homogenizer (EmulsiFlex-C3), it is of extreme importance to resuspend the cell pellet as thoroughly as possible. This can be achieved by repeated cycles of mechanical manual mixing of the suspension with the serological pipette, vortexing, and extensive pipetting with electronic motorized pipette controller (pipette aid). No culture clumps must be present in the suspension before putting them into the homogenizer. Otherwise, either air bubbles are likely to be generated (this will prevent the instrument from working properly) or the thin tubing, through which the suspension is passed, may become clogged (causing serious damage to the equipment).
18. Other French press-like devices or microfluidizers can be used as EmulsiFlex-C3 substitutes to efficiently rupture the cells. Sonication can also be considered as an alternative, although considerably less convenient, particularly taking into account the large volume of the cell suspension (>100 mL) and the need to manually prevent sample overheating, unless water bath sonicator (e.g. Bioruptor from Diagenode) is available.

19. Cell homogenate should be dispensed into 2–3 tubes, which should be cautiously counterbalanced using laboratory (preferably analytical) scales prior to high-speed ultracentrifugation. Lysis buffer can be used for balancing purposes.
20. While transferring the supernatant to the glassware after ultracentrifugation, it is advisable to keep a residual amount of the supernatant over the insoluble protein fraction pellet. This will avoid disturbing of the pellet and reduce contamination of the sample used for purification.
21. Instead of using ÄKTAxpress for Tt_Xrn1 enrichment via immobilized metal affinity chromatography, this initial protein purification step can be performed entirely manually in a batch format. Following prewashing of the appropriate resin (such as Ni-NTA Agarose or Ni-NTA Superflow) with the lysis buffer in 50 mL Falcon tubes, the protein extract can be incubated with the beads in a cold cabinet/cold-room with head-over-tail rotation for 1–2 h. After protein binding, the resin may be settled by gravity or with the aid of low-speed centrifugation (300–500× *g*) and the flow-through removed. Washing steps and bound protein recovery with several portions of the elution buffer containing imidazole at high concentration can be also carried out in the tube. However, to increase the efficacy of nonspecific proteins removal, it is highly recommended to transfer the beads after binding step in a small volume of the flow-through to the gravity-flow columns with a porous bed support (i.e. frit) able to retain the beads (such as Poly-Prep chromatography columns) and only then proceed with washes and consecutive elutions.
22. It must be stressed that in the protocol presented herein, only 1 mL out of approximately 8 mL of the pooled eluate from the nickel resin was used for subsequent purification steps. Therefore, the total yield of purified enzyme obtained from a single lysate can be increased several-fold provided that SEC and IEX procedures are repeated for the remainder of the eluate collected during affinity chromatography step. This could be advantageous if the potential user aims at obtaining highly concentrated enzyme. Following several consecutive purifications, the protein can be subjected to concentration using ultrafiltration devices (such as Centricon, Amicon, or Vivaspin) with proper molecular weight cut-off.
23. It should be emphasized that the affinity-purified Tt_Xrn1-6xHis migrates in Superdex 200 Increase 10/300 GL SEC column as two peaks. The first peak, referred herein as Tt_Xrn1_A (maximum of $A_{280\ nm}$ at ~8.7 mL; *see* Fig. 1d) is likely a protein aggregate, while the second one, termed Tt_Xrn1_B (maximum of $A_{280\ nm}$ at ~11.8 mL; *see* Fig. 1d)

corresponds to the native, properly folded enzyme. We purified Tt_Xrn1_A and Tt_Xrn1_B separately by IEX (*see* Fig. 1e) and employed both in biochemical assays. Curiously, proteins collected from both peaks proved to be active towards various RNA substrates (*see* Fig. 2a,b). Notably, while Tt_Xrn1_A is less abundant than Tt_Xrn1_B (*see* Fig. 1e), the former appears to contain less degradation products arising from proteolysis of the full-length protein (*see* Fig. 1e,f).

24. The use of a series of RNA oligonucleotides bearing specifically modified 5′-ends is more expensive than using total RNA, but guarantees more accurate evaluation of the purity of the obtained enzyme. Since Xrn1 is preferentially active towards monophosphorylated 5′-ends, efficient decay of such substrate and stability of its counterparts with other 5′-end modifications (e.g. hydroxyl group, triphosphate, cap structures) allows to rule out a contamination with any other bacterial nucleases.
25. The assay employing total RNA is based on monitoring degradation of ribosomal RNAs (rRNAs), which constitute the overwhelming majority of the total RNA and can be easily visualized in simple agarose gels stained with ethidium bromide. Several rRNAs are contained within large polycistronic precursors, which are synthesized in a 5′-triphosphorylated form. Functional ribosomal RNAs are generated via endo- and exoribonucleolytic processing [22], wherein the former converts 5′-terminal triphosphate to monophosphate, making mature rRNA targets susceptible to Xrn1 enzymes, contrary to mRNAs equipped with the 5′ cap structure. Theoretically, this differentiation in 5′ modification status could be employed for rRNA elimination prior to mRNA-directed high-throughput sequencing approaches. However, this method for ribodepletion and mRNA enrichment is not routinely used in laboratory practice due to the fact that Xrn1 frequently stalls on internal secondary structures [23, 24] and polyguanosine tracts, widely present in ribosomal RNAs and thus Xrn1 does not degrade rRNA with 100% efficiency (Fig. 2b). Remaining rRNA degradation intermediates would be amplified in sequencing libraries more readily than mRNAs due to large quantities, which would adversely affect the efficiency of mRNA sequencing.
26. Using 5′-monophosphorylated in vitro transcribed mRNA allows to prove that such molecules are substrates for Xrn1 efficient digestion, whereas 5′ hydroxyl group-, triphosphate-, or cap-1-bearing IVT mRNAs are protected from degradation mediated by Xrn1, similar to synthetic oligoribonucleotide counterparts.
27. The concentration of oligonucleotides used for the reactions should be adjusted for convenient visualization of reaction

products in the gel. This depends on the intensity of FAM fluorescence and the sensitivity of FAM detection. Digestion of 5′-monophosphorylated oligonucleotides by Xrn1 is processive and rapid. Taking this under consideration, Xrn1 dilution as well as the reaction time points should be optimized to determine the conditions allowing to reliably monitor the course of the reaction over time.

28. Denser time-course of the reaction may be performed, if desired, to provide more detailed information about the reaction kinetics (e.g. by collecting samples at 0′, 15′, 30′, and 60′ time points). In such case, transfer 5 μL aliquots from the reaction mixture to 5 μL of F-dye at each time point and freeze them in liquid nitrogen.
29. Tt_Xrn1 can be used to determine the onset of decapping during in vivo mRNA decay. Coding transcripts are modified in the nucleus at the 3′-end by the addition of a pA-tail, which is originally 60 adenosines long and gradually removed in the cytoplasm. It is believed that substantial shortening of the pA-tail leads to decapping. mRNAs with pA-tails as short as 20 or less adenosines are more likely to undergo decapping [25]. Therefore, removal of uncapped mRNAs using Xrn1-mediated digestion should lead to substantial alteration in the pA-tail profiles of individual mRNAs, with the transcripts eliminated in the digested sample being most likely uncapped. To perform such profiling, the polyadenylated mRNA fraction should be used for sequencing. Such samples can be obtained using widely available kits. The pA+ fraction is commonly used for RNA sequencing library construction [13, 18]. In contrast to rRNAs, mRNAs contain few secondary structures, and are thus good substrates for Tt_Xrn1, that can degrade the entire decapped mRNA fraction. The pA-tail profiles are most convincingly established using Direct RNA Sequencing (DRS) from Oxford Nanopore Technology (ONT), rather than Illumina-based TAILseq. Although both technologies give reliable pA-tail profiles of yeast mRNAs, DRS reads are much longer and can span almost the entire mRNA. This is a major advantage, as in the rare cases of the presence of strong secondary structures that could inhibit Tt_Xrn1 activity on a given mRNA, the DRS dataset can be additionally analyzed for the length of reads mapping to the transcriptome reference in the control and Tt_Xrn1 digested sample. With good quality data, this could be used as an additional criterion to select the decapped mRNA fraction. For a protocol of DRS library synthesis and data analysis, see ref. [13]. 100–200 ng of polyadenylated yeast RNA is sufficient to produce a DRS library of satisfactory quality [13].

30. Though the capping reaction may be very efficient [7, 12], small fractions of 5′-triphosphate-containing mRNAs are always present in the in vitro transcription output. Trace amount of such transcripts are highly immunogenic [5]. One approach for removal of immunogenic 5′-triphosphates involves their conversion to 5′-OH by phosphatases (e.g. CIAP), which leaves the mRNA body intact [26, 27]. However, the 5′-OH mRNAs are not translated and thus reduce the yield of protein produced from transcripts (including mRNA vaccines) delivered to cells. Alternatively, the in vitro transcribed mRNA pool can be cleared of the uncapped fraction in two-step enzymatic treatment: (a) the first step involves the use of 5′ RNA polyphosphatase to convert the 5′-triphosphorylated to 5′-monophosphorylated RNAs, which then become the preferred Xrn1 substrates; (b) subsequent digestion with Tt_Xrn1 is employed to remove all mRNA molecules unprotected by the cap. Thus, Xrn1-mediated degradation of the uncapped fraction creates a homogenous capped sample, which allows for efficient translation upon delivery of the transcript to the cells. In brief, up to 10 μg of purified in vitro-capped mRNA is mixed with 2 μL of 5′ RNA polyphosphatase 10× buffer, 0.5 μL of RiboLock™ RNase Inhibitor, and 1 μL of 5′ RNA polyphosphatase (note that the specific proportions of enzymes depend on the in vitro capping efficiency and should be first experimentally established) in a final volume of 20 μL. The mixture is then incubated at 37 °C for 30 min and the polyphosphatase-treated mRNA purified using an RNA cleanup kit. Elution is performed with the volume of water adjusted to obtain concentration of 250 ng/μL or higher. Next, 10 μg of polyphosphatase-treated mRNA is diluted with RNase-free water to 39 μL in a 1.5 mL tube, followed by addition of 5 μL of 10× NEBuffer 3, 1 μL of RiboLock™ RNase Inhibitor, and 5 μL of Tt_Xrn1. The reaction mixture is incubated at 37 °C for 60 min and the Xrn1-treated mRNA subjected to purification using an RNA cleanup kit. Ultimately, the eluted sample contains a homogenous capped mRNA fraction.

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Chapter 8

Production of Fully Capped mRNA for Transfection into Mammalian Cells: A Protocol for Enzymatic Degradation of Uncapped Transcripts After In Vitro mRNA Synthesis

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Abstract

The functionality of messenger RNA, such as stability and translation, is determined by several elements. In Eukaryotes, the 5′ end of the mRNA is modified to contain a 5′ cap structure, the presence of which protects the mRNA from degradation by 5′ to 3′ exoribonucleases and promotes mRNA translation. The in vitro synthesis of RNA has recently attracted ample attention for its application as a source of therapeutic agents or research tools. Although in vitro mRNA synthesis (IVT) methodology is well established and is still being improved, not all synthesized RNA molecules have the expected properties. For example, the co-transcriptional addition of a 5′ cap is frequently incomplete. Yet, the uncapped mRNA molecules are undesirable and must be removed before further processing. Here, we present a protocol for the enzymatic removal of uncapped RNA molecules. This approach offers an excellent opportunity to enrich IVT products with fully competent, 5′ cap-containing mRNA molecules.

Key words In vitro transcription, IVT, mRNA, Cap, N1-methyl-pseudouridine

1 Introduction

In vitro transcription (IVT) is a simple technique allowing template-directed synthesis of high-quality mRNAs. Such synthetic molecules are single-stranded RNA transcripts engineered to mimic naturally occurring RNA and use the translational machinery of the cell to produce proteins. Recent advances in biotechnology and medicine enabled the usage of IVT mRNAs as vaccines and therapeutics [1]. It is anticipated that this advancement will aid in preventing and treating a wide range of diseases, encompassing both infectious and genetic conditions. In the research laboratory, IVT mRNA can be used as a tool to study gene function and activity as an alternative to plasmid DNA, small interfering RNA, and viral vectors. Modifications of mRNA elements influence the immunogenicity, stability, and efficiency of the translation process

Maxime Wery (ed.), *mRNA Decay: Methods and Protocols*, Methods in Molecular Biology, vol. 2863,
https://doi.org/10.1007/978-1-0716-4176-7_8,

[2]. Detection of mRNA introduced into cells by the innate immune system as nonself nucleic acids can lead to its degradation and translation inhibition.

Eukaryotic mRNA is composed of five functional regions: the 5′ cap, the 5′ untranslated region (UTR), the open reading frame (ORF), the 3′ UTR, and the poly(A) tail [3]. In mammals, the 5′ cap primarily consists of an N7-methylguanosine linked to the first nucleotide through an unusual 5′−5′ triphosphate bond and a 2′-O-methyl group at the first nucleotide. This structure, known as cap 1, regulates the nuclear export of mRNA, protects it from exonucleases, and initiates protein translation by binding to eukaryotic translation initiation factor 4E (eIF4E) [4]. The 5′-ppp and 5′-pp ends, created during the incomplete capping process, are sensed by RIG-I and lead to the activation of the immune response [5, 6]. Another key component of mRNA is nucleotides. IVT mRNA containing unmodified uridine is recognized as exogenous nucleic acid by pattern recognition receptors (PRRs) [7]. Replacement of uridine by modified nucleosides suppresses the recognition of in vitro synthesized mRNA by PRRs and increases its stability and translational capacity [7, 8]. Capping and introducing N1-methyl-pseudouridine increases the efficiency of mRNA translation.

Here, we describe a detailed protocol for synthesizing capped mRNA with N1-methyl-pseudouridine (me1Ψ-UTP), which can be used to transfect cells. The procedure consists of the following steps: (a) in vitro transcription, (b) phenol/chloroform/isoamyl alcohol extraction of RNA, (c) removal of uncapped mRNA, and (d) analysis of synthesized mRNA on agarose gel (Fig. 1). The results of IVT mRNA encoding EGFP protein (Fig. 2) and the effect of cap and modified uridine presence in an IVT product (Fig. 3) are shown.

2 Materials

2.1 In Vitro Transcription

1. HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs). The kit contains the T7 RNA Polymerase Mix, the 10× T7 reaction buffer, 100 mM ATP, 100 mM CTP and 100 mM GTP as well as FLuc control template (linearized control template which can be used to verify RNA synthesis) (*see* **Note 1**).
2. N1-Methylpseudo-UTP.
3. CleanCap Reagent (TriLink BioTechnologies) (*see* **Note 2**).
4. RiboLock RNase Inhibitor.
5. Linear template DNA (*see* **Note 3**).
6. dsDNase.

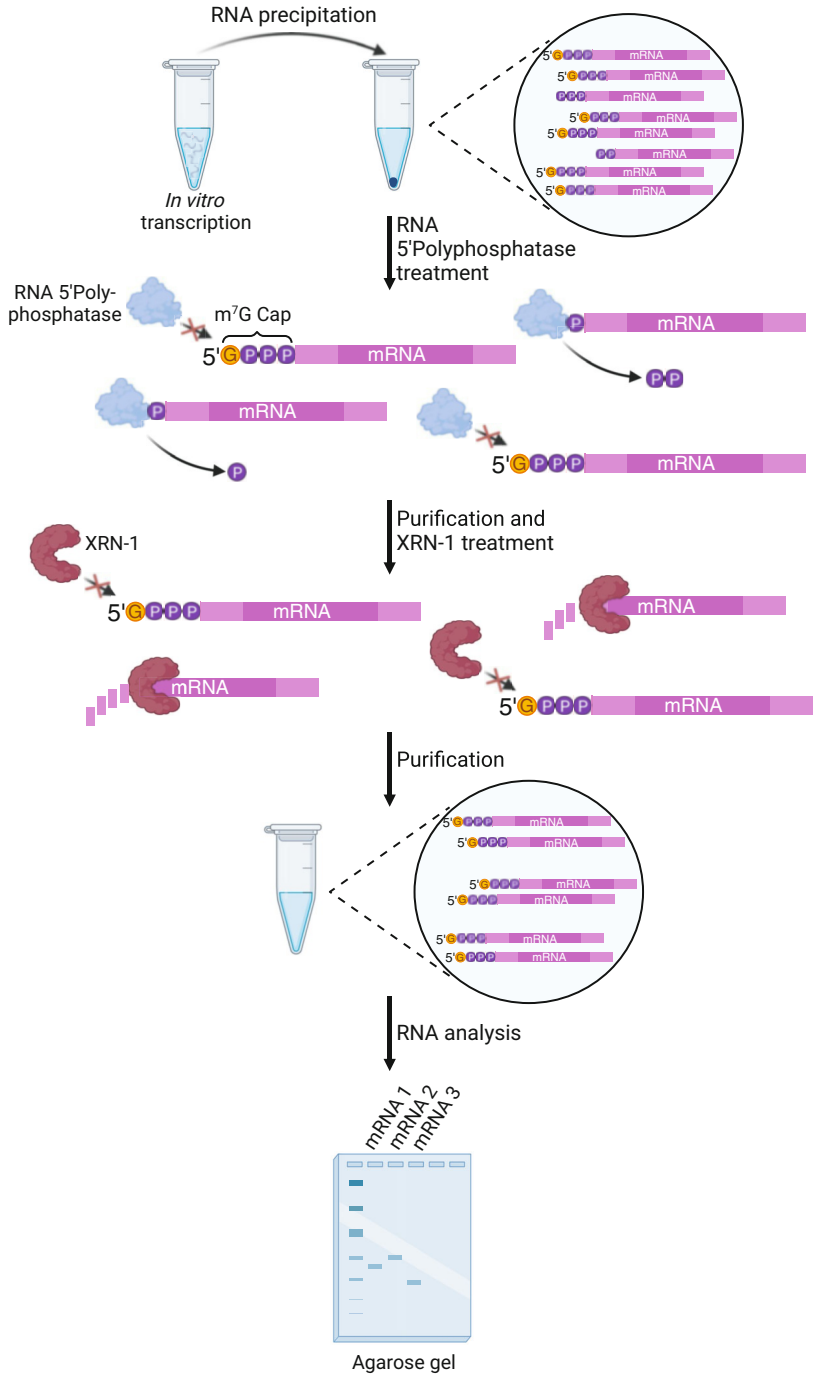

Fig. 1 Scheme of the procedure. The mRNA is synthesized using the in vitro transcription process. The product is purified and precipitated. The RNA 5′Polyphosphatase removes the phosphates from 5′-di- and triphosphorylated RNA but does not dephosphorylate monophosphorylated or 5′-capped RNA. After purification, the product is treated with XRN-1 enzyme, which degrades uncapped mRNA substrates marked by a 5′ monophosphate. After additional purification, the mRNA can be analyzed using agarose gel electrophoresis. (Created with BioRender.com)

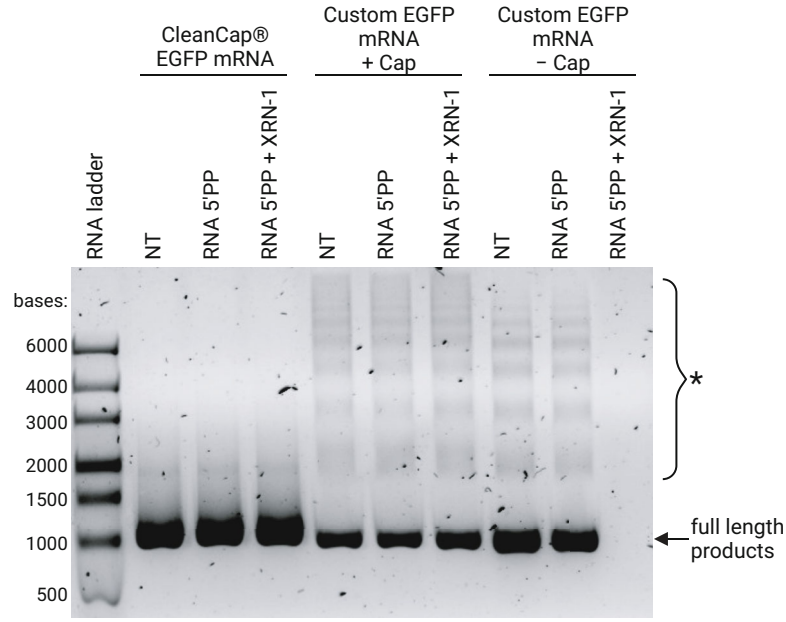

Fig. 2 Treatment of in vitro transcribed mRNA with RNA 5′ Polyphosphatase and XRN-1. Commercial CleanCap Enhanced Green Fluorescent (EGFP) Protein mRNA (TriLink BioTechnologies) and in vitro transcribed mRNA encoding EGFP with and without CleanCap (Cap) were left untreated (NT), treated with RNA 5′ Polyphosphatase (RNA 5′PP) or treated with RNA 5′PP and XRN-1 according to the procedure described above. Then, mRNAs were analyzed on 1% denaturating agarose gel. * 3′-extended products (*see* **Note 12**). The gel was overexposed to reveal unintended products

7. Nuclease-free water.
8. PCR thermocycler.
9. Vortex mixer.
10. Benchtop centrifuge.

2.2 Phenol/ Chloroform/Isoamyl Alcohol Extraction of RNA

1. ROTI Phenol/chloroform/isoamyl alcohol: 25:24:1 ratio, pH 4.5–5.
2. Chloroform.
3. 3 M ammonium acetate pH 5.2.
4. GlycoBlue coprecipitant.
5. 75% (v/v) and 100% (v/v) ethanol.
6. Nuclease-free water.
7. Vortex mixer.
8. Centrifuge (*see* **Note 4**).
9. UV-Vis spectrophotometer or fluorometer.

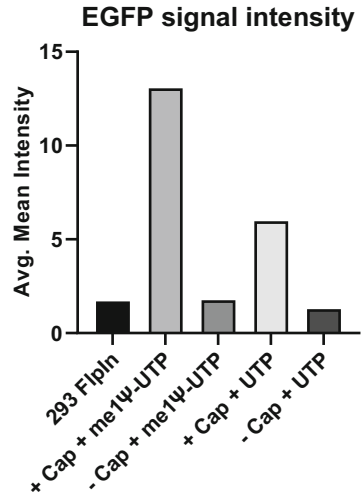

Fig. 3 Composition of IVT mRNA influences the efficiency of translation. Flp-In 293 T-REx cells (293 FlpIn) were left untreated or transfected with in vitro transcribed mRNA encoding the enhanced green fluorescent protein (EGFP). mRNA was synthesized with or without CleanCap (Cap) resulting in a Cap 1 structure and N1-methyl-pseudouridine (me1Ψ-UTP) or unmodified UTP. The green fluorescence intensity of cells was monitored 24 h after mRNA transfection

2.3 Removal of Uncapped mRNA

1. RNA 5′ Polyphosphatase (Lucigen) (*see* **Note 5**).
2. XRN-1 with 10× NEBuffer 3 (New England Biolabs) (*see* **Note 6**).
3. ROTI Phenol/Chloroform/Isoamyl alcohol: 25:24:1 ratio, pH 4.5–5.
4. Chloroform.
5. 3 M ammonium acetate pH 5.2.
6. GlycoBlue coprecipitant.
7. 75% (v/v) and 100% (v/v) ethanol.
8. Nuclease-free water.
9. PCR thermocycler.
10. Vortex mixer.
11. Benchtop centrifuge.

2.4 Analysis of Synthesized mRNA on Agarose Gel

1. 10× NBC buffer: 0.5 M boric acid, 10 mM sodium acetate, 50 mM NaOH.
2. UltraPure Agarose.
3. Nucleic acid staining dye (*see* **Note 7**).
4. 37% formaldehyde.

5. 2× Loading dye: 90% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol in 20 mM EDTA, pH 8.0.
6. Nuclease-free water.
7. Agarose gel electrophoresis system.
8. Blue light illuminator or UV transillumination.

3 Methods

3.1 In Vitro Transcription

1. Thaw the components of the HiScribe T7 High Yield RNA Synthesis Kit and the CleanCap Reagent. Keep the T7 RNA Polymerase Mix and CleanCap Reagent on ice. Mix and spin down the reagents to the bottom of the tubes.
2. In a 0.2 mL PCR tube, set up the reaction at room temperature by mixing 4.85 μL of nuclease-free water, 0.5 μL of 10× T7 Reaction Buffer, 0.5 μL of 100 mM ATP, 0.5 μL of 100 mM GTP, 0.5 μL of 100 mM CTP, 0.5 μL of 100 mM N1-methyl-pseudouridine, 0.4 μL of 100 mM CleanCap Reagent, 1 μL of 250–500 ng/μL linear template DNA, 0.25 μL RiboLock RNase Inhibitor and 1 μL of T7 RNA Polymerase Mix. Pipet up and down gently to mix and spin down (*see* **Note 8**).
3. Incubate at 37 °C for 2 h in a PCR thermocycler.
4. Dilute the reaction mixture with nuclease-free water up to 44 μL. Add 5 μL of 10× dsDNase buffer and 1 μL of dsDNase. Pipet up and down gently to mix and spin down. Incubate at 37 °C for 15–30 min in a PCR thermocycler.
5. At this step, samples can be frozen at −80 °C or further processed.

3.2 Phenol/Chloroform/Isoamyl Alcohol Extraction

1. Add nuclease-free water to the reaction mixture to bring the total volume up to 0.5 mL. Add 0.5 mL of Phenol/Chloroform/Isoamyl alcohol (under the hood). Vortex for 2 min.
2. Centrifuge at room temperature for 5 min at 14,000× *g*.
3. Under the hood, tilt the tube at 45° and carefully transfer the upper aqueous phase (~0.5 mL) to a fresh 1.5 mL tube. Be sure not to carry over any phenol during pipetting.
4. Add 0.5 mL of chloroform to the aqueous phase. Vortex for 2 min.
5. Centrifuge at room temperature for 5 min at 14,000× *g*.
6. Tilt the tube at a 45° angle and transfer the upper aqueous phase (~0.45 mL) to a fresh 2 mL tube. Add 45 μL of 3 M ammonium acetate and 1 μL of GlycoBlue. Vortex shortly and add 1 mL of 100% ethanol.

7. Place the tube at −80 °C for 1 h or at −20 °C overnight to precipitate the RNA.
8. Centrifuge the sample at 4 °C for 30 min at 16,000× *g* to pellet the RNA.
9. Carefully remove the supernatant without disturbing the pellet.
10. Add 1 mL of 75% ethanol and flip gently the tube to mix.
11. Centrifuge the sample at 4 °C for 3 min.
12. Carefully remove the supernatant. Spin the tube briefly and remove as much as possible of the remaining ethanol.
13. Air-dry the pellet under the hood for 5–10 min (*see* **Note 9**).
14. Resuspend the pellet in 30–50 μL of nuclease-free water and measure the concentration of mRNA.

3.3 Removal of Uncapped mRNA

1. Thaw the RNA 5′ Polyphosphatase 10× Reaction Buffer and thoroughly mix it.
2. In a 0.2 mL PCR tube, set up the reaction on ice by mixing 43.75 μL of IVT mRNA sample dissolved in nuclease-free water (up to 50 μg), 5 μL of RNA 5′ Polyphosphatase 10× Reaction Buffer, 0.25 μL of RiboLock RNase Inhibitor, and 1 μL of RNA 5′ Polyphosphatase.
3. Thoroughly mix and spin down the reaction mixture.
4. Incubate at 37 °C for 30 min in a PCR thermocycler.
5. Purify and precipitate the treated RNA as described in Subheading 3.2 (*see* **Note 10**). Resuspend the pellet in 43 μL of nuclease-free water.
6. Thaw the NEBuffer 3 and thoroughly mix it.
7. In a 0.2 mL PCR tube, set up the reaction on ice by mixing 43 μL of IVT mRNA sample after RNA 5′ Polyphosphatase treatment, 5 μL of NEBuffer 3, and 2 μL of XRN-1 enzyme.
8. Thoroughly mix the reaction.
9. Incubate at 37 °C for 1 h in a PCR thermocycler.
10. Purify and precipitate the treated RNA as described in Subheading 3.2 (*see* **Note 10**).
11. mRNA samples can be frozen at −80 °C (*see* **Note 11**).

3.4 Analysis of Synthesized mRNA on Agarose Gel

1. Prepare 1% agarose gel in 1× NBC buffer.
2. When the gel has cooled down to ~65 °C, under the hood add 2.5 mL of 37% formaldehyde and 5 μL of GelRed Nucleic Acid Gel Stain per 100 mL of gel, and pour the gel into the gel tray. Let it cool down for 30–45 min.

3. Add 0.5–1 μg of RNA diluted in nuclease-free water (total volume of 5 μL) to 5 μL of 2× Loading dye. Dilute 1 μL of RiboRuler High Range RNA Ladder in 4 μL nuclease-free water and add 5 μL of 2× Loading dye. Vortex briefly and spin down.
4. Heat the samples to 70 °C for 10 min to denature the RNA. Chill quickly on ice and load on the gel.
5. Run the gel in 1× NBC buffer with a constant voltage of 80 V for 1–1.5 h.
6. Remove the gel from the electrophoresis chamber. The gel can be visualized with a blue light illuminator or a UV transillumination.

4 Notes

1. This protocol has been optimized for use with the HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs). Other kits and T7 polymerases are available on the market, however, proper optimization may be required when using the other reagents.
2. The CleanCap Reagent selection depends on the initiation sequence. TriLink has developed multiple CleanCap analogues with different initiating nucleosides (AG, GG, and AU) for in vitro transcription.
3. Linearized plasmid DNA (pDNA), PCR products, or cDNA can be used as templates for in vitro transcription as long as they contain a double-stranded T7 RNA polymerase promoter region in the correct orientation. pDNA needs to be linearized by restriction digestion downstream of the insert to generate blunt ends or 5′-overhangs. 3′-overhangs generate spurious transcripts and must be avoided.
4. Centrifuge with maximum centrifugal force of at least 16,000× *g* and cooling function is required.
5. The protocol was optimized for use with the RNA 5′ Polyphosphatase from Lucigen. Other companies offer enzymes with the same specificity; however, proper optimization may be required. We noticed uncapping of capped IVT mRNA when using a 5′-polyphosphatase enzyme from another manufacturer.
6. The protocol was optimized for use with the XRN-1 enzyme from New England Biolabs. Other companies may offer enzymes with the same specificity; however, proper optimization may be required.

7. Any nucleic acid stain that allows the detection of RNA in agarose gel using the available equipment can be used.
8. The in vitro transcription can be upscaled up to 50 μL without loss of the reaction efficiency.
9. Do not overdry the pellet, making RNA difficult to redissolve.
10. A spin column-based purification kit can be used to separate pure mRNA.
11. Aliquots should be made to avoid unnecessary freeze–thaw cycles of purified mRNA.
12. If the 3′ end of the mRNA is sufficiently complementary to the synthesized transcript (in *cis*), the T7 RNA polymerase may produce incorrect products, extending the 3′ end of the RNA with the sequence complementary to the intended RNA product [9].

Acknowledgments

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Chapter 9

Transcriptome-Wide Analysis of the 5′ Cap Status of RNA Using 5′ Monophosphate-Dependent Exonuclease Digestion and RNA Sequencing

Maxime Wery, Ugo Szachnowski, Sara Andjus, and Antonin Morillon

Abstract

Eukaryotic mRNAs carry an N7-methylguanosine (m^7G) cap structure at their 5′ extremity, which protects them from the degradation by 5′-3′ exoribonucleases and plays a pivotal role in mRNA metabolism, promoting splicing, nuclear export, and translation. Decapping, the enzymatic process that removes this structure, is a key event during cytoplasmic mRNA 5′-3′ decay, leading to the degradation of the transcript body by Xrn1. In this chapter, we describe a procedure to assess the cap status of RNA at the transcriptome level. It is based on a treatment of total RNA extracts with a 5′ monophosphate-dependent exonuclease, which like Xrn1 specifically degrades decapped RNAs harboring 5′ monophosphate extremities, but not RNAs with intact m^7G cap. The digested RNAs are then analyzed by RNA sequencing.

Key words mRNA decay, Decapping, Dcp2, Terminator 5′-Phosphate-Dependent Exonuclease, Ribosomal RNA depletion, RNA-Seq, Xrn1-sensitive Unstable Transcripts

1 Introduction

The addition of a 5′ N7-methylguanosine (m^7G) cap structure is the first co-transcriptional modification of eukaryotic mRNA, taking place as soon as the first nucleotides of the nascent transcript exit RNA polymerase II [1, 2]. The cap protects the RNA from the action of 5′-3′ exoribonucleases, Rat1/XRN2 in the nucleus and Xrn1 in the cytoplasm [3], which specifically degrade RNA with 5′ monophosphate ends [4, 5]. In addition, the cap also promotes pre-mRNA splicing, nuclear export, and translation [6].

Decapping corresponds to the enzymatic removal of the m^7G cap structure, yielding RNA with a 5′ monophosphate. In yeast, this process depends on a single enzyme made of the catalytic subunit Dcp2 and the regulator subunit Dcp1 [7, 8], assisted by several accessory factors defined as decapping enhancers or activators [9]. Decapping is critical for the control of mRNA turnover

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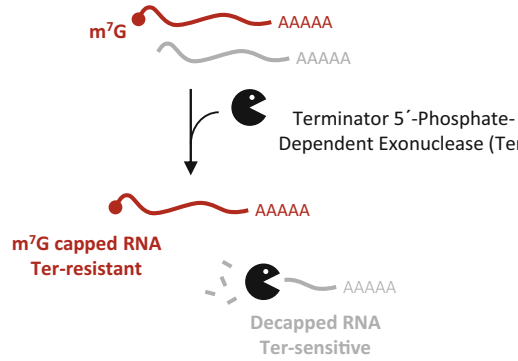


Fig. 1 Schematic representation of the action of the Terminator 5′-phosphate-dependent exonuclease, which degrades RNAs that are decapped (gray), but not those with an intact m^7G cap (red)

[9, 10] and constitutes a pivotal event during the general mRNA 5′-3′ decay [11–13] as well as specialized mRNA decay pathways, such as Nonsense-Mediated mRNA Decay [14, 15], microRNA-induced mRNA degradation [16] or deadenylation-independent degradation of specific transcripts [17, 18]. Apart from mRNAs, decapping also regulates the abundance of cytoplasmic long non-coding (lnc)RNAs in budding yeast [19]. In fact, decapping inactivation results in the accumulation of most Xrn1-sensitive Unstable Transcripts (XUTs) [20], a family of cryptic lncRNAs identified in budding and fission yeasts and that are extensively degraded by Xrn1 [21–24].

To characterize the pathway leading to RNA degradation by Xrn1 and to decipher the functional role of decapping in this process, it is important to discriminate capped vs. decapped RNA decay intermediates. To that purpose, here we describe a procedure to assess the cap status of RNAs at the transcriptome-wide level. It is based on an in vitro treatment of total RNA extracts with a 5′-phosphate-dependent exonuclease called "Terminator," which specifically degrades RNA with 5′ monophosphate extremities (Fig. 1). In parallel, the same RNA extracts undergo a classical depletion of ribosomal (r)RNAs instead of the Terminator treatment. Both Terminator-digested and rRNA-depleted RNAs are then analyzed by RNA sequencing.

The procedure described here uses yeast RNA, but it can be easily applied to other eukaryotic models as the enzymatic treatment is performed in vitro, following total RNA extraction (for example, *see* [25]).

2 Materials

Prepare all solutions with ultrapure, deionized, sterile water and store them at room temperature, unless specified. For the resuspension and dilution of RNA samples, only use water which is guaranteed nuclease-free. Clean working surfaces and pipettes with RNase removal solution. Wear gloves to avoid contaminating RNA samples with RNases. Use RNase-free filter tips for pipetting to avoid cross-contamination between samples.

2.1 RNA Digestion with the Terminator 5′-Phosphate-Dependent Exonuclease

1. Total RNA extracted from the yeast strains of interest. *See* **Note 1**.
2. ERCC RNA spike-in mix. Dilute 1 μL at 1:100 in nuclease-free water just before the experiment.
3. Terminator 5′-Phosphate-Dependent Exonuclease. *See* **Note 2**.
4. SUPERase-In RNase inhibitor (20 U/μL) or equivalent.
5. Phenol:chloroform:iso-amyl alcohol (125:24:1) solution (pH 4.3–4.7).
6. Safe-lock, RNase-free 1.5 mL tubes.
7. Refrigerated microcentrifuge.
8. Ethanol.
9. GlycoBlue coprecipitant (15 mg/mL).
10. 3 M sodium acetate, pH 5.5.
11. Nuclease-free water.
12. RNA 6000 Pico kit or High Sensitivity RNA ScreenTape, ladder, and sample buffer (Agilent).
13. 2100 Bioanalyzer or 4200 TapeStation system (Agilent).

2.2 Depletion of rRNA

1. ERCC spike-in RNA, diluted 1:100 in nuclease-free water just before the experiment.
2. RiboMinus Eukaryote Kit v2 (Invitrogen). The kit contains the probe mix, the 2× hybridization buffer, and the RiboMinus magnetic beads.
3. RiboMinus Concentration Module (*see* **Note 3**). The kit contains the columns, the binding buffer (L3), the wash buffer (W5), and an aliquot of RNase-free water. Before use, add 6 mL of ethanol to 1.5 mL of wash buffer.
4. Ethanol.
5. Nuclease-free water.
6. RNase-free microtubes.
7. Thermal mixer.

8. Magnetic stand for microtubes.
9. RNA 6000 Pico kit or High Sensitivity RNA ScreenTape, ladder, and sample buffer (Agilent).
10. 2100 Bioanalyzer or 4200 TapeStation system (Agilent).

2.3 Library Preparation

1. TruSeq mRNA stranded mRNA library preparation kit (Illumina; *see* **Note 4**). The kit contains the Fragment, Prime, Finish Mix (FPF), the First Strand Synthesis Act D Mix (FSA), the resuspension buffer (RSB), the Second Strand Marking Master Mix (SMM), the A-Tailing Mix (ATL), the Ligation Mix (LIG), the Stop Ligation Buffer (STL), the PCR Primer Cocktail (PPC), and the PCR Master Mix (PMM).
2. SuperScript II reverse transcriptase.
3. IDT for Illumina TruSeq RNA UD indexes (Illumina).
4. AMPure XP beads.
5. Ethanol.
6. Nuclease-free water.
7. RNase-free 0.2 mL tubes.
8. Nuclease-free LoBind 1.5 mL microtubes.
9. Thermal cycler.
10. Thermal mixer.
11. Magnetic stand for microtubes.
12. Vacuum concentrator (SpeedVac or equivalent).
13. Qubit dsDNA HS Assay Kit.
14. Qubit fluorometer.
15. High Sensitivity DNA ScreenTape, ladder, and sample buffer (Agilent).
16. 4200 TapeStation system (Agilent).

2.4 Software

1. Trim Galore (https://github.com/FelixKrueger/TrimGalore).
2. HISAT2 V2.2.0 (https://daehwankimlab.github.io/hisat2/).
3. Samtools (https://www.htslib.org/).
4. FeatureCounts v2.0.0 (https://subread.sourceforge.net/featureCounts.html).
5. DESeq2 [26].
6. R (version 3 or higher) and R Studio.
7. Visualization tool, e.g. the Integrative Genomics Viewer (IGV) [27] or VING [28].

3 Methods

3.1 Terminator Digestion

This section describes the procedure for the preparation of Terminator-digested RNA to be used for subsequent construction of RNA-Seq library. However, if the experiment aims at assessing the cap status of a single transcript, the user might consider RT-qPCR or Northern blotting instead of RNA-Seq (*see* **Note 5**).

1. In a clean RNase-free 1.5 mL microcentrifuge tube, mix 15 μL of total RNA (100 ng/μL, i.e. 1.5 μg), 3 μL of a 1:100 dilution of ERCC spike-in RNA, 2.5 μL of 10× buffer A, 3 μL of nuclease-free water, 0.5 μL of SUPERase-In RNase inhibitor (10 U), and 1 μL of Terminator 5′-Phosphate-Dependent Exonuclease (1 U). Homogenize by pipetting up and down. *See* **Notes 6** and **7**.
2. Incubate for 1 h at 30 °C.
3. Add 225 μL of nuclease-free water.
4. Add 250 μL of Phenol:chloroform:iso-amyl alcohol solution. Vortex at full speed for 30 s.
5. Centrifuge for 5 min at room temperature at full speed.
6. Transfer 200 μL of the upper (aqueous) phase into a new RNase-free 1.5 mL microcentrifuge tube. Add 2 μL of GlycoBlue coprecipitant (15 mg/mL), 20 μL of 3 M sodium acetate pH 5.5 and 600 μL of 100% ethanol. Mix by vortexing.
7. Precipitate overnight at −80 °C.
8. The next day, centrifuge for 30 min at 4 °C at full speed.
9. Remove the ethanol using a micropipette.
10. Wash the RNA pellet with 750 μL of ice-cold 75% ethanol.
11. Centrifuge for 15 min at 4 °C at full speed.
12. Repeat **steps 9–11** (i.e. two washes with 75% ethanol).
13. Completely remove the ethanol using a micropipette and then dry the RNA pellet keeping the tubes open on the bench or under hood for 5 min.
14. Resuspend the RNA pellet in 15 μL of nuclease-free water.
15. Determine the concentration using the Qubit RNA High Sensitivity kit.
16. Optional: analyze 1 μL (diluted at 1 ng/μL) of Terminator-digested RNA using an RNA 6000 Pico chip or a High Sensitivity RNA ScreenTape. Figure 2 shows the expected profile (*see* **Note 8**).
17. Store at −80 °C until construction of libraries.

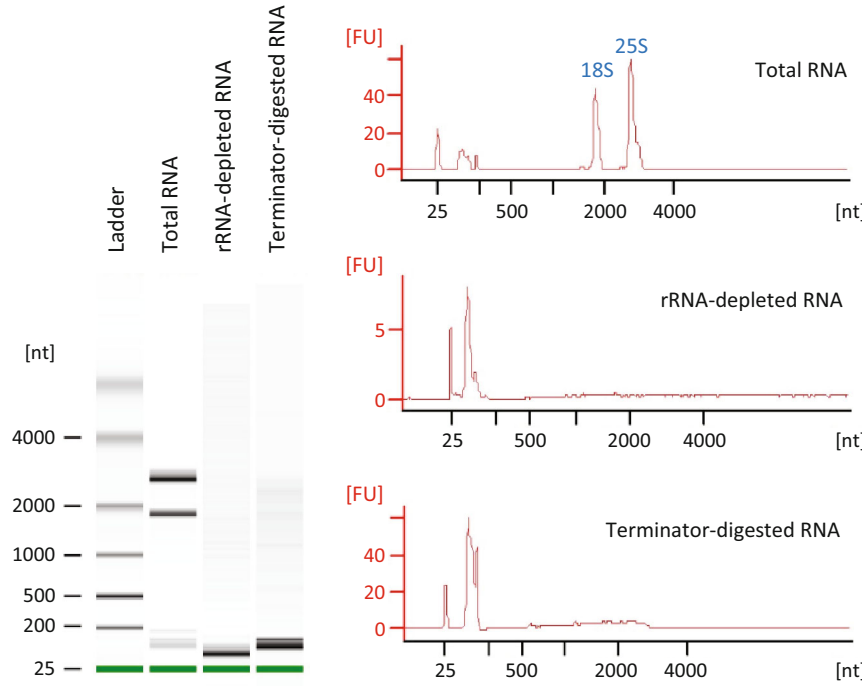


Fig. 2 Samples (1 ng) of total RNA, rRNA-depleted RNA, and Terminator-digested RNA were analyzed using an RNA 6000 Pico chip in a 2100 Bioanalyzer system

3.2 rRNA Depletion

1. Preheat the 2× hybridization buffer at 50 °C to bring salts into solution.
2. In a new RNase-free 1.5 mL tube, mix 1 μg of total RNA (10 μL at 100 ng/μL) with 2 μL of 1:100 diluted ERCC RNA spike-in.
3. Add 34 μL of nuclease-free water, 4 μL of probe mix and 50 μL of preheated 2× hybridization buffer (*see* **Note 9**). Mix by pipetting or gentle vortexing.
4. Incubate the tube with the RNA/probe mix (100 μL) at 70 °C for 10 min to denature the RNA.
5. Immediately transfer the tube at 37 °C and incubate for 20 min.
6. While the sample is cooling at 37 °C, resuspend the RiboMinus magnetic beads by thorough vortexing. Prepare 200 μL of 1× hybridization buffer per sample by diluting 2× buffer with an equal volume of nuclease-free water.
7. For each sample, pipet 500 μL of beads into a new, RNase-free 1.5 mL tube. Place the tube with the beads on a magnetic stand for 1 or 2 min until the solution clears. Gently aspirate and discard the supernatant without disturbing the beads.

8. Wash the beads by resuspending them in 500 μL of nuclease-free water. Place the tube on the magnetic stand until the solution clears and discard the supernatant without disturbing the beads.
9. Repeat **step 8** (second wash).
10. After the second wash, resuspend the beads in 200 μL of prepared 1× hybridization buffer. Place the resuspended beads at 37 °C in a thermomixer and incubate for at least 5 min.
11. After the 20 min incubation at 37 °C (**step 5**), briefly centrifuge the RNA/probe mix to collect the sample at the bottom of the tube. Transfer the RNA/probe mix (100 μL) in the tube with the beads preheated at 37 °C. Mix well by pipetting up and down or vortexing at low speed.
12. Incubate the RNA/probe/beads mix at 37 °C for 15 min, under mild agitation (750 rpm) to keep the beads in suspension.
13. Briefly centrifuge the tube and then place it on the magnetic stand until the solution clears. Transfer 300 μL of supernatant (corresponding to the rRNA-depleted RNA fraction) to a new RNase-free 1.5 mL tube.
14. Add 300 μL of binding buffer L3 to the rRNA-depleted RNA and 900 μL of 100% ethanol (*see* **Note 10**). Mix by vortexing.
15. Transfer up to 600 μL of the sample to the spin column (with the collection tube).
16. Centrifuge at 12,000× *g* for 1 min at room temperature. Discard the flow-through, and reinsert the column into the same collection tube.
17. Repeat **steps 15** and **16** until the entire sample is processed.
18. Wash the column with 600 μL of wash buffer (W5). Centrifuge the column at 12,000× *g* for 1 min at room temperature. Discard the flow-through.
19. Discard the collection tube and place the column into a clean collection tube, supplied with the kit.
20. Centrifuge the column at maximum speed for 2 min at room temperature to remove any residual wash buffer. Place the column in a clean, 1.5 mL recovery tube.
21. Add 16 μL of nuclease-free water to the middle of the column. Incubate at room temperature for 1 min.
22. Centrifuge the column at maximum speed for 1 min at room temperature. The recovery tube contains the purified rRNA-depleted RNA.
23. Place the rRNA-depleted RNA on ice. Determine the concentration using the Qubit RNA High Sensitivity kit.

24. Optional: analyze 1 μL of rRNA-depleted RNA (diluted at 1 ng/μL) using an RNA 6000 Pico chip or a High Sensitivity RNA ScreenTape. Figure 2 shows the expected profile (*see* **Note 11**).
25. Store at −80 °C or proceed to the construction of libraries.

3.3 Library Construction

This section describes the procedure for the construction of RNA-Seq libraries from both Terminator-digested RNA and rRNA-depleted RNA. It has been adapted from the standard TruSeq mRNA stranded mRNA library preparation protocol from Illumina.

3.3.1 RNA Fragmentation and Priming

1. Thaw the Terminator-digested and rRNA-depleted RNA samples on ice.
2. Dry the RNA samples using a vacuum concentrator (*see* **Notes 12** and **13**).
3. Resuspend the dried RNA in 17 μL of 1× Fragment, Prime, Finish Mix by gently pipetting the entire volume and then transfer in an RNase-free 0.2 mL tube (*see* **Note 14**).
4. Place the tube in a thermal cycler (with the lid set to 100 °C). Run the following program: 94 °C for 3 min, 4 °C hold (*see* **Note 15**).
5. Remove the tube from the cycler as soon as it reaches 4 °C and centrifuge briefly. Place the tube on ice and proceed immediately to first strand cDNA synthesis.

3.3.2 First Strand cDNA Synthesis

1. Add 1 μL of SuperScript II to 9 μL of First Strand Synthesis Act D Mix (FSA) per library. Mix thoroughly by gently pipetting. Centrifuge briefly.
2. Add 8 μL of this mix (FSA + SuperScript II) to each tube containing the fragmented and primed RNA. Gently pipette up and down to mix thoroughly. Centrifuge briefly.
3. Place the tube in a thermal cycler (with the lid set to 100 °C). Run the following program: 25 °C for 10 min, 42 °C for 15 min, 70 °C for 15 min, hold at 4 °C.
4. Remove the tube from the cycler when it reaches 4 °C. Centrifuge briefly, place the tube on ice and proceed immediately to second strand cDNA synthesis.

3.3.3 Second Strand cDNA Synthesis and Cleanup

1. Add 5 μL of resuspension buffer (RSB) to each tube.
2. Add 20 μL of Second Strand Marking (SMM) Master Mix. Mix thoroughly by gently pipetting the entire volume up and down. Centrifuge briefly.
3. Place the tube in a thermal cycler. Incubate at 16 °C for 1 h with the lid closed and set to 30 °C (*see* **Note 16**).

4. Remove the tube from the cycler. Transfer in a 1.5 mL tube and let stand to bring the sample to room temperature.
5. Vortex the AMPure XP beads until they are well resuspended (*see* **Note 17**).
6. Add 90 µL of AMPure XP beads to a 1.5 mL tube containing 50 µL of cDNA. Gently pipette the entire volume up and down 10 times.
7. Incubate at 22 °C (or room temperature) for 5 min in a thermomixer, under moderate agitation (600 rpm).
8. Pulse-spin, then place the tube on a magnetic stand at room temperature for 2–3 min.
9. Remove and discard the supernatant.
10. With the tube still on the magnetic stand, add 500 µL of freshly prepared 80% ethanol without disturbing the beads.
11. Incubate for 1 min, pulse-spin, place the tube on the magnetic stand and remove all of the supernatant.
12. Repeat **steps 10** and **11** (i.e. two washes).
13. Open the tube and dry the beads at 37 °C for 4–5 min (*see* **Note 18**).
14. Add 20 µL of resuspension buffer (RSB). Thoroughly resuspend the beads by gently pipetting the entire volume up and down.
15. Incubate at 22 °C (or room temperature) for 5 min in the thermomixer, under moderate agitation (600 rpm).
16. Pulse-spin, then place the tube on the magnetic stand for 5 min.
17. Transfer 17.5 µL of the supernatant to a new tube. It is possible to stop here. If so, store the tube at −20 °C.

3.3.4 Adenylation of 3′ Ends

1. Add 12.5 µL of thawed A-tailing (ATL) Mix. Gently pipette the entire volume up and down to mix thoroughly.
2. Place the tube in a thermal cycler (with the lid set to 100 °C). Run the following program: 37 °C for 30 min, 70 °C for 5 min, hold at 4 °C.
3. Remove the tube from the cycler as soon as it reaches 4 °C and centrifuge briefly. Place the tube on ice and proceed immediately to the ligation of adapters.

3.3.5 Ligation of Adapters and Cleanup

1. For each library, premix 2.5 µL of resuspension buffer (RSB), 2.5 µL of the selected RNA UD index adapter and 2.5 µL of Ligation Mix (LIG) in a separate 0.2 mL tube. Each sample should be barcoded with a different index. *See* **Notes 19** and **20**.

2. Preheat a thermal cycler at 30 °C (lid set to 100 °C).
3. Briefly centrifuge the tube containing the A-tailed cDNA.
4. Transfer the A-tailed DNA (30 µL) into the tube containing the RSB/Index/LIG premix. Gently pipette the entire volume up and down to mix thoroughly. Centrifuge briefly.
5. Place the tube on the preheated thermal cycler. Incubate at 30 °C for 10 min.
6. Remove the tube from the cycler. Add 5 µL of Stop Ligation Buffer (STL). Gently pipette the entire volume up and down to mix thoroughly. Centrifuge briefly. Transfer in a 1.5 mL tube.
7. Vortex the AMPure XP beads until they are well dispersed (*see* **Note 17**), then add 42 µL of beads to the sample. Gently pipette the entire volume up and down to mix thoroughly.
8. Proceed to the cleanup of the ligated cDNA as described in **steps 7–17** of Subheading 3.3.3, with the following modifications. After the second wash in 80% ethanol, dry the beads for maximum 2 min at 37 °C (*see* **Note 18**). Resuspend the dried beads in 52.5 µL of Resuspension Buffer (RSB). After elution, transfer 50 µL of the cleared supernatant to a new 1.5 mL tube.
9. Perform a second cleanup by adding 50 µL of AMPure XP beads to the sample as described above. For the elution, resuspend the dried beads in 23 µL of Resuspension Buffer (RSB), and finally recover 20 µL of the cleared supernatant into a new 0.2 mL tube. It is possible to stop here. If so, store the tube at −20 °C.

3.3.6 Amplification of DNA Fragments and Cleanup

1. Add 5 µL of thawed PCR primer cocktail (PPC) to the sample.
2. Add 25 µL of thawed PCR Master Mix (PMM). Gently pipette the entire volume up and down to mix thoroughly.
3. Place the tube in a thermal cycler. Run the following program (lid closed and set to 100 °C): 98 °C for 30 s, 12 cycles (98 °C for 10 s, 60 °C for 30 s, 72 °C for 30 s), 72 °C for 5 min, hold at 4 °C.
4. Remove the tube from the cycler.
5. Vortex the AMPure XP beads until they are well dispersed (*see* **Note 17**), then add 50 µL of AMPure XP beads to the tube containing the amplified DNA. Gently pipette the entire volume up and down to mix thoroughly.
6. Proceed to the cleanup of the amplified libraries using 50 µL of AMPure XP beads as described in Subheading 3.3.5. For the elution, resuspend the dried beads in 34 µL of Resuspension Buffer (RSB). Recover 30 µL of the cleared supernatant to a new tube.
7. Store the purified libraries at −20 °C.

3.4 Quality Controls

1. Quantify the libraries using the Qubit dsDNA HS Assay Kit. Use 2 μL of each library for the quantification.
2. Dilute each library at 2 ng/μL in Resuspension Buffer or nuclease-free water. Load 1 μL of the diluted library on an Agilent DNA High Sensitivity chip or an Agilent High Sensitivity D1000 ScreenTape, following the manufacturer's instructions. This analysis will give the size distribution and the molarity of the diluted library (Fig. 3).

3.5 Sequencing and Data Processing

1. Prepare a pool of libraries by mixing equimolar amounts of each diluted library. Refer to your sequencing facility for instruction and to define the sequencing parameters (*see* **Note 21**).
2. After demultiplexing, trim the reads using Trim Galore.
   For single-end sequencing:

```
trim_galore --gzip --length 20 --trim-n reads_1.fastq.gz
```

   The trimmed file is named "reads_1_trimmed.fq.gz".
   For paired-end sequencing:

```
trim_galore --gzip --length 20 --trim-n --paired reads_1.fastq.gz reads_2.fastq.gz
```

   The trimmed files are named "reads_1_val_1.fq.gz" and "reads_2_val_2.fq.gz".
3. Map the reads on the *S. cerevisiae* reference genome (R64-2-1, including the 2-micron plasmid) and ERCC spike-in RNA sequences using HISAT2 v2.2.0 with default parameters and maximum intron size of 5000 (*see* **Note 22**).
   First, build genome index:

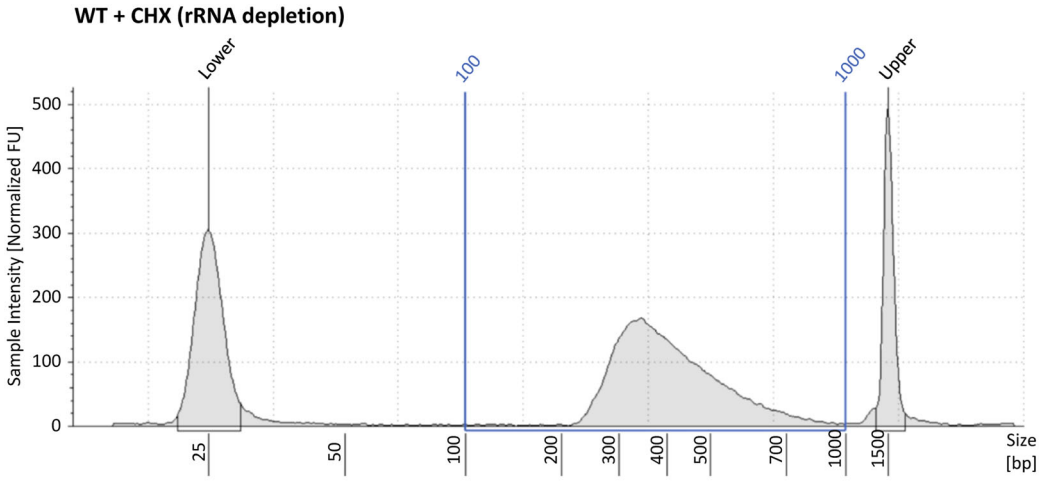
```
mkdir genome_index
hisat2-build genome.fa genome_index/index
```

   Map the reads, filtering out those with mapping score <30 and sort bam file (this requires Samtools).
   For single-end reads:

```
hisat2 --max-intronlen 5000 -x genome_index/index -U reads_1_trimmed.fq.gz | samtools view -bh -q 30 -F 0x100 -F 0x800 | samtools sort -o reads.bam -
```

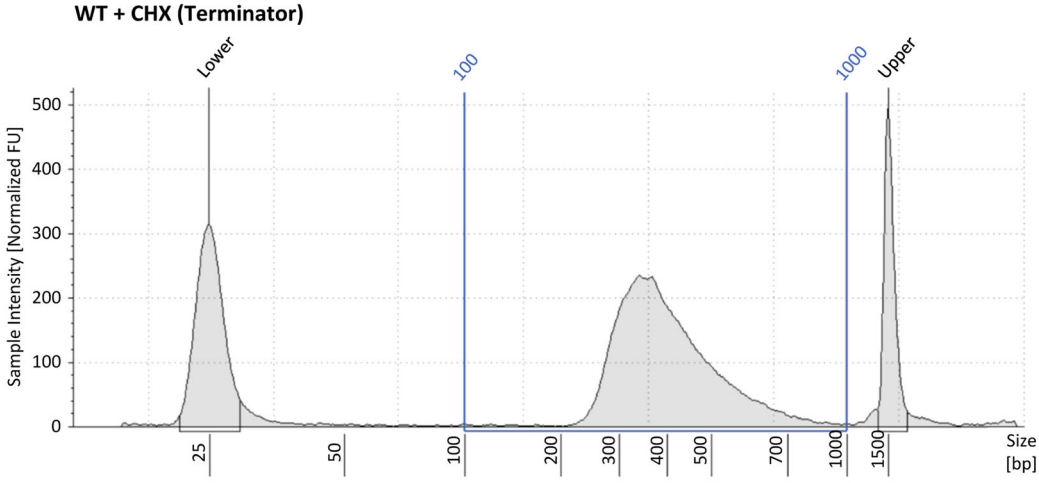
   For paired-end reads:

```
hisat2 --max-intronlen 5000 -x genome_index/index -1 reads_1_val_1.fq.gz -2 reads_2_val_2.fq.gz | samtools view -bh -q 30 -F 0x100 -F 0x800 -f 0x2 | samtools sort -o reads.bam -
```



Region Table

From (bp)	To (bp)	Average size (bp)	Concentration (pg/µL)	Region molarity (pmol/L)	% of total
100	1000	415	768	3120	91.42



RegionTable

From (bp)	To (bp)	Average size (bp)	Concentration (pg/µL)	Region molarity (pmol/L)	% of total
100	1000	409	971	3970	92.25

Fig. 3 Samples (2 ng) of final RNA-Seq libraries constructed from rRNA-depleted RNA and Terminator-digested RNA were analyzed using a High Sensitivity D1000 ScreenTape in a 4200 TapeStation system

Index the bam file:

```
samtools index reads.bam
```

4. Obtain gene counts using featureCounts v2.0.0 (*see* **Note 23**).
   For single-end reads:

```
featureCounts -O --fraction -M -s 2 -a annotation.gtf -o
counts.tsv reads.bam
```

   For paired-end reads:

```
featureCounts -O --fraction -M -s 2 -p -a annotation.gtf -o
counts.tsv reads.bam
```

5. Normalize gene counts using the estimatesizefactorsformatrix () function from the DESeq2 package (*see* **Note 24**).

```
sf <- DESeq2::estimateSizeFactorsForMatrix(counts)
norm_counts <- t(t(counts)/sf)
```

6. Compute normalized densities for each sample as gene count / gene length.

```
norm_densities <- norm_counts / gene_length
```

Figures 4 and 5 show illustrative results obtained upon analysis of the 5′ cap status of mRNAs and XUTs in yeast cells treated with cycloheximide (CHX), a translation elongation inhibitor that was previously proposed to interfere with decapping [29] and that was recently shown to lead to a global stabilization of XUTs [30]. Firstly, we controlled that both ERCC spike-in RNAs and snoRNAs behave similarly (the corresponding dots are mostly dispersed on the equality diagonal), which indicates that the latter are globally not affected by CHX treatment nor Terminator digestion (Fig. 4a, b). Secondly, differential expression analysis using DESeq2 identified significantly upregulated mRNAs and XUTs in CHX-treated cells (Fig. 4c). The same analysis using the data obtained from Terminator-digested RNAs highlights the subset

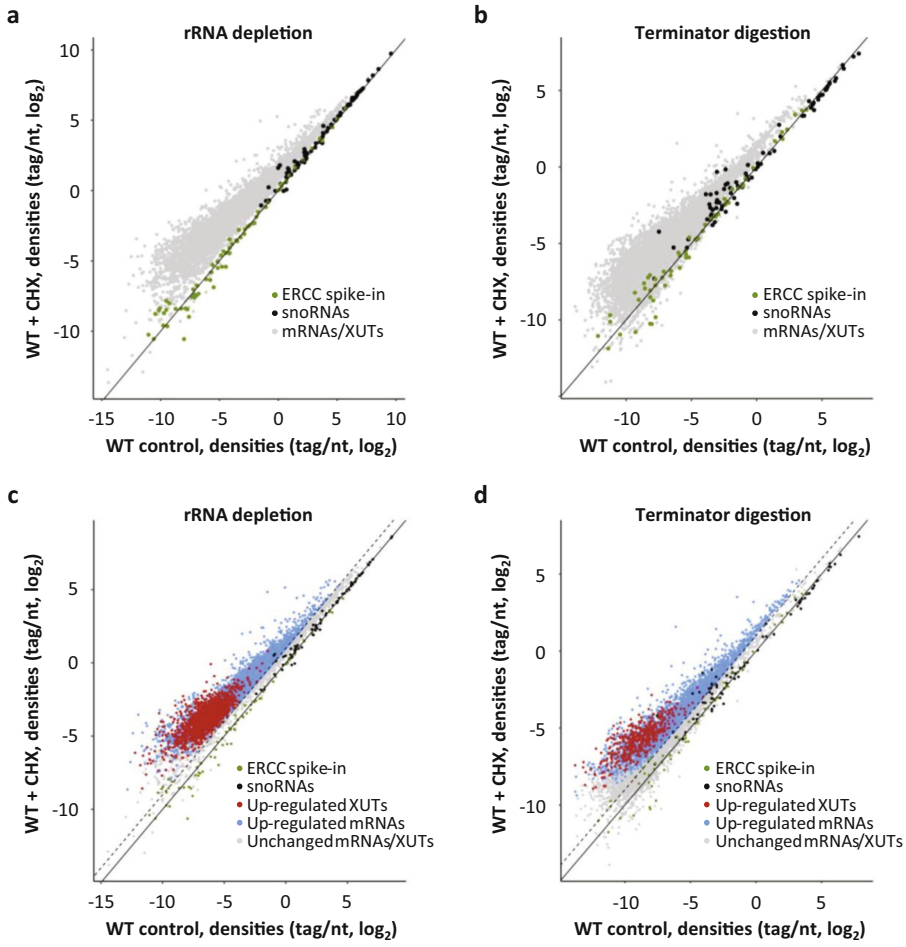


Fig. 4 (**a**) Scatter-plot of RNA-Seq signals for ERCC spike-in RNAs (green) and snoRNAs (black) in CHX-treated and control WT cells, for libraries prepared from rRNA-depleted RNA. The gray dots represent mRNAs and XUTs. Black diagonal: equality ($x = y$). (**b**) Same as above for libraries prepared from Terminator-digested RNA. (**c**) Same as (**a**) highlighting CHX-sensitive mRNAs (blue) and XUTs (red) that significantly accumulate in CHX-treated cells (fold-change >2, *P*-value <0.05 upon differential expression analysis using DESeq2). The gray dots correspond to CHX-insensitive mRNAs and XUTs. Dashed diagonal: fold-change (y/x) = 2. (**d**) Same as (**c**) for libraries prepared from Terminator-digested RNAs. The blue and red dots correspond to the mRNAs and XUTs, respectively, that accumulate in CHX-treated cells as Terminator-resistant, i.e. capped RNAs. *See* **Note 25**

of mRNAs and XUTs that accumulate in CHX-treated cells as capped RNAs (Fig. 4d). Finally, snapshots allow to visualize RNA-Seq signals for selected XUTs that exist in CHX-treated cells as decapped or capped RNA (Fig. 5a, b).

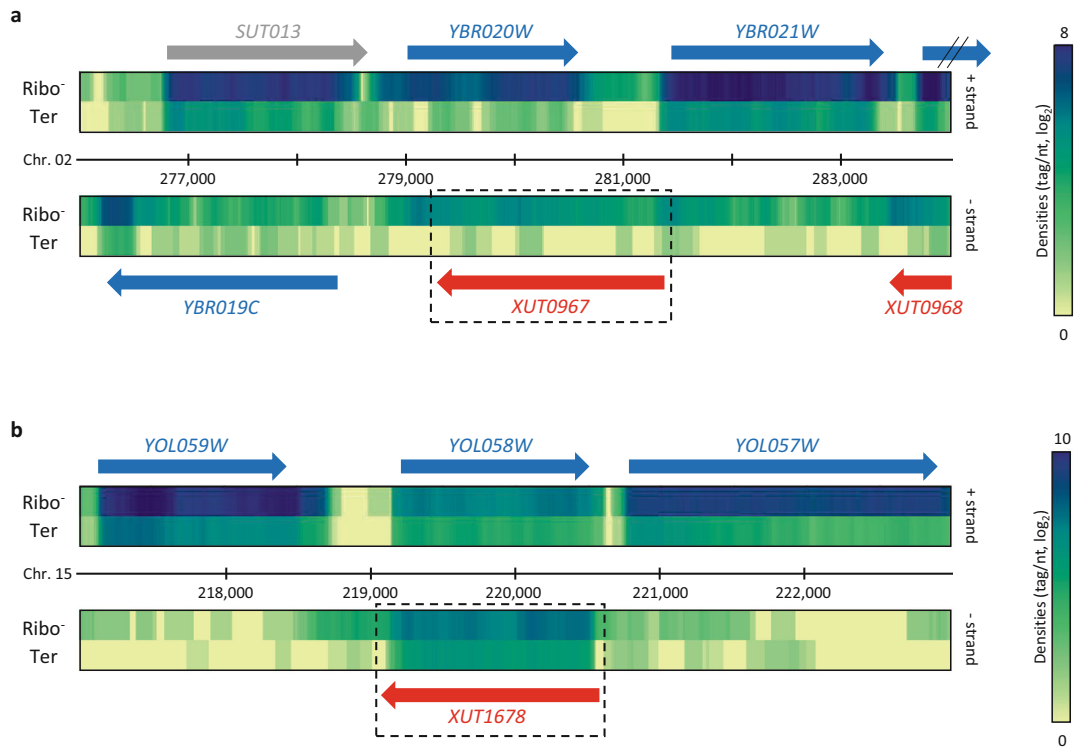


Fig. 5 (**a**) Snapshot of RNA-Seq signal along the *YBR020W/XUT0967* locus in CHX-treated WT cells, for libraries prepared from rRNA-depleted RNA (Ribo$^-$) or Terminator-digested RNA (Ter). The signals for the + and − strands are visualized as heat maps in the upper and lower panels, respectively, using the VING software [28]. The dashed box highlights that *XUT0967* is not detected anymore upon Terminator digestion, indicating that it is mostly decapped in CHX-treated cells. (**b**) Same as above for the *YOL058W/XUT1678* locus. *XUT1678* is still detected following Terminator digestion, indicating that at least a fraction of this transcript exists as capped RNA in CHX-treated cells

4 Notes

1. Different methods for total RNA extraction from yeast exist. Elsewhere in this volume, we provide a protocol adapted from the standard hot-phenol procedure. Regarding the yeast strains to test, we recommend including a decapping mutant (such as the conditional *dcp2-7* allele) and an *xrn1Δ* mutant, which will accumulate capped and decapped decay intermediates, respectively [20].
2. Commercial XRN-1 could be used as an alternative to the Terminator 5′-Phosphate-Dependent Exonuclease. Both enzymes specifically degrade RNA with 5′ monophosphate. Recombinant Xrn1 could also be produced in-house, as described by Drążkowska and colleagues in this volume.

3. Using the RiboMinus concentration module (or equivalent column-based purification kit) is critical to purify rRNA-depleted RNA and remove any trace of the hybridization buffer following rRNA depletion using the RiboMinus Eukaryote Kit v2. Ethanol precipitation is not appropriate for that. We failed to obtain libraries when we used rRNA-depleted RNA that was purified by ethanol precipitation instead of the RiboMinus concentration module.
4. Kits from other manufacturers can be used to prepare the RNA-Seq libraries. In our case, as rRNA depletion is performed using the RiboMinus kit, we construct libraries using the TruSeq mRNA stranded library preparation kit, which is much cheaper than the TruSeq Total RNA stranded library preparation kit. Both kits contain essentially the same components for the construction of the libraries per se. The only exception is the concentration of the Fragment, Prime, Finish mix which is provided as a 1× solution in the TruSeq mRNA stranded library preparation kit and as a 2× solution in the TruSeq Total RNA stranded library preparation kit.
5. If the digested RNA has to be analyzed by Northern blotting, we recommend increasing the quantity of input material (10 μg of total RNA). Increase the quantity of the enzyme proportionally.
6. It is important to control that all RNA samples are precisely diluted to the same concentration before starting to reduce the impact of pipetting errors. We recommend diluting the samples to 100 ng/μL.
7. We recommend preparing a premix that contains all the components of the reaction (including the diluted ERCC spike-in RNA), except the total RNA. Prepare this premix with a minimum excess of 10% to account for potential pipetting errors.
8. Upon Terminator treatment, the prominent peaks corresponding to the 25S and 18S rRNAs should disappear, as these RNAs carry 5′ monophosphate extremities.
9. We recommend preparing a premix (90 μL per sample) that contains the diluted ERCC spike-in RNA, the probe mix, the preheated 2× hybridization buffer, and nuclease-free water. Include a minimum excess of 10% to account for pipetting errors.
10. The binding step is performed with a 60% ethanol concentration to ensure recovery of all RNA species, including those of <200 nt, which are typically not retained using standard silica binding conditions. This is essential to keep snoRNAs, which can be used for normalization.

11. Following rRNA-depletion, the intensity of the peaks corresponding to rRNAs should strongly decrease.
12. For the construction of libraries, we use 50–100 ng of rRNA-depleted RNA as starting material. For the Terminator-digested RNA, we use the rest of the sample.
13. Low or mid-heating during the concentration process does not alter RNA integrity. To avoid overdrying the RNA, take the samples out of the concentrator as soon as the last trace of liquid disappears.
14. The Fragment, Prime, Finish mix contains random hexamers for RT priming and serves as the first strand cDNA synthesis reaction buffer.
15. The fragmentation time can be adjusted. The Illumina protocol recommends 8 min at 94 °C. We use to decrease this time to 3 min in order to generate larger RNA fragments.
16. If the temperature of the lid cannot be set to 30 °C, leave it open.
17. The AMPure XP beads should be brought at room temperature at least 30 min before use.
18. The beads must be dried, but avoid overdrying them. Remove all ethanol after the second wash. Stop drying as soon as the pellet of beads appears cracked (this reduces the efficiency of elution). It can take 4–5 min for 90 μL of beads during the first cleanup in Subheading 3.3.3, but it takes less than 2 min for smaller volumes (42.5 or 50 μL of beads) in Subheadings 3.3.5 and 3.3.6.
19. Check the Illumina protocol and/or Illumina website for information about the compatibility between indexes, or ask for instructions at the facility that will sequence your libraries. Carefully write the correspondence between each sample and its associated index. This information will be essential for RNA-Seq data demultiplexing.
20. The premixes can be prepared during the previous step (adenylation of 3′ ends) and kept on ice. The ligation mix should be added last.
21. For libraries prepared from yeast RNA, we usually use paired-end sequencing (2 × 50 nt). Sequencing using a NovaSeq 6000 system (as it is the case for the data described in this chapter) requires unique dual (UD) indexes to be used during libraries preparation (*see* Subheadings 2.3 and 3.3.5).
22. The value of maximum intron size can be adjusted.
23. The value of the "s" parameter depends on the type of library: 0 for unstranded, 1 for same strand, 2 for opposite strand.

24. Usually, we first normalize gene counts on the ERCC RNA spike-in signal to control that snoRNAs expression is not affected in the mutant/condition analyzed. If so, snoRNA counts are then used for normalization. SnoRNAs are not affected upon decapping or Xrn1 inactivation [20], but this is not true in other conditions, for example, when the RNA helicase Mtr4 is depleted [31].
25. The raw data used to prepare these figures can be accessed from the NCBI Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) using accession numbers GSE203277 and GSE203280.

Acknowledgments

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Part IV

Labeling-Based Methods

Chapter 10

RNA Decay Assay: 5-Ethynyl-Uridine Labeling and Chasing

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Abstract

Eukaryotic RNA synthesis and degradation are intricately regulated, impacting on gene expression dynamics. RNA stability varies in individual transcripts and is modulated by *trans*-acting factors such as microRNAs, long noncoding RNAs, and RNA-binding proteins, which determine protein output and subsequent cellular processes. To measure RNA decay rate, accurate and reliable methodologies are essential in the field of RNA biology. Transcription inhibition and metabolic labeling enable comprehensive investigations on RNA decay, offering critical insights into dynamic regulation of RNA decay. Transcription shut-off has been employed widely by using various approaches, such as treatment with chemical inhibitors or generation of temperature-sensitive mutants of RNA polymerases. However, it has limitations, providing a static view and lacking real-time dynamics as well as precise measurement of decay rate. Metabolic labeling, a method of incorporating modified nucleotides into RNA transcripts, complements shut-off approaches, allowing selective monitoring of newly synthesized RNA and tracing decay intermediates. The purpose of the protocol described in this chapter is to assess the kinetics and statics of newly synthesized RNA and its decay by 5-ethynyl uridine labeling.

Key words mRNA decay, Metabolic labeling, qPCR

1 Introduction

In eukaryotic cells, the process of RNA synthesis and degradation is tightly regulated through various steps at both the transcriptional and posttranscriptional levels [1]. The ultimate abundance of mature RNA is determined by the delicate balance between its synthesis and degradation [2]. This balance can be altered by external or internal trans-acting factors such as RNA-binding proteins and regulatory noncoding RNAs, which subsequently lead to changes in cellular dynamics [3]. The rate at which RNA is degraded determines how quickly each transcript can adapt to a new steady-state level after changes in transcription [4]. Precisely

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regulating the decay of particular transcripts is essential for their regulatory dynamics, allowing cells to respond and adapt to environmental cues, ensuring proper gene expression and cellular functions [5, 6].

In mammalian cells, a subset of RNAs undergo rapid degradation, restricting their protein output and facilitating prompt adjustments in response to cellular signals. Conversely, other RNAs persist in the cytoplasm for longer time, optimizing protein production per transcript but impeding swift expression changes under altered cellular conditions [7]. Therefore, measurement of RNA decay is essential when assessing RNA abundance [8, 9]. Comprehending the impact of these mechanisms on gene expression necessitates reliable methodologies for measuring decay rates.

Recent studies highlighted long noncoding RNAs (lncRNAs) as significant regulators of mRNA and miRNA decay, alongside RNA binding-proteins (RBPs). RBPs play a crucial role in influencing lncRNA stability, functions, and their target RNAs at various stages [10, 11]. This regulatory process occurs in both the cytoplasm and nucleoplasm, involving molecular mechanisms such as deadenylation and decapping. The 3′ poly(A) tail on RNAs is crucial for protein synthesis and is tightly associated with cytoplasmic poly(A)-binding protein (Pab1/PABPC1), facilitating RNA translation and protecting RNAs against degradation [12]. However, deadenylation catalyzed by the CCR4–NOT and the PAN2/3 complex leads to translational repression, marking a key step in mRNA decay [13]. Subsequently, deadenylated mRNA undergoes degradation via decapping by DCP1/DCP2 and exoribonuclease XRN1 [14] or 3′-5′ exonucleolytic decay by exosome complex [15]. Longer poly(A) tails enhances translational efficiency whereas deadenylation promotes RNA decay [16]. Decapping is also activated in specialized 5′-3′ RNA decay pathways, such as Nonsense-Mediated mRNA Decay (NMD) and AU-rich element (ARE) RNA-mediated decay [17]. NMD targets mRNAs containing premature termination codons, initiating mRNA decapping and decay [18, 19]. AREs, recognized by RBPs tristetraprolin (TTP) and butyrate response factor 1 undergo 3′-5′ exonucleolytic decay [20]. However, RBPs such as HuR inhibit the decay of mRNA that contains AREs [21].

Various methods have been used to inhibit transcription. Initially, radiolabeling and inducible/repressible promoters were common but lacked transcriptome-wide applicability [5, 22, 23]. Chemical inhibitors like flavopiridol (an inhibitor of the activity of CDKs phosphorylating RNA polymerase II, thus halting transcription), actinomycin D (a DNA intercalator preventing the movement of RNA polymerase II), and α-amanitin (an inhibitor of RNA polymerase II), have become preferred for high-throughput analyses upon transcription inhibition [24–26]. Transcription shut-off lacks real-time dynamics and precise decay rate measurement,

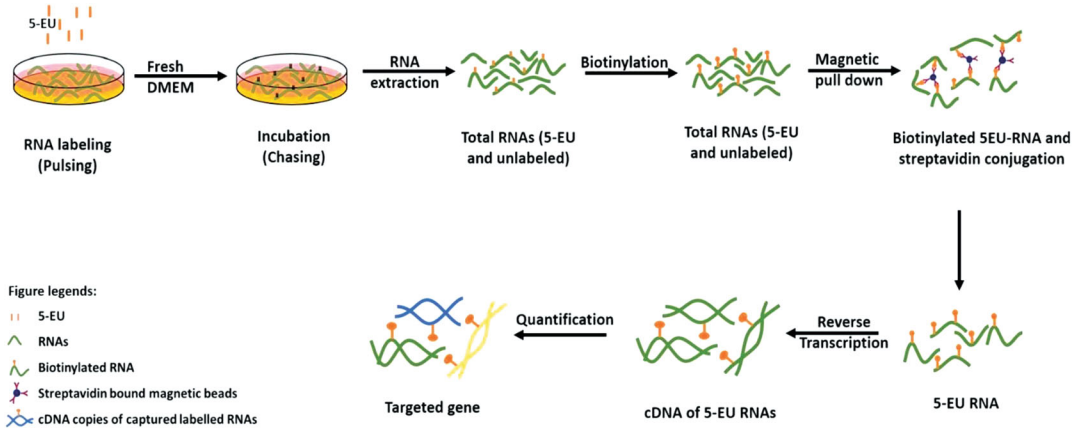

Fig. 1 Schematic representation of 5-EU-labeled RNA and its isolation

with challenges including cellular stress responses and difficulty in distinguishing individual degradation pathways [23, 27]. To overcome these challenges, metabolic labeling has demonstrated the highest adaptability and is widely used to study RNA decay [28, 29]. It enables thorough transcriptome-wide investigations, offering valuable insights into gene expression dynamics by analyzing RNA turnover under various conditions [23]. This method involves incorporating modified nucleotide analogs such as 5-ethynyl uridine (5-EU) and 4-thiouridine (4-SU) into newly synthesized RNA, easily detectable and trackable using click chemistry or other labeling techniques (Fig. 1). This facilitates selective labeling and monitoring of RNA turnover, providing insights into gene expression dynamics [30, 31]. By using metabolic labeling, this approach can selectively label and monitor newly synthesized RNA molecules and trace their decay.

2 Materials

2.1 Mammalian Cell Culture and RNA Labeling

1. Dulbecco's Modified Eagle Medium (DMEM) (*see* **Note 1**).
2. Fetal bovine serum (FBS).
3. 10,000 U/mL Penicillin/streptomycin.
4. Dimethylsulfoxide (DMSO) (*see* **Note 2**).
5. 100 or 150 mm culture dishes.

2.2 RNA Extraction, RNA Biotinylation and Purification of Biotinylated RNA, Reverse Transcription and qPCR

1. 1× Phosphate-Buffered Saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4.
2. Trizol.
3. Chloroform.
4. Isopropanol.
5. 200 proof, anhydrous 99.9% ethanol.

6. 200 mM 5-EU stock solution. Dissolve 5 mg of 5-EU in 93 µL of DMSO. Vigorously vortex the solution; it will be dark brown. Store at −20 °C (*see* **Note 2**).
7. 2× 5-EU buffer: 100 mM HEPES pH 7.5, 10 mM EDTA.
8. 10 mM biotin azide. Dissolve 1 mg of biotin azide in 162.2 µL of DMSO. Vortex and store at −20 °C in the dark (*see* **Note 3**).
9. 25 mM copper (II) sulfate ($CuSO_4$). First, prepare a 100 mM stock solution by dissolving 16 mg of $CuSO_4$ in 1 mL of nuclease-free distilled water. Take 50 µL from this stock solution and add 150 µL of nuclease-free distilled water. Store at 4 °C for further use.
10. 400 mM Tris[(1-hydroxy-propyl-1H-1,2,3-triazol-4-yl) methyl]amine (THPTA). Dissolve 17.38 mg of THPTA in 100 µL of nuclease-free distilled water, mix well, and store at −20 °C for further use.
11. 400 mM sodium ascorbate (reducing agent). Dissolve 396.22 mg of sodium ascorbate in 5 mL of nuclease-free distilled water. Divide into aliquots and store at −20 °C in the dark. Freeze–thaw repeatedly but discard if it yellows.
12. GlycoBlue coprecipitant.
13. 7.5 M Ammonium Acetate.
14. Nuclease-free distilled water.
15. Dynabeads MyOne Streptavidin T1 or NEB Streptavidin Magnetic Beads (*see* **Note 4**).
16. Bead binding buffer (BBB): 1 M NaCl, 100 mM Tris-HCl pH 7.0, 10 mM EDTA, 0.2% (v/v) Tween-20.
17. RNaseOFF RNase inhibitor (40 U/µL).
18. Wash buffer 1: 0.4 M NaCl, 100 mM Tris-HCl pH 7.0, 10 mM EDTA, 0.2% (v/v) Tween-20.
19. Elution buffer: 10 mM Tris pH 7.4, 1 mM EDTA pH 8.0.
20. RNase-free 1.5 mL tubes.
21. Refrigerated centrifuge.
22. Thermomixer and classic vortex.
23. UV spectrophotometer.
24. Magnetic stand.
25. 100 mM dNTP.
26. 10 µM N9 Random primer.
27. 5× RNA buffer: 250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM dithiothreitol.
28. Maxima Reverse Transcriptase (200 U/µL).
29. RNaseOFF RNase inhibitor (40 U/µL).

30. PowerTrack™ SYBR Green Master Mix for qPCR. This kit includes the 2× SYBR Green Master Mix and the 40× Yellow Sample Buffer.
31. RNase-free PCR tubes.
32. PCR thermal cycler.
33. 48 well or 96 well or 384 well qPCR plates.
34. qPCR instrument.

3 Methods

3.1 Mammalian Cell Culture and RNA Labeling

1. Expand mammalian cell lines in a 100 or 150 mm culture dish using DMEM supplemented with 10% (v/v) fetal bovine serum and 100 U/mL penicillin/streptomycin. Grow cells up to 80% confluence. After 80% confluence, transfer the cells to a new culture dish and seed them again (*see* **Note 5**).
2. Add 200 mM of 5-EU stock solution (0.2 mM final) into the media and incubate the cells at 37 °C for 1 h for pulsing (*see* **Note 6**).
3. After incubation, change the 5-EU-containing medium by a medium lacking 5-EU and harvest the cells at 0, 1, 2 and 3 h depending on the estimated half-life.

3.2 Total RNA Extraction by the Trizol Method

1. Wash the cells twice with 10 mL of PBS and then add 1 mL of Trizol to lyse the cells.
2. Transfer the lysate to a 1.5 mL RNase-free tube by scrapper and pipet.
3. Add 200 μL of chloroform and shake vigorously for 10 s.
4. Incubate for 10 min at room temperature.
5. Centrifuge the sample for 15 min at 12,000× *g*, 4 °C.
6. Transfer the aqueous phase (400 μL) containing RNA to a new tube.
7. Add 0.5 mL of isopropanol to the aqueous phase, shake vigorously for 10 s, and then incubate for 10 min at 4 °C.
8. Centrifuge for 15 min at 12,000× *g* at 4 °C and discard the supernatant.
9. Resuspend the pellet in 1 mL of ice-cold 75% ethanol.
10. Invert the sample briefly, then centrifuge for 5 min at 7500× *g* at 4 °C, and discard the supernatant. Make sure to discard all the supernatant.
11. Open the tubes and let the RNA pellet dry at room temperature for 10 min in clean-bench and add 50 μL of nuclease-free distilled water.

12. Measure the RNA concentration and then dilute the RNA with nuclease-free distilled water to have a concentration of 1–5 μg/μL for the click reaction.

3.3 Biotinylation of RNA

1. Thaw the 10 mM biotin azide, THPTA, and sodium ascorbate stock at room temperature.
2. Prepare the click reaction mixture as follows: 1 μL (5 μg) of total RNA, 25 μL of 2× 5-EU buffer (1× final concentration), 4 μL of 25 mM $CuS0_4$ (2 mM final concentration) 2.5 μL of 10 mM biotin azide (0.5 mM final concentration), and 14.75 μL of nuclease-free distilled water (*see* **Notes 7** and **8**).
3. After adding each component, mix the cocktail gently by pipetting up and down.
4. Add 1.25 μL of 400 mM THPTA (10 mM final concentration), immediately mix the cocktail by gently pipetting it and incubate for 3 min.
5. After the 3 min incubation with THPTA, add 1.5 μL of 400 mM sodium ascorbate (12 mM final concentration). This initiates the click reaction between 5-EU-labeled RNA and biotin azide. It should turn dark brown (*see* **Notes 7** and **8**).
6. Incubate this reaction mixture cocktail for 2 h by gently mixing (50–70 rpm) at room temperature (21–25 °C).

3.4 Precipitation of Biotinylated RNAs

1. Add 1 μL of GlycoBlue coprecipitant, 50 μL of 7.5 M ammonium acetate, and 700 μL of chilled 100% ethanol to the biotinylated RNA sample. Mix it by inverting the tubes or gently pipetting up and down.
2. Incubate the tube overnight at −70 to −80 °C.
3. Centrifuge the tube at 13,000× *g* for 20 min at 4 °C.
4. Remove the supernatant without disturbing the RNA pellet and then add 700 μL of chilled 75% ethanol. Vortex the tube briefly and centrifuge it at 13,000× *g* for 5 min at 4 °C.
5. Repeat **step 4** once, and discard all the supernatant without disturbing the RNA pellet. Let the pellet dry in open tubes for 10 min at clean bench, and resuspend it in 30 μL of nuclease-free distilled water.
6. Measure the RNA concentration using a UV spectrophotometer. Typical RNA yield after this procedure is approximately 70% of the starting material. At this step, RNA can be stored at −20 °C or can be used further (*see* **Note 9**).

3.5 Binding of Biotinylated RNA to Magnetic Beads

1. Prepare 20 μL of Dynabeads MyOne Streptavidin T1 in 1.5 mL tubes.

2. Centrifuge the beads mix at 2000× *g* for 1 min at 4 °C and remove the supernatant.
3. Wash the beads with 500 μL of BBB. Place the tube on a magnet for 2 min and discard the supernatant.
4. Repeat **step 3**, for a total of two washes.
5. After the second wash, resuspend the beads in 200 μL of BBB.
6. Prepare the RNA-binding reaction mix in a 1.5 mL tube as follows: 75 μL of 2× BBB, 1 μL of RNase inhibitor (40 U), 30 μL of RNA (from **step 5** of Subheading 3.4), and 44 μL of nuclease-free distilled water. The final volume is 150 μL.
7. Heat the RNA-binding reaction mix at 70 °C for 5 min.
8. Add 20 μL of beads suspension to the preheated RNA binding reaction mixture.
9. Incubate the RNA-binding reactions at room temperature (21–25 °C) for 2 h while incubating them on a thermomixer. This will prevent settling of the beads (*see* **Note 10**).
10. After the incubation, wash the beads with 200 μL of wash buffer 1 (or 10 volumes of resuspend beads). Spin down and discard the supernatant. Repeat this step five times.
11. Wash three times with 200 μL of 1× PBS. Spin down and discard the supernatant.

3.6 Elution of Captured Beads from RNA

1. Elute the RNA by resuspending the beads in 30 μL of elution buffer and then incubating at 92 °C for 4 min with shaking at 450 rpm at thermomixer.
2. Place the tube on the magnet for 1 min and save the supernatant, which contains the 5-EU-labeled RNA.
3. Measure the RNA concentration using a UV spectrophotometer. At this step, RNA can be either stored −20 °C or can be used further.

3.7 cDNA Synthesis from 5-EU-Labeled RNA and qPCR

1. Add the following components to an RNase-free PCR tube: 12 μL of 5-EU-labeled RNA, 0.75 μL of 10 mM dNTPs (final concentration 0.5 mM), 0.75 μL of 10 μM N9 random primer (final concentration 0.5 mM), and 1.5 μL of nuclease-free distilled water. The total volume is 15 μL.
2. Incubate at 65 °C for 5 min and then at 4 °C for 5 min.
3. To perform cDNA synthesis (reverse transcription), add the following components to the 15 μL from step 1: 4 μL of 5× RT buffer, 0.5 μL of reverse transcriptase (100 U), and 0.5 μL of RNase inhibitor (20 U). The total volume is now 20 μL.
4. Run the following cycle for reverse transcription: 10 min at 25 °C followed by 30 min at 50 °C; then inactivate the RT at 85 °C for 5 min.

5. Store the cDNA at −20 °C for further use.
6. For the qPCR, mix the following components: 2 μL of cDNA, 10 μL of 2× SYBR Green Master Mix, 0.5 μL of 40× Yellow Sample Buffer, 1 μL of 100 pM forward primer, 1 μL of 100 pM reverse primer, and 5.5 μL of nuclease-free distilled water. The total volume is 20 μL.
7. Run the following program: 2 min at 50 °C; 10 min at 95 °C; 40 cycles (15 s at 95 °C, 1 min at 60 °C); melting curve analysis: 95 °C 15 s, 60 °C 1 min, 95 °C 1 s- Step and hold 0.05 s- 1 cycle.

4 Notes

1. Dulbecco's Modified Eagle Medium (DMEM) contains pyruvate and glutamine. Researchers can modify or change the product according to their study.
2. Since 5-EU has a good solubility in DMSO, we used DMSO to solubilize 5-EU. It is known that DMSO may affect the cell viability and cellular process including RNA decay and protein synthesis. Thus, before starting the experiment, researchers should take this into consideration and include a DMSO control to eliminate interference in their study due to the addition of DMSO. However, 5-EU is readily soluble in alcohol, water, or aqueous buffers.
3. It is recommended to dissolve biotin azide in high-quality, anhydrous dimethylformamide (DMF) or DMSO.
4. Dynabeads MyOne Streptavidin T1 are magnetic beads coupled to streptavidin. Streptavidin binds to biotin and this interaction is very strong to isolate biomolecules of interest. Researchers can use other streptavidin-coated magnetic beads as well.
5. Please see the necessary requirements for the growth of the cells of interest. Cells may be seeded at density of $5 \times 10^5 - 1 \times 10^6$ per mL reaching 40–50% confluence at the time of 5-EU pulsing. To prepare working DMEM media, separate out 55 mL of DMEM and mix 50 mL of FBS (10% working concentration) and 5 mL of 10,000 U/mL of penicillin and streptomycin (100 U/mL or 1% working concentration).
6. Pulses ranging from 20 min to 24 h should be optimized, depending on the experimental condition, objective, and cell type.

7. The timing for addition of the THPTA and sodium ascorbate is crucial, and it must be followed as closely as possible. This is because THPTA functions as a ligand to stabilize the copper catalyst and prevent its oxidation back to inactive Copper (II) species. If THPTA is added after the reduction step, there may be insufficient THPTA present to effectively coordinate with and stabilize Copper (I) ions, compromising the catalytic activity of the system. Sodium ascorbate is a reducing agent that converts Copper(II) ions to Copper(I) ions, which are the active catalytic species for the CuAAC click reaction. If THPTA is added after the reduction step, it may not efficiently stabilize the Copper (I) ions generated by sodium ascorbate, potentially leading to the formation of inactive copper species or aggregation of copper salts.
8. THPTA and sodium ascorbate should be added last. The $CuSO_4$:THPTA ratio should be 1:5.
9. This step could be used as a stop point. Store the resuspended RNA at −20 °C for future use or store it at 4 °C overnight to proceed to the next step.
10. You may optimize an incubation time from 30 min to 2 h.

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Chapter 11

Assessment of mRNA Decay and Calculation of Codon Occurrence to mRNA Stability Correlation Coefficients after 5-EU Metabolic Labeling

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Abstract

mRNA translation and decay are tightly connected. This chapter describes a method to assess the influence of each codon identity on mRNA stability in cultured cells. The technique involves metabolic labeling of the nascent mRNAs by addition of the nucleoside analog 5-ethynyluridine (5-EU), purification of the RNA at different time-points after chase of the 5-EU, then biotinylation with Click chemistry, pull-down, and sequencing. The transcripts' half-lives are calculated from the expression level of each mRNA at the different time-points. Finally, the method describes the calculation of the Codon occurrence to mRNA Stability correlation Coefficient, or CSC, as a correlation between the codon occurrence in a transcript and the transcript half-life, for each codon.

Key words Metabolic labeling, mRNA decay, Transcriptomics, Codon optimality, CSC

1 Introduction

Messenger RNA (mRNA) stability plays a pivotal role in the cellular regulation of gene expression. Several factors influence the stability of mRNAs—co-transcriptional modifications, posttranscriptional modifications, RNA binding proteins, and *cis* elements such as the 5′ and 3′ untranslated regions (UTRs) [1–4]. Importantly, the vulnerability of each mRNA transcript to degradation is further determined by the efficiency of its translation. During translation elongation, the ribosome's speed is governed by the availability of transfer RNAs (tRNAs) matching each codon, a phenomenon known as codon optimality [5]. It has been shown in several organisms that mRNAs enriched with more optimal codons exhibit heightened stability, whereas those with a less optimal codon composition have shorter half-lives and are more readily targeted and metabolized by the cell's mRNA degradation machinery [6–9]. The influence of codon identity on mRNA stability can be

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quantified as a correlation between the occurrence of each codon in a transcript and the mRNA half-life. This metric is called CSC for Codon occurrence to mRNA Stability correlation Coefficient (in short Codon Stability Coefficient) and may be used as a proxy for codon optimality [10].

To study codon optimality-mediated mRNA decay, one must assess the half-life of each individual transcript on a global scale. In the past, metabolic labeling of macromolecules, such as mRNA transcripts, was a major approach to determining the kinetics of decay [11, 12]. However, these approaches were only suited towards bulk analysis and were unable to provide information on the level of individual transcripts [13]. The subsequent use of reporters allowed for the quantification of individual molecule's half-lives but only on a limited set of transcripts. Transcription inhibitors, such as actinomycin D, also have a long history of use to gain information about dynamic changes in RNA turnover. However, such drugs have the potential to significantly perturb the cell, possibly resulting in pleiotropic consequences including a global shutdown to mRNA translation [14, 15], potentially resulting in erroneous results.

With the advent and subsequent decreasing costs of next-generation sequencing, metabolic labeling has reemerged as an attractive approach to monitor the transcription and decay of any RNA in a dynamic living system, and is a preferred method that minimally disturbs the cell [16]. Various chemical derivatives of uridine can be readily incorporated within RNA strands and are thought to have minimal impact on RNA function or cellular processes [17–19]. For example, providing cells with the modified nucleoside, 5-ethynyluridine (5-EU), allows for its incorporation into newly transcribed RNAs. By pulsing in 5-EU over a time interval followed by a uridine chase, and purifying the labeled RNA at specific time points, all transcripts can be tracked until they decay. Indeed, the addition of biotin via Click chemistry permits the isolation of 5-EU labeled transcripts followed by sequencing (Fig. 1a, b). This workflow allows for the precise calculation of the half-life of each individual mRNA. While this approach may have its own flaws [20–22], it aims to address the biological issues associated with other assays. Optimal strategy will involve leveraging several techniques such as metabolic labeling and reporter assay approaches to validate the obtained results [23]. While several strategies exist [24, 25], here we present one method to label and purify RNAs with 5-EU in mammalian cells, followed by sequencing, analysis of the data, and calculation of mRNA half-lives and CSCs.

(a) An extended pulse of 5-EU on cultured cells allows for metabolic labeling of the RNAs, followed by a chase with uridine, to track the labeled RNAs as they decay over several timepoints.

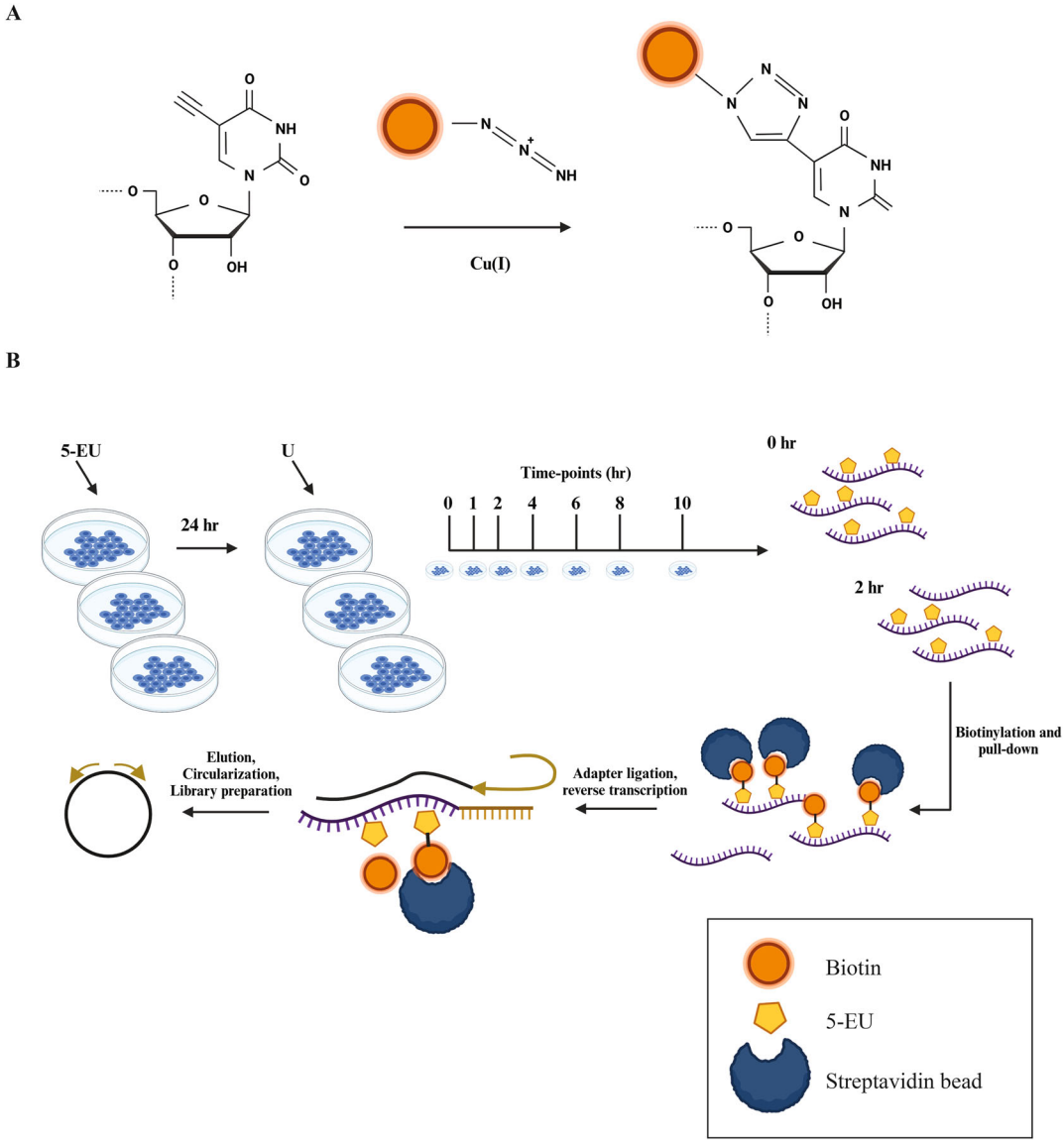

Fig. 1 Workflow of the method. (**a**) Schematic of the uridine analog 5-ethynyluridine 5-EU in RNA. The cycloaddition catalyzed by copper between the alkyne (ethynyl) of 5-EU and the azide (N3) of biotin azide (Click chemistry) leads to a covalent bond between RNA and biotin. (**b**) Cells take up 5-EU that is incorporated into nascent RNAs. After a chase with uridine, cells are collected at different time-points to track the decay of the labeled RNAs (the population of labeled RNA will decrease over time). The RNAs are processed, biotinylated, and pulled down. After adapter ligation and reverse transcription, the cDNAs are eluted, circularized, and amplified before sequencing. Analysis of the reads at each time-point allows for determination of the mRNAs half-lives and calculation of Codon Stability Coefficients. (Created with BioRender.com)

(b) 5-EU labeled spike-in RNAs are prepared by in vitro transcription to add right after RNA isolation.

(c) After ribosomal RNA depletion and controlled fragmentation to prepare for library generation, 5-EU labeled mRNAs are biotinylated by Click chemistry.

(d) Labeled RNAs are pulled down and adapter ligation and reverse transcription are performed on the beads.

(e) The cDNA is eluted from the beads, circularized, and amplified before sequencing.

(f) Simple steps are proposed to analyze the sequencing data and determine the levels of protein-coding transcripts at each time-point, using examples of tools available.

(g) mRNA half-lives are calculated after fitting an exponential decay model, and the calculation of CSCs is described.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C) and analytical grade reagents. Some reagents or kits materials are hazardous materials. Use equipment and practices appropriate for handling such materials. Diligently follow all waste disposal regulations when disposing of waste materials.

2.1 5-EU "Pulse"-Chase and RNA Isolation

1. Cell culture reagents for the cells of interest (*see* **Note 1**).
2. Click-iT® Nascent RNA Capture Kit (Thermofisher Scientific).
3. 5-ethynyluridine (5-EU) solution: use 5-EU powder from the Click-iT® Nascent RNA Capture Kit or another source, to make a 200 mM 5-EU solution in sterile DMSO or DNase/RNase-free water (*see* **Notes 2** and **3**).
4. Uridine solution: use uridine powder to make a 1 M uridine stock in sterile DMSO or DNase/RNase-free water, aliquot and freeze at −20 °C.
5. Trizol.
6. Chloroform.
7. Glycogen or glycoblue.
8. Isopropanol, ethanol.
9. 2× SDS extraction buffer: 1% SDS, 2 mM EDTA pH 8, 40 mM Tris-HCl pH 7.5.
10. 3 M sodium acetate pH 5.2.
11. Phenol, saturated, pH 4.5.

12. Phenol/chloroform/LET: stir the same volume of phenol and LET (100 mM LiCl, 20 mM EDTA, 25 mM Tris-HCl pH 8) for 30 min at room temperature, then let sit for 30 min (in a chemical hood). Decant the bottom layer (phenol) using a separatory fennel, then repeat (stir/sit) with another volume of LET. Decant the phenol phase (as well as a thin layer of LET) then add chloroform in proportions 1:1, stir well, aliquot, and freeze. Aliquots are kept at 4 °C after thawing.

2.2 In Vitro Transcription of Spike-Ins

1. Plasmid (typically pBluescript) containing a sequence exogenous to the genome of the cells to be studied (such as Firefly luciferase), under the control of T7 RNA polymerase promoter, for in vitro transcription of RNA spike-in (*see* **Note 4**).
2. Restriction enzyme to linearize the plasmid downstream of the spike-in sequence.
3. Optional: DNA gel purification kit such as the GenElute kit (Sigma).
4. T7 RNA polymerase in vitro transcription kit such as the HiScribe® T7 High Yield RNA Synthesis Kit (NEB).
5. 5-ethynyl uridine triphosphate (5-EUTP).
6. DNase such as TURBO DNase.

2.3 mRNA Purification, Fragmentation, and Click Chemistry-Mediated Biotinylation

1. Ribosomal RNA depletion kit (*see* **Note 5**).
2. RNA Clean & Concentrator™ Kit (Zymo Research).
3. 2× alkaline fragmentation buffer: 2 mM EDTA, 100 mM $NaCO_3$ pH 9.2 (= 15 parts Na_2CO_3, 110 parts $NaHCO_3$) (*see* **Note 6**).
4. Click-iT® Nascent RNA Capture Kit (Thermofisher Scientific).
5. Ammonium acetate.
6. 10 mM Tris-HCl, pH 8.
7. 2× denaturing loading dye: 98% formamide, 10 mM EDTA, 300 μg/mL bromophenol blue.
8. RNA ultramers to use as size markers for PAGE — to design based on the estimated size of RNA after fragmentation (*see* **Note 7**). Examples of short size are the following:

   34 nt: 5′- AUGUACACGGAGUCGAGCUCAACCCG CAACGCGA-(Phos)-3′

   70 nt: 5′- AUGUACACGGAGUCGAGCUCAACCCG CAACGCGAACCGAUCCUAGAGGUCUAGCAA GAUCGGCCAUGAAU-(Phos)-3′
9. Low molecular weight RNA and DNA ladders such as Low Range ssRNA Ladder

(NEB) and Ultra Low Range DNA Ladder or GeneRuler Low Range DNA Ladder (Thermofisher Scientific).

10. 12% polyacrylamide, 7 M urea, 1× TBE gels, homemade or commercially available such as Novex™ TBE-Urea Gels (*see* **Note 8**).
11. SYBR™ Gold Nucleic Acid Gel Stain (10,000× Concentrate in DMSO).
12. RNA gel extraction buffer: 300 mM NaOAc pH 5.2, 1 mM EDTA, 0.25% (w/v) SDS.
13. Optional: sodium hydroxyde 1 N.

2.4 Pull-Down and On-Beads Library Preparation

1. RNase Inhibitor such as SUPERase•In™ RNase Inhibitor.
2. T4 polynucleotide kinase (T4 PNK).
3. Preadenylated linker: Universal miRNA Cloning Linker 5′--rAppCTGTAGGCACCATCAAT–NH_2-3′ (NEB).
4. T4 RNA Ligase 2, truncated (T4 Rnl2tr).
5. Reverse transcriptase such as Superscript IV or the SuperScript III First-Strand Synthesis SuperMix (ThermoFisher scientific).
6. Reverse transcription primer (*see* **Note 7**): 5′-(Phos)-AGATCGGAAGAGCGTCGTGTAGGGAAAGATCGGACT GTAGAACTCTGAACGTGT-(SpC18)-CACTCA-(SpC18)-CCTTGGCACCCGAGAATTCCAATCTATT-GATGGTGCCTACAG-3′

2.5 Library Preparation and Sequencing

1. DNA gel extraction buffer: 300 mM NaCl, 10 mM Tris pH 8, 1 mM EDTA.
2. CircLigase ssDNA ligase (LGC Biosearch Technologies).
3. High fidelity polymerase such as Phusion polymerase (NEB), or NEBnext Ultra II Q5 next-generation master mix.
4. Forward PCR primer (Illumina Truseq small RNA PCR primer RP1):

   5′-AATGATACGGCGACCACCGAGATCTACACGTTC AGAGTTCTACAGTCCGA-3′
5. Reverse indexed PCR primers (Illumina Truseq small RNA PCR index primers RPI):

   5′-CAAGCAGAAGACGGCATACGAGAT[BARCODE] GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA-3′ (*see* **Note 9**).
6. 6× non-denaturing loading dye: 10 mM Tris pH 8, 1 mM EDTA, 15% (w/v) ficoll 400, 0.25% bromophenol blue.
7. 8% polyacrylamide, 1× TBE gels, homemade or commercially available such as Novex™ TBE Gels, 8% (*see* **Note 8**).

2.6 Equipment

1. Nanodrop.
2. Magnetic rack.
3. Rotisserie microfuge rotator.
4. Thermomixer.
5. XCell SureLock Mini-Cell (ThermoFisher Scientific) or Mini-PROTEAN® handcast system (Biorad).
6. Gel transilluminator.
7. 0.5 mL tubes.
8. Microfuge spin filter tubes.

3 Methods

See **Note 10**.

3.1 5-EU "Pulse"-Chase and RNA Isolation

1. Seed cells in 60 mm diameter dishes. When they reach 40% confluency, replace the media by 5-EU containing media at a final concentration of 0.2 mM (*see* **Notes 11** and **12**).
2. After 24 h, wash the cells once with media without 5-EU, and then replace with media containing uridine at a final concentration of 5 mM (*see* **Note 13**)—this is the chase. For the 0 h time-point (= 24 h labeling), skip this step and collect the cells directly for RNA extraction (*see* **Notes 14** and **15**).
3. At time-points determined for your experiment (*see* **Note 16**), remove and discard the media from a 60 mm dish and add 2 mL of Trizol. Resuspend and lyse the cells by pipetting up and down several times, and transfer each 1 mL to a 1.5 mL microtube (the same RNAs will be pooled together after resuspension) (*see* **Note 17**). Incubate at room temperature for 5 min. The samples can be frozen at −80 °C at this stage.
4. Add 200 μL chloroform for each 1 mL of Trizol, vortex for 15 s, and then sit at room temperature for 3 min. Spin at 12,000× *g* for 15 min at 4 °C.
5. Carefully transfer the aqueous phase to a new 1.5 mL tube, add 1 μL of glycogen, mix, and then add 500 μL of isopropanol. Mix well, incubate 10 min at room temperature, and then spin for 10 min at 12,000× *g* at 4 °C.
6. Remove the supernatant and then extract a second time: resuspend the pellet in 400 μL of 1× SDS extraction buffer and then add sodium acetate pH 5.2 to a final concentration of 0.3 M (made in DNase/RNase-free water). Add 400 μL of phenol/chloroform/LET, vortex 5 min, and spin at 16,000× *g* for 5 min at 4 °C.

7. Extract the aqueous phase again with 400 µL chloroform (vortex 5 min and then spin for 5 min at 4 °C). Precipitate with 40 µL of 3 M sodium acetate, 1 µL of glycogen, and 1 mL of EtOH 100% at −20 °C (1 h to overnight).
8. Spin at 16,000× *g* for 20 min at 4 °C.
9. Wash twice with 75% EtOH (in DNase/RNase-free water).
10. Air-dry for 10 min then resuspend in 25 µL of DNase/RNase-free water.
11. Measure RNA concentration with nanodrop.

3.2 In Vitro Transcription of Spike-Ins (See Note 4)

1. Linearize the plasmid containing the sequence to transcribe for spike-in with the appropriate restriction enzyme (after the spike-in sequence, the T7 polymerase will run off). Digest up to 10 µg with 2 µL of enzyme (typically 40 units) for 3–4 h at the appropriate temperature.
2. Check that the linearization is complete by running on a 1% agarose gel and, if needed, purify the linearized plasmid from the gel (using a kit such as GenElute kit from Sigma). If the digestion is complete, the DNA can be purified by alternative methods such as phenol/chloroform extraction (*see* **Note 18**). Measure the concentration of the template DNA.
3. Combine 1 µg of linearized plasmid with reaction buffer (final 0.75×) from the in vitro transcription kit (here HiScribe), 1.5 µL of each ATP, GTP and CTP 100 mM (7.5 mM final), 1 µL of UTP 100 mM (5 mM final), 0.5 µL of 5-Ethynyl-UTP 100 mM (2.5 mM final), 1 µL of DTT 0.1 M (5 mM final), and 1.5 µL of T7 RNA polymerase mix, in a total volume of 20 µL. Incubate at 37 °C for 2 to 4 h.
4. DNase treat for 15 min at 37 °C. Add DNase/RNase-free water to a total volume of 300 µL and then extract with 300 µL of phenol/chloroform/LET. Follow **steps 6** to **8** of Subheading 3.1.
5. Resuspend in DNase/RNase-free water and measure concentration. Dilute to an appropriate concentration (1–1000 ng/µL), aliquot, and keep at −80 °C. Thaw an aliquot before use.

3.3 mRNA Purification, Fragmentation, and Click Chemistry-Mediated Biotinylation

1. Add 1 to 5 ng of each 5-EU-tagged spike-in to 5 µg of each sample (*see* **Note 19**). It is important to precisely add the same amount of spike-in control to each sample.
2. Treat 5 µg of RNA + 1 ng of spike-in with 2 units of TURBO DNase (or other DNase), in a final volume of 50 µL with 1× DNase buffer, for 30 min at 37 °C (*see* **Note 20**).
3. Bring the volume up to 200 µL with DNase/RNase-free water, add 200 µL of 2× SDS extraction buffer, and resuspend.

4. Perform phenol/chloroform extraction as in **steps 6** to **10** of Subheading 3.1. Resuspend in 20 μL of DNase/RNase-free water.
5. Enrich the RNA samples for mRNAs, by performing ribosomal RNA depletion or poly(A) mRNA selection (*see* **Note 5**).
6. Purify the mRNA samples using the RNA Clean & Concentrator™ Kit from Zymo Research and following the manufacturer's instructions, including the DNase treatment. Elute twice with 11 μL water (total 20–22 μL).
7. Fragment the RNA: combine the RNA with 20 μL of 2× alkaline fragmentation buffer (*see* **Note 6**). Incubate at 95 °C for 20–25 min (*see* **Note 21**). Put on ice and stop immediately with 562 μL of stop/precipitation solution (60 μL of 3 M sodium acetate, 2 μL glycoblue, 500 μL of water). Precipitate with 800 μL of isopropanol (on dry ice for 1 h or at −80 °C overnight).
8. Centrifuge at 16,000× *g* for 30 min at 4 °C. Wash with 75% EtOH (in DNase/RNase-free water). Dry and resuspend in 15.75 μL of RNase-free water.
9. Perform biotinylation of the labeled RNA by using the reagents and following the instructions from the Click-iT® Nascent RNA Capture Kit. Use 2.5 μL of biotin azide (0.5 mM final) per sample. Mix gently and incubate at 24 °C in a thermomixer at 300–400 rpm for 30 min. For a negative control without biotinylation, omit the biotin azide in the reaction.
10. Add 1.5 μL of glycogen/glycoblue and 50 μL of ammonium acetate 7.5 M, mix, and then add 900 μL of EtOH 100% and precipitate at −80 °C overnight.
11. Centrifuge the RNA at 16,000× *g* for 20 min at 4 °C. Wash twice with 75% EtOH (in DNase/RNase-free water). Resuspend in 10 μL of Tris-HCl 10 mM pH 8.
12. Add 10 μL of 2× denaturing loading dye. Also prepare a size ladder (low molecular weight DNA or RNA ladder) as well as the RNA size oligonucleotide markers (1 μL of ladder or 1 μL of a mix of RNA ultramers each at 10 μM, combined to 9 μL of Tris-HCl 10 mM pH 8 and 10 μL of dye). Denature for 2 min at 80 °C.
13. Run on denaturing polyacrylamide gel (TBE-urea) at 200 V for about 70 min (*see* **Note 8**).
14. Decast the gel and stain with 1× SYBR Gold in 1× TBE for up to 15 min (*See* **Note 22**). You can visualize the gel using a gel imaging system.
15. Using a gel transilluminator, use a clean razor to cut the RNA fragments with the help of the different ladders and size markers (Fig. 2a). Transfer the gel slices into a 0.5 mL tube with a

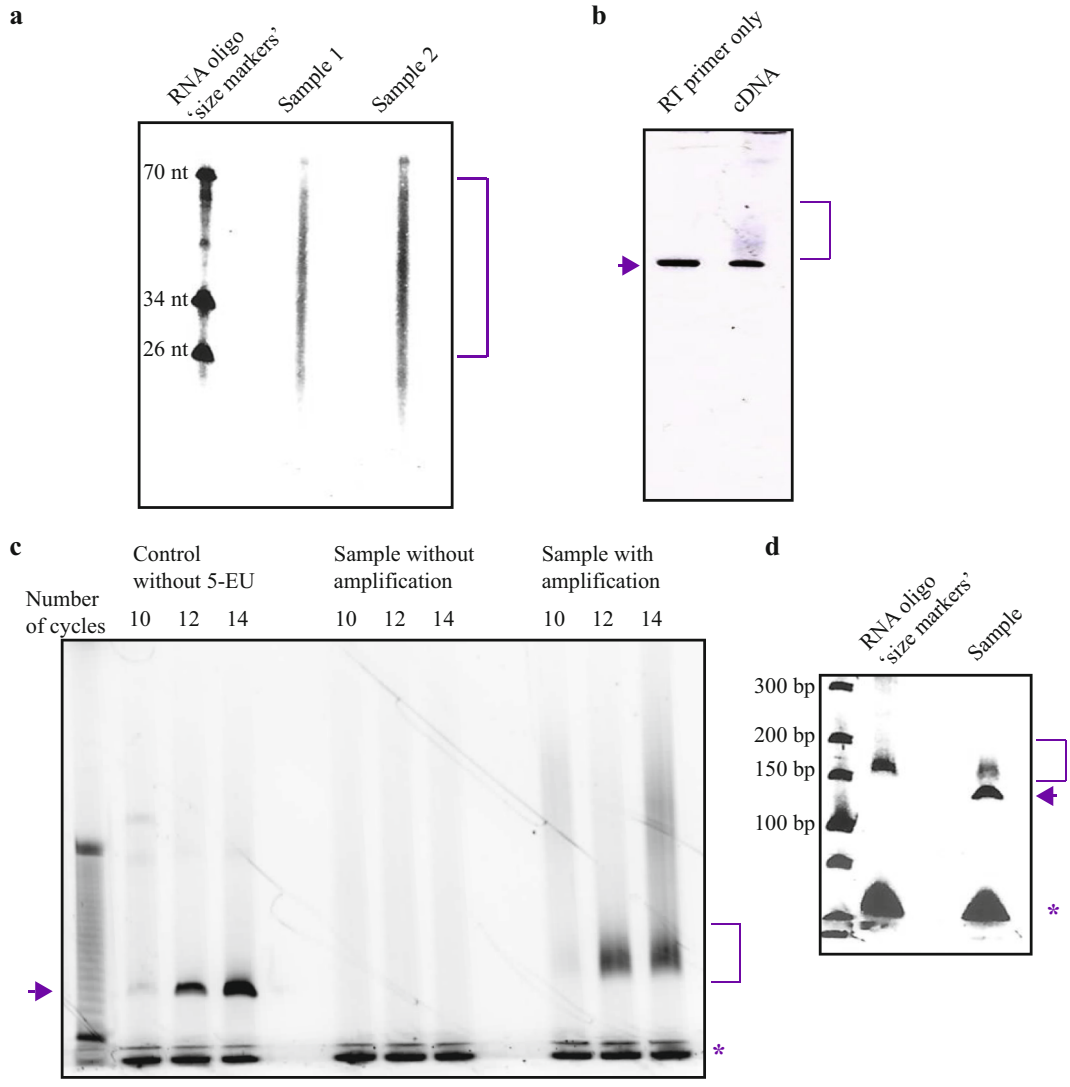

Fig. 2 Intermediate steps in the method (for Subheadings 3.1, 3.2, 3.3, 3.4, and 3.5). (**a**) Example of urea PAGE for two samples after alkaline fragmentation. In this experiment, the RNAs were fragmented to a short size between 30 and 70 nucleotides (*see* **Note 21**). RNA oligonucleotides of known length help determine the precise size of the RNA fragments and can be processed through the rest of the experiment as size markers (*see* **Note 23**). (**b**) Example of cDNA PAGE. There is an excess of reverse transcription primer that is to be avoided when cutting off the cDNA band (arrow). Running the reverse transcription primer alone (first lane) helps to determine the correct size. (**c**) Example of libraries obtained after several cycle numbers (PAGE). A sample without metabolic labeling mainly shows a background band derived from the unextended reverse transcription primer (arrow) [37]. The middle sample didn't amplify and had to be troubleshooted. The last sample shows a correct amplicon band with increasing intensity as the number of cycles increases (brackets). The optimal number of cycles in this example is 12, as more cycles lead to re-annealed partial duplex library products (high molecular weight). The asterisk shows excess PCR primers. (**d**) Electrophoresis of a final library product from an RNA sample that was fragmented to a short size. There is a lot of contamination derived from the unextended reverse transcription primer (arrow) - it is important to avoid this product when excising the library band from the gel, especially when the RNA fragments are short like in this example. The RNA size markers processed until the end of the experiment help determine the correct size of the library. The asterisk shows excess of the PCR primers

20-Ga hole punctured in the bottom and the lid cut off. Nest this tube into a 1.5 mL tube (*see* **Notes 21** and **23**).

16. Spin at 16,000× *g* for 2 min at room temperature to force the gel through the hole and then transfer any remaining gel pieces.
17. Add 500 µL of RNA gel extraction buffer and then freeze on dry ice for 30 min.
18. Rock overnight at room temperature on a rotisserie microtube rotator.
19. The next day, transfer the gel slurry (using a 1 mL pipette with tip cut off) to a microfuge spin filter tube and spin for 2 min at 16,000× *g*.
20. Transfer the flow-through to a new 1.5 mL tube, add 1.5 µL of glycogen/glycoblue, and precipitate with 700 µL of isopropanol (1 h on dry ice or overnight at −80 °C).
21. Centrifuge at 16,000× *g* for 30 min at 4 °C.
22. Dry the pellet and resuspend in 10 µL of Tris-HCl 10 mM pH 8.

3.4 Pull-Down and On-Beads Library Preparation

1. Perform pull-down of the biotinylated labeled RNAs following the protocol of the Click-iT® Nascent RNA Capture Kit. Wash 50 µL of streptavidin beads, twice, with 500 µL of Wash buffer 2, and then resuspend them in 50 µL of Wash buffer 2. Each time, fully resuspend the beads (brief vortexing), place on magnetic rack for 1 min, and discard the supernatant (*see* **Note 24**).
2. Add RNase-free water to each RNA sample to a total of 123 µL. Combine 125 µL of RNA binding buffer, 2 µL of SUPERase•In™ RNase Inhibitor, and the 123 µL of RNA (total 250 µL). Denature for 5 min at 68–70 °C.
3. Add the beads and rock on a rotisserie microtube rotator at room temperature for 30 min.
4. Wash five times with 500 µL of Wash buffer 1 then three times with 500 µL of wash buffer 2 (*see* **Note 25**).
5. Resuspend the beads in 20 µL of Wash buffer 2.
6. Dephosphorylate the RNA on the beads. Add 20 µL of Tris-HCl 10 mM pH 8 to each beads/RNA sample. Denature at 80 °C for 90 s and then equilibrate at 37 °C. Add 5 µL of 10× PNK reaction buffer, 1 µL of SUPERase•In™ RNase Inhibitor, and 1 µL of T4 PNK. Incubate for 1 h at 37 °C in a thermomixer, at interval or constant mixing (*see* **Note 26**). Inactivate at 70 °C for 10 min.
7. Place on magnet, remove the supernatant, and then wash the beads once with 500 µL of Wash buffer 2.

8. Resuspend the beads in 9 μL Tris-HCl 10 mM pH 8.
9. 3′ linker ligation: combine the 9 μL beads/RNA with 1 μL of preadenylated linker (0.5 μg/μL) and denature at 80 °C for 90 s. Cool down to room temperature. Set up the ligation reaction: beads/RNA + 2 μL of 10× Rnl2 buffer, 6 μL of PEG 8000 (part of the Rnl2 kit), 1 μL of SUPERase•In™ RNase Inhibitor, and 1 μL of T4 Rnl2(Tr) RNA ligase. Incubate at 25 °C for 2 h in a thermomixer at 1000 rpm (*see* **Note 27**).
10. Place the beads on a magnetic rack and remove the supernatant. Wash the beads once with 500 μL of Wash buffer 1 and then once with 500 μL of Wash buffer 2.
11. Resuspend in 10–12 μL of Tris-HCl 10 mM, pH 8.
12. Reverse transcription: reverse transcriptases such as Superscript III or IV can be used, following the manufacturer's instructions and with the reverse transcription primer described in Subheading 2.4. We have used the SuperScript III First-Strand Synthesis SuperMix with the following protocol: add 2 μL of the reverse transcription primer resuspended at 1.25 μM, and 2 μL annealing buffer. Denature at 65 °C for 5–10 min then place on ice. Add 20 μL of 2× first-strand reaction mix and 4 μL of SSIII/RNAseOUT mix. Incubate at 50 °C in a thermomixer at 900 rpm for 1 h.
13. cDNA elution: heat at 90 °C for 8 min in a thermomixer at 1200 rpm to inactivate the reverse transcriptase and to elute the cDNA from the beads. Collect the cDNA (around 35 μL) by placing the beads on a magnetic rack.
14. Precipitate by adding water to 300 μL, 30 μL of 3 M sodium acetate (0.3 M final), 2 μL of glycogen/glycoblue, and 500 μL isopropanol (−80 °C overnight or 1 h on dry ice).

3.5 Library Preparation and Sequencing

1. Centrifuge the samples at 16,000× *g* for 30 min at 4 °C, wash with 75% EtOH, and then resuspend the cDNA in 10 μL of 10 mM Tris-HCl pH 8.
2. cDNA gel purification: add 10 μL of 2× denaturing sample buffer to each sample. Include a control with the reverse transcription primer only (*see* **Note 28**) and a low molecular weight DNA ladder (1 μL + 9 μL of 10 mM Tris-HCl pH 8 + 10 μL of 2× denaturing sample buffer). Run on a denaturing polyacrylamide gel as in **steps 12** to **15** of Subheading 3.3. Excise the product band while avoiding the unextended primer (Fig. 2b).
3. Transfer the gel slices into 0.5 mL tubes with a 20-Ga hole punctured in the bottom and the lid cut off. Nest this tube into a 1.5 mL tube. Spin at 16,000× *g* for 2 min at room temperature to force the gel through the hole then transfer any remaining gel pieces.

4. Add 400 µL of DNA gel extraction buffer and then freeze on dry ice for 30 min.
5. Rock overnight at room temperature on a rotisserie microtube rotator.
6. The next day, transfer the gel slurry (using a 1 mL pipette with tip cut off) to a microfuge spin filter tube and spin for 2 min at 16,000× *g*.
7. Transfer the flow-through to a new 1.5 mL tube, add of 1.5 µL glycogen/glycoblue, and precipitate with of 700 µL isopropanol (1 h on dry ice or overnight at −80 °C).
8. Centrifuge at 16,000× *g* for 30 min at 4 °C.
9. Dry the pellet and resuspend in 15 µL of Tris-HCl 10 mM pH 8.
10. Circularization of the cDNA: add 2 µL of 10× CircLigase Buffer, 1 µL of 1 mM ATP, 1 µL of 50 mM $MnCl_2$, and 1 µL of CircLigase (all components are in the CircLigase kit). Incubate for 1 h at 60 °C and then heat inactivate at 80 °C for 10 min.
11. Precipitate by adding 20 µL of 3 M sodium acetate, 140 µL of water, 2 µL of glycogen/glycoblue, and 400 µL of isopropanol. Centrifuge at 16,000× *g* for 30 min at 4 °C.
12. Dry the pellet and then resuspend in 50 µL of water.
13. Amplify the cDNA, using a tenth of the circularized cDNA input for each 20 µL PCR reaction containing a final concentration of 0.2 mM dNTPs, 0.5 µM forward and reverse library primers, 1× polymerase buffer, and 0.2 µL of Phusion polymerase or high-fidelity DNA polymerase (1 min initial denaturation at 98 °C and then cycles of 20 s at 98 °C, 20 s at 65 °C, and 20 s at 72 °C) (*see* **Note 29**). First, assess the number of cycles required to obtain a prominent product band but few high molecular products that originate from reannealed partial duplex library products (Fig. 2c): prepare a master mix and then aliquot it into each PCR tube that you will remove from the thermocycler after the elongation step of cycles 8, 10, 12, and 14 (*see* **Note 30**). Once you determine the best number of cycles, use the rest of the cDNA to perform several amplifications (with the same number of cycles) that you will pool together after purification to obtain the final libraries (*see* **Note 31**).
14. Gel purify the library. Add 4 µL of 6× non-denaturing loading dye to each PCR product and separate on an 8% non-denaturing polyacrylamide TBE gel (handmade without urea, or precast such as Novex™ TBE Gels, *see* **Note 8**), similar to **steps 12** to **15** of Subheading 3.3. Include a low molecular weight DNA Ladder. Run at 180 V (around 50–60 min) in 1×

TBE. Stain with SYBR gold in 1× TBE. Excise the product from lanes where the PCR is not saturated (= avoid the large molecular weight products), but avoid any small product band derived from the unextended reverse transcription primer around 145 bp. The intensity of the library product should increase in intensity with more PCR cycles (Fig. 2c, d).

15. Extract with DNA gel extraction buffer as in **steps 3** to **9**.
16. Resuspend the final libraries in 15 μL of 10 mM Tris-HCl pH 8.
17. Check the libraries with a Bioanalyzer then sequence on an Illumina instrument such as NextSeq or NovaSeq in single-end mode (*see* **Note 9**).

3.6 Reads Processing and Alignment

Below are examples of analyses to process the reads. Several tools exist for each step and can be used depending on the user's preference.

1. Check the quality of the reads with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and process them if needed. Trim the adapter sequence in 3′ using tools such as cutadapt [26] or fastx-clipper (FASTX-Toolkit, http://hannonlab.cshl.edu/fastx_toolkit/). From a folder that contains your fastq files, you can run (*see* **Note 32**):

```
$ sh fastqc_bash.sh
```

with fastqc_bash.sh:

```
#!/bin/bash
for x in *.fastq.gz
do
echo "Processing sample $x"
fastqc $x
cutadapt -a CTGTAGGCACCATCAAT -m 18 \
-o ${x%%.fastq.gz}_clip.fastq.gz $x 1>>processing.log
done
```

2. Generate the sequence and annotation files to map the reads. We recommend Gencode for human or mouse references. Below is an R code from A. Darnell [27] to select for the set of canonical protein-coding genes or transcripts, for mouse. To estimate codon effects, only the coding transcripts are of interest. Furthermore, we restrict to canonical sequences for simplification but a transcriptome index that includes all the transcripts isoforms can also be used (skip the filtering step based on canonical annotation), if you want to estimate all the splice variants.

First, download the annotation and fasta files, for example, for mouse: gencode.vM34.annotation.gff3 and gencode.GRCm39.primary_assembly.genome.fa

```
> library(BSgenome.Mmusculus.UCSC.mm39) # example for mouse genome
> library(GenomicFeatures)
> gffFile <- 'gencode.vM34.annotation.gff3'
> annotations <- import.gff3(gffFile)
> annotationsdf <- tibble::as_tibble(annotations)
```

Select transcripts on tag "appris_principal" or "Ensembl_canonical":

```
> ccdstx <- (
 annotationsdf
 %>% filter(!is.na(ccdsid))
 %>% filter(str_detect(tag, "appris_principal"))
 %>% filter(gene_type == 'protein_coding')
 %>% filter(type == 'transcript')
 %>% mutate(ccdsnum = as.numeric(substring(ccdsid, 5)))
 %>% mutate(txnum = as.numeric(substring(ID, 8)))
 %>% group_by(gene_id)
 %>% filter(ccdsnum == min(ccdsnum))
 %>% filter(txnum == min(txnum))
)
```

Generate gff3 file:

```
> ccds <- annotations[
 (annotations$transcript_id %in% ccdstx$transcript_id) |
 (annotations$gene_id %in% ccdstx$gene_id & annotations$type == 'gene')]
> export.gff3(ccds, 'gencode.transcript.canonical.gff3')
```

Generate fasta file:

```
> ccds <- annotations[annotations$transcript_id %in% ccdstx$transcript_id
 & annotations$type == 'exon']
> ccds <- split(ccds, ccds$transcript_id)
> ccdsseqs <- extractTranscriptSeqs(Mmusculus, ccds)
> names(ccdsseqs) <- names(ccds)
> writeXStringSet(ccdsseqs, file = 'gencode.transcript.canonical.fa', format='fasta')
```

Generate fasta file of the coding sequences (CDS) for calculation of CSCs in Subheading 3.7:

```
> ccds <- annotations[annotations$transcript_id %in% ccdstx
$transcript_id
 & annotations$type == 'CDS']
> ccds <- split(ccds, ccds$transcript_id)
> ccdsseqs <- extractTranscriptSeqs(Mmusculus, ccds)
> names(ccdsseqs) <- names(ccds)
> writeXStringSet(ccdsseqs, file = 'gencode.transcript.cano-
nical.CDS.fa', format='fasta')
```

3. Map the reads to the genome or transcriptome using a general aligner like STAR [28] or a quasi-mapper (pseudo-aligner) like Salmon [29], respectively (*see* **Note 33**). Because we need to determine the set of transcripts and their expression level at each time-point, rather than the precise location that reads come from within the transcripts or genome, the use of pseudo-aligners is fine for this analysis (*see* **Note 34**) [18]. An example is given for each. Note that the sequences of the spike-ins are added to the reference files to process them alongside the endogenous transcripts.

   For Salmon:

   Generate the index:

```
$ cat gencode.transcript.canonical.fa spike.in.fa gencode.as-
sembly.genome.file.fa > gentrome_canonical.fa (see Note 35)
$ grep "^>" < gencode.assembly.genome.file.fa | \
cut -d " " -f 1 > decoys.txt
$ sed -i.bak -e 's/>//g' decoys.txt
$ conda activate salmon
(salmon) $ salmon index -t gentrome_canonical.fa -d decoys.txt
\
-p 8 -i salmon_canonical_index --gencode -k 20
```

   Run salmon for each trimmed fastq file:`(salmon) $ sh sal-mon_canonical.sh`

   With bash salmon_canonical.sh:

```
#!/bin/bash
for x in *_clip.fastq.gz
do
echo "Processing sample $x"
salmon quant -i salmon_canonical_index -l A -r $x -p 8 \
--validateMappings \
-o    salmon_quants_canonical/${x%%_clip.fastq.gz}_quant_-
cano
done
```

For STAR:
Generate the index:

```
$ cat 'gencode.transcript.canonical.gff3' 'spike.in.gff3' >
'gencode.transcript.canonical.SI.gff3'
$ STAR --runThreadN 8 --runMode genomeGenerate --genomeDir
STAR_index --genomeFastaFiles gencode.assembly.genome.file.fa
spike.in.fa --sjdbGTFfile gencode.transcript.canonical.SI.
gff3 --sjdbGTFtagExonParentTranscript Parent
```

Run STAR for each trimmed fastq: `$ sh STAR.sh`

With bash STAR.sh:

```
#!/bin/bash
for x in *_clip.fastq.gz
do
echo "Processing sample $x"
STAR --runThreadN 8 --genomeDir STAR_index --readFilesIn $x --
readFilesCommand gunzip -c --outSAMtype BAM SortedByCoordinate
--quantMode TranscriptomeSAM GeneCounts --outFileNamePrefix
STAR_ouput/${x%%_clip.fastq.gz}
done
```

4. Optional: If using an aligner like STAR, index the generated bam files with Samtools and load them into the Integrative Genomics Viewer to check the reads coverage over specific genes [30]. The density of reads will decrease with increasing time-points (Fig. 3a).

```
$ samtools index Aligned.sortedByCoord.out.bam
```

5. Get the number of counts/reads or TPM (Transcripts per million) for each transcript (*see* **Note 36**).
   For Salmon:

```
$ sh extract_TPM_counts_cano.sh
```

With bash extract_TPM_counts_cano.sh:

```
#!/bin/bash
for fn in salmon_quants_canonical/Sample*;
do
samp=`basename ${fn}`
echo "Processing sample ${samp}"
awk '{print $1 "\t" $4 "\t" $4}' ${fn}/quant.sf > \
```

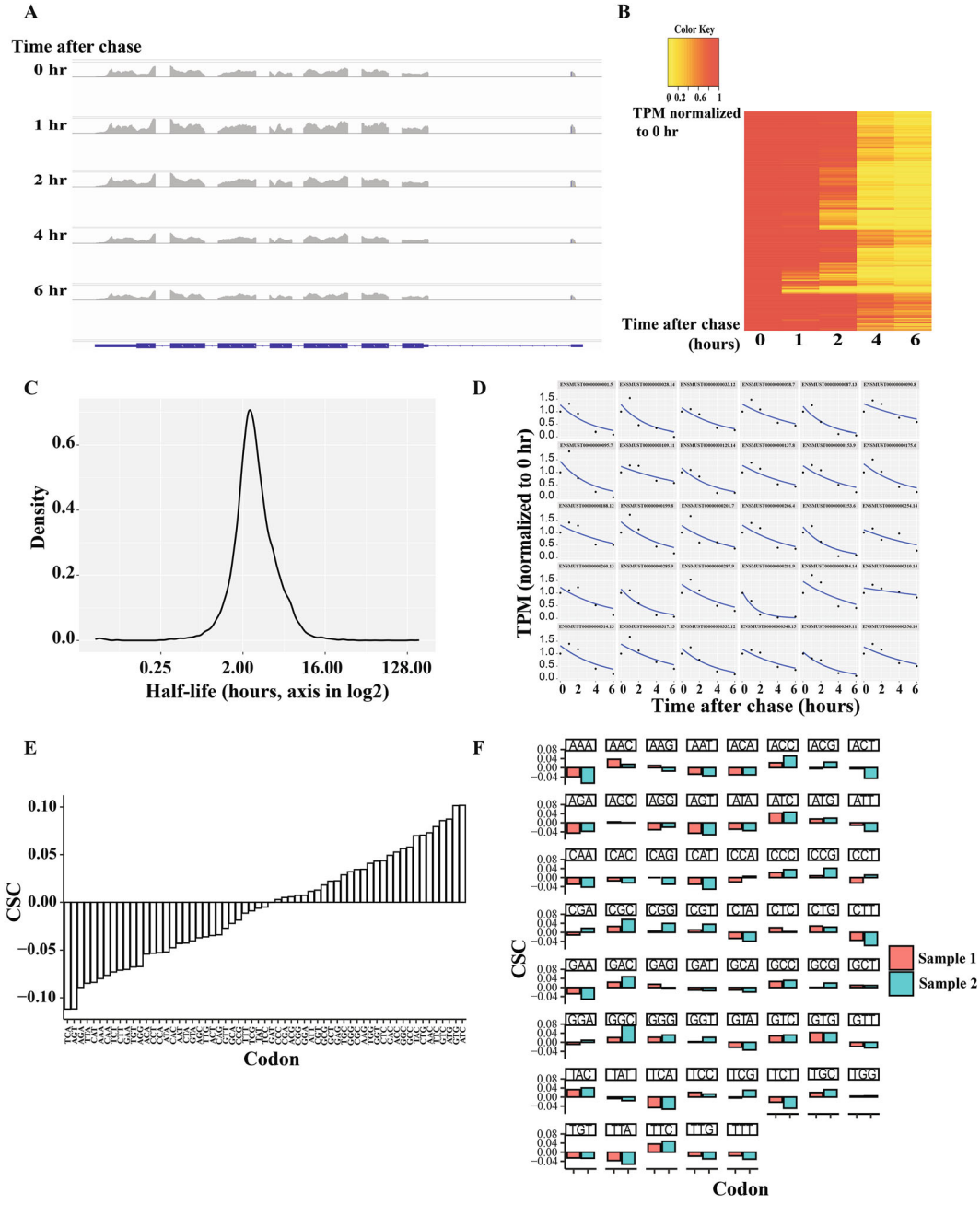

Fig. 3 Data analysis and plotting. (**a**) Reads coverage over a given transcript visualized in IGV (Integrative Genomics Viewer). The features of the transcript are shown at the bottom (UTRs, exons, introns). The number of reads decreases over the time-course as the mRNAs are degraded. The mRNA in this example is quite stable so there is still a lot of coverage at the latest time-point. (**b**) Heatmap of the TPMs (normalized to the 0 h time-point) of all the transcripts over the time-course—we can see the range of decay rates between transcripts. (**c**) Example of the distribution of half-lives values (mouse cells). (**d**) Estimation of the accuracy of the decay model fitting for a set of transcripts, by plotting the regression fit over the expression level at each time-point. In this example, several transcripts seem to increase in expression at 1 hour of chase (*see* **Note 14**), which affects the modeling accuracy. (**e**) Histogram of the CSCs calculated for a type of mammalian cells. (**f**) CSCs plotted side by side for two different samples, for each codon

```
TPM_cano/${samp}_TPM_Reads.txt
done
```

For STAR, use the "ReadsPerGene.out.tab" files that contain the counts for each gene, or run Salmon on the "Aligned.toTranscriptome.out.bam" files to get the TPM.

3.7 Calculation of Half-Lives and CSCs

With a chase experiment, we can fit an exponential decay model $A_{(t)} = A_0 \times e^{-kt}$ to the data to extract the half-life values, with $A_{(t)}$ the abundance of each transcript at time t and A_0 the abundance at time-point $t = 0$ h. The half-life $T_{1/2}$ is the time t at which $A_{(t)} = A_0/2$ so that $½ = e^{-kT1/2}$ and $T_{1/2} = (\log(2))/k$, with k estimated from the fitted nonlinear least squares (nls) regression model (*see* **Note 37**). The following steps are simple codes run with R in RStudio.

1. Normalize the data to the spike-ins and to the 0 h time-point which is similar to the "Spike-in Normalized Reads per Million Mapped Reads in the negative control" method (reads(transcript),t X reads(spike-in),t_0) / (reads(spike-in),t x reads(transcript),t_0)), using counts or TPM.

```
> library(tidyverse)
> setwd("path_to_file")
> files ← c("file_0hr_TPM_Reads.txt", "file_t_TPM_Reads.txt",
...)
```

Get the averaged value for the spike-ins at each time-point and use these values for normalization:

```
> lapply(seq_along(files), function(x) {
 dt <- read.table(files[x], header = T)
 e <- dplyr::filter(dt, grepl("spike_in_1_ID|spike_in_2_ID",
Name))
 SI <- mean(e$TPM)
})
```

For each file, assign the output values from the previous code as a "mean(spike-in)" at each time-point:

```
> table_t <- read.table('"file_t_TPM_Reads.txt"', header=T) %>
%
 dplyr::filter(!grepl("spike_in_1_ID",Name)) %>%
 dplyr::filter(!grepl("spike_in_2_ID",Name)) %>%
 mutate(TPM_nSI_t = TPM*mean(spike-in),t/mean(spike-in),t0) %>
%
 dplyr::select(-c(TPM))
```

Then merge:

```
> list <- list(table_t0, table_t, ...)
> all <- Reduce(function(x, y) merge(x, y, all=TRUE), list)
```

We also filter out transcripts with 0 number of reads/TPM at all time-points and with less than 1 TPM or 5 reads at time-point 0 h (*see* **Note 38**).

```
> all_filt <- all[rowSums(all[, -1])>0, ] %>% filter
(TPM_nSI_t0 >=1)
```

Normalization to the 0 h time-point:

```
> all_filt_n <- all_filt %>%
 mutate(TPM_t0_n = TPM_nSI_t0/TPM_nSI_t0) %>%
 mutate(TPM_t_n = TPM_nSI_t/TPM_nSI_t0) %>%
 ... %>%
 dplyr::select(c(Name, TPM_t0_n, TPM_t_n, ...))
```

2. Optional: plot a heatmap of the filtered and normalized TPMs or counts over the time course to visualize the overall decay of all transcripts, which can be compared between samples if the experiment was performed with different conditions (Fig. 3b).

```
> library(gplots)
> color.palette <- colorRampPalette(c("yellow", "red"))
> heatmap.2(as.matrix(all_filt_n[2:6]),col=color.palette,col-
Row= NULL, scale="none", trace="none", dendrogram="none",
margin=c(7, 5), labRow=FALSE, cexCol=0.9,density.info="-
none",breaks = seq(0, 1, length.out = 20),Colv=F)
> dev.off()
```

3. Fit a nonlinear least squares regression model to the data to extract the k constant. First, reshape the data:

```
> all_filt_n_m <- reshape2::melt(all_filt_n)
> all_filt_n_m <- all_filt_n_m %>%
 mutate(time_point = ifelse(grepl("t0", variable) , "0",
 ifelse(grepl("t", variable) , "t numerical value", ... ))) %>%
 dplyr::select(c("Name", "time_point", "value"))
> all_filt_n_m$time_point <- as.numeric(all_filt_n_m$time_-
point)
```

Then select a small number of transcripts (50) to estimate the intercept (should be close to 1 as we normalized to the 0 h time-point) and slope k from a linear regression model, to use as the "start" parameter for the nonlinear model:

```
> filt_50 <- all_filt_n_m %>%
 group_by(Name) %>%
 filter(value>0) %>%
 filter(n() == "number of time-points") %>%
 mutate(log = log(value))
filt_50 <- filt_50[order(filt_50$Name),] %>%
 head(50 * "number of time-points")
> lm_50 <- dlply(filt_50, "Name", function(df)
 lm(log(value) ~ time_point, data = df))
> lm_50_table <- ldply(lm_50, coef)
> lm_50_table <- lm_50_table %>% mutate(A0 = exp(lm_50_table$`
(Intercept)`)) %>%
 mutate(k = -(lm_50_table$time_point))
```

Make sure it is in correct order and run the nonlinear least squares fitting model:

```
> all_filt_n_m_name <- all_filt_n_m[order(all_filt_n_m
$Name),]
> all_filt_n_m_name$Name <- as.factor(all_filt_n_m_name$Name)
> library(nlme)
> nls <- nlsList(value ~ A * exp(-k*time_point)| Name, data =
all_filt_n_m_name,
  start=list(A=1, k=0.5), control=nls.control(maxiter =
10000, minFactor=0))
```

(*see* **Note 39**)

```
> nls_fit <- summary(nls)
> write.table(nls_fit$coefficients, 'nls_coefficients.txt',
sep='\t', quote=FALSE)
```

4. Extract the residuals sum of squares to filter out transcripts with a model that doesn't fit the data closely.

```
> res <- nls_fit$residuals
```

Remove NULL values (from errors in nls):

```
> res2 <- Filter(Negate(is.null), res)
> res3 <- do.call(data.frame, res2)
```

```
> res4 <- as.data.frame(t(res3))
> res4 <- tibble::rownames_to_column(res4, 'Name')
> res5 <- res4 %>% mutate(sum_squares = (res4$V1)^2 + (res4
$V2)^2 + ...) %>%
 dplyr::select(c(Name, sum_squares))
```

Check the distribution of the residuals sums of squares and determine the threshold above which to exclude the corresponding transcripts from the analysis:

```
> ggplot(res5, aes(x=sum_squares)) + geom_density() + sca-
le_x_continuous(trans='log2')
```

5. Extract the half-life values $T_{1/2}$ and filter out the transcripts with a high discrepancy between the data and the model (elevated sum of squares from **step 4**). Also filter out the $T_{1/2}$ with a negative value (inferred from transcripts whose expression increases with increasing time-points) (*see* **Note 40**).

```
> coeff <- read.table('nls_coefficients.txt', header=TRUE)
> coeff <- tibble::rownames_to_column(coeff, "Name") %>%
 dplyr::select(c(Name, Estimate.A, Estimate.k)) %>%
 mutate(T12 = log(2)/Estimate.k)
> T12 <- Reduce(function(x,y) merge(x,y, by = "Name"), list
(coeff, res5)) %>%
 filter(sum_squares <= 1) %>%
 filter(T12 > 0) %>%
 dplyr::select(c("Name", "T12"))
```

Plot the distribution of half-lives (Fig. 3c):

```
> T12 %>% ggplot(aes(x=T12)) + geom_density() + scale_x_con-
tinuous(trans='log2') +
 xlab("Half-life (hr) - axis log2")
```

Check the decay modeling fit on a small set (30) of transcripts (Fig. 3d):

```
> fit_plot <- Reduce(function(x,y) merge(x,y, by = "Name"),
list(all_filt_n_m_name, T12)) %>%
 head(30 * "number of time_points")
> ggplot(data=fit_plot, aes(x=time_point, y=value)) + geom_s-
mooth(method="nls", formula = y ~ A * exp(-k*x), method.args=
list(start=list(A=1, k=0.5), control=nls.control(maxiter =
1000)), se=FALSE) +
 geom_point() +
 facet_wrap(~Name)
```

The data can also be plotted for a transcript of interest:

```
> fit_plot %>% filter(Name == "transcript_ID") %>%
  ggplot(aes(x=time_point, y=value)) + geom_smooth(meth-
od="nls", formula = y ~ A * exp(-k*x), method.args= list
(start=list(A=1, k=0.5), control=nls.control(maxiter =
1000)), se=FALSE) +
 geom_point()
```

6. Calculate the codon stability coefficients CSCs. First, calculate the occurrence of each codon in each open reading frame:

```
> require(Biostrings)
> ORFs <- readDNAStringSet("gencode.transcript.canonical.CDS.
fa") %>% as.data.frame()
> ORFs <- tibble::rownames_to_column(ORFs, "Name")
> colnames(ORFs)[2] <- "sequence"
> T12_CDS <- merge(T12, ORFs, by = "Name")
> T12_CDS$sequence <- DNAStringSet(T12_CDS$sequence)
>  T12_CDS[, 4:67] <- oligonucleotideFrequency(T12_CDS$se-
quence, width = 3, step = 3, as.prob = TRUE, as.array =
FALSE, fast.moving.side = "right", with.labels = TRUE, sim-
plify.as = "matrix") %>% as.data.frame()
```

The CSC is the Pearson correlation between half-life and codon occurrence:

```
> CSC <- function(x,y) {
 correl_df <- data.frame()
 correl = cor.test(x, y, method = "pearson")
 correl_df[1,1] <- correl$estimate
 colnames(correl_df) <- c("CSC")
 return(correl_df)
}
> T12_Codons <- T12_CDS %>%
dplyr::select(-c(Name, sequence, TAA, TAG, TGA))
> corr <- vector(mode = "list", length = 61)
for (i in 2:62) {
 corr[[i-1]] <- CSC(T12_Codons[,1], T12_Codons[,i])
}
> Codon_vector <- colnames(T12_Codons[,2:62])
> CSC <- do.call("rbind", corr)
> CSC$Codon <- Codon_vector
```

Plot the CSCs as a histogram (Fig. 3e):

```
> ggplot(CSC, aes(x=reorder(Codon, CSC), y=CSC)) + geom_col
(colour = "black", fill="white") + theme_classic() + labs
(x="Codon") + theme(axis.text.x = element_text(angle=90,
size=7))
```

CSCs from replicates or different samples can be compared and plotted together (Fig. 3f):

```
> CSC_merge <- Reduce(function(x,y) merge(x,y, by='Codon'),
list(CSC, CSC_2))
colnames(CSC_merge)[2] <- "CSC_1"
colnames(CSC_merge)[3] <- "CSC_2"
> CSC_merge_m <- CSC_merge %>% melt() %>%
 mutate(Sample = ifelse(grepl("_1", variable) , "Sample 1",
"Sample 2"))
> CSC_merge_m %>%
 ggplot(aes(x=Sample, y=value, fill=Sample)) +
 facet_wrap(~ Codon) + geom_col(colour="black")+ theme_classic
() +
 labs(x="Codon") + theme(axis.text.x = element_text(angle=90,
size=7), strip.text = element_text(size=7, margin = margin
()))
```

4 Notes

1. The protocol described here is designed for adherent mammalian cells. For other cell types, modify the protocol accordingly. For budding or fission yeast that don't express a nucleoside transporter, a uracil analog can be used instead of a uridine analog (it will be metabolized by the enzyme uracil phosphoribosyltransferase). Alternatively, an engineered yeast strain expressing the human equilibrative nucleoside transporter (hENT1) can be used [31].
2. Other ribonucleoside analogs can be used for metabolic labeling of nascent RNAs, such as 4-thiouridine (4sU) or bromouridine (BrU), with adapted methods for pull-down [19, 32, 33].
3. Commercially available RNase/DNase-free water can be used. Alternatively, RNase-free water can be made by treating water with diethyl pyrocarbonate (DEPC). Add to ultrapure water and stir covered in a chemical hood overnight, then autoclave twice to inactivate the DEPC.

4. It is recommended to use different spike-in sequences for precise normalization and determination of mRNA half-lives. Moreover, use RNAs from the same in vitro transcription reaction for all the samples that are to be compared: make a batch reaction, aliquot and freeze at −80 °C. Alternatively, some groups have used a labeled spike-in of whole genome reads from a nontarget organism to have a variety of length distributions and sequence compositions of spike-in [18, 34].
5. We prefer ribosomal RNA depletion over poly(A) selection, to be able to sequence mRNAs that have shortened tails, as poly (A) metabolism is critical in mRNA decay pathways [6]. However both methods will give mostly similar results when comparing the stability of different mRNAs (stable versus unstable), while poly(A) selection will lead to an overall increase in the absolute values of half-lives [6]. We have published data obtained using previous Ribo-Zero depletion kits from Illumina that have been discontinued and replaced by Ribo-Zero Plus rRNA Depletion kit (that targets human, mouse, rat and bacterial rRNAs). We recommend using kits that have an RNA input range up to 5 μg such as RiboMinus™ Eukaryote System v2 (Thermofisher Scientific). Alternatively, and especially for organisms that are not covered by commercially available rRNA depletion kits, you can manually design and produce biotinylated oligonucleotides that target the ribosomal RNAs of interest and capture them with streptavidin-coated beads [35].
6. Make the alkaline fragmentation buffer fresh, as it will equilibrate with gaseous CO_2 over time and raise the pH. Alternatively, store in tightly capped, single-use aliquots at room temperature.
7. Order the RNA size markers, the reverse transcription primer and the PCR primers from suppliers like IDT with a purification option such as PAGE purification. For the RNA size markers, design RNA ultramers that encompass the length of RNA after fragmentation (*see* **Note 21**).
8. If handmaking the polyacrylamide gels, use Mini-PROTEAN® handcast systems (Biorad) and 40% acrylamide/bis-acrylamide, 19:1 solution. Mix TBE (1× final), polyacrylamide, and ultrapure water. For denaturing gels, stir urea at room temperature—the reaction is endothermic and it will take some time to dissolve. Degas for 10 min in a flask under vacuum, then add ammonium persulfate (APS; 100 μL of 10% APS for 20 mL) and tetramethylethylenediamine (TEMED; 15 μL for 20 mL) before pouring. Prerun the gels for 15 min before running the samples. It is important to rinse the wells off the urea before loading the samples, using a syringe and needle.

9. Order the reverse primer with different barcodes corresponding to your different samples, so that the libraries can be pooled and sequenced simultaneously during a single run (multiplexing). The sequences can be found on the Illumina Adapter Sequences document under "Truseq Small RNA." Refer to your sequencing facility to decide how many samples you can pool together. We recommend a minimum output of 40 million reads per sample.
10. It is recommended to perform the whole experiment at least three times (biological replicates), for reproducibility of the results, especially if the half-lives and CSCs have to be compared between different conditions.
11. Cells seeding, culture conditions, and confluency, will have to be determined depending on your cell line and desired output. These conditions worked well for adherent cells such as Chinese Hamster Ovary cells [7]. We also labeled mouse oligodendrocyte precursor cells after 40 h of culture, that were seeded in 10 cm dishes at two million cells per dish, or at five million cells per dish for differentiation to oligodendrocytes [9].
12. Prewarm enough complete media for all your cells. Just before use, thaw and add the required amount of 5-EU to the whole volume of media, and distribute to your different dishes. Plan a volume of media that is sufficient to supply nutrients for the whole duration of the labeling.
13. Labeling time may have to be determined empirically based on your cell line and culture conditions. 24 hours labeling was determined based on an approach to steady state experiment performed with CHO cells. Prolonged culture with a high concentration of 5-EU has been suggested to cause inhibition of cell growth [17].
14. Ideally, two "0 h time-points" should be collected—before replacing the media with uridine, and just after starting the chase. This would allow for comparison before/after media change, which induces a replenishment of nutrients that can perturb the cell metabolism.
15. An extra sample can also be processed at 0 h, to use as a negative control for which the biotinylation step will be skipped.
16. Plan enough time-points - we strongly encourage more than three time-points to fit an accurate decay model and estimate half-lives with confidence, as well as more time-points close to each other towards the beginning of the chase. For example, 0 h, 1 h, 2 h, 4 h, 6 h, and 10 h should work well for mammalian cells.

17. You don't need to wash the cells before addition of Trizol, but tilt the plate and aspirate as much media as possible. After addition of Trizol, pipet up and down as soon as possible, and manually tilt and rotate the plate to collect and lyse all the cells.
18. Refer to the in vitro transcription kit manufacturer's instruction for details on how to obtain a pure template for transcription.
19. A good ratio of total sample to spike-in control is important to allow enough read coverage over the spike-in, but avoiding that the majority of sequencing reads are spike-in derived. Reasonable ratios are suggested to be from 1:1000 to 1:50 of spike-in to the total input RNA, although we prefer considering the input RNA obtained after depletion of the ribosomal RNAs that make up most of the RNA sample [36].
20. To increase the final yield of the experiment, you can perform the DNase treatment and ribosomal RNA depletion in duplicates for each sample, and combine them after ribosomal RNA depletion in **step 6** of Subheading 3.3. Use 2 × 5 μg of each RNA sample.
21. The alkaline hydrolysis step should be tested prior and the duration determined empirically for your experiment. It is used to randomly fragment the RNAs in early steps of library preparation. It was designed similar to fragmentation of the total RNA that is processed in parallel to ribosome protected fragments in ribosome profiling experiments [37]. However, it is aimed to get longer fragments than the 30 nt fragments obtained in these experiments, ideally around 100 nt. The incubation time range given here is based on different mammalian cells, and is shorter than what has been used for yeast. We show examples of RNA fragmented between 30 to 70 nt in Fig. 2. If really short, the cDNA fragments obtained after ligation and reverse transcription will be difficult to separate from the reverse transcription primer (same with the final libraries) and you will get a lot of contaminant when excising from the gels. Furthermore, small reads will be filtered out during analysis. Be aware that uneven fragmentation of RNA can be a source of bias leading to differential representation of specific regions of the RNAs [38].
22. Pre-wet your hands and handle the gels with care when de-casting, imaging, and cutting. Thicker gels (1.5 mm or 1 mm compared to 0.75 mm) will be easier to handle.
23. You can cut the RNA size markers and collect them in a separate tube to carry along as a control for the next PAGE. In this case, modifications to the protocol should be made for this sample as follows:

Don't perform the pull-down **steps 1** to **5** of Subheading 3.4. Instead, resuspend in 10 μL of Tris-HCl 10 mM pH 8 at the end of **step 22** of Subheading 3.3 and then perform the dephosphorylation in **step 6** (add 33 μL of water and start from the denaturation step). Skip **steps 7–8**, and instead precipitate by adding 39 μL of water, 1 μL of glycoblue, 10 μL of 3 M sodium acetate, and 300 μL of isopropanol. Resuspend in 9 μL of Tris-HCl 10 mM pH 8. Perform the linker ligation (**step 9** of Subheading 3.4) and then instead of **steps 10–11**, add 338 μL of water, 40 μL of 3 M sodium acetate, 1.5 μL of glycoblue and precipitate with isopropanol. Gel purify the ligated RNA oligonucleotides, excising the ligation product and not the unligated linker. Resuspend in 12 μL of Tris-HCl 10 mM pH 8. Perform reverse transcription as in **step 12** of Subheading 3.4, then replace **step 13** by RNA hydrolysis: add 2.2 μL of 1 N sodium hydroxyde and incubate at 98 °C for 20 min. Follow the next steps similar to the other samples.

24. The Click-iT® Nascent RNA Capture Kit uses Dynabeads™ MyOne™ T1 streptavidin beads. We have also used Dynabeads™ MyOne™ C1 streptavidin beads, which gave similar results with this protocol. However, while T1 and C1 have the same size, they differ in hydrophobicity (based upon a tosylactivated bead versus a carboxylic acid bead, respectively) and may have different binding capacity. We recommend using the same type of beads for experiments that will be compared to each other.
25. The composition of the wash buffers is proprietary and assays with homemade buffers have not been successful in our hands. The number of washes was adapted from the kit's instruction manual as the wash buffer 2 tends to be limiting. Tubes can be rocked for a few minutes on a rotisserie microtube rotator during the washes.
26. Unlike 4sU, the covalent bond between the alkyne group of the 5-EU and the biotin azide generated by the Click chemistry reaction is not easily reversed. Therefore, all the steps from pull-down of the labeled RNA to synthesis of the cDNA are performed on beads. Make sure to shake the samples during each incubation on a thermomixer to prevent the beads from settling down—either constant shaking at 900–1000 rpm or intermittent shaking (for example, 15 s at 1000 rpm, every minute). Cleavable biotin azide molecules have been developed and could be tested to elute the RNA from the beads [39].
27. To try to increase the ligation efficiency, you can incubate at 25 °C for 1–2 h then overnight at 16 °C in a thermomixer at 1000 rpm.

28. The cDNA signal can be weak, use the lane where only the reverse transcription primer was run as a reference, to make sure to cut above the unextended primer (band at 100 nucleotides). It is important to avoid the unextended primer or this background will comprise a greater fraction of the final libraries.
29. The cDNA amplification can also be performed with NEBNext® Ultra™ II Q5® Master Mix, following the manufacturer's protocol.
30. Make sure to remove the tubes from the thermocycler before the next denaturation step.
31. The number of cycles given here is an estimate based on experiments with different mammalian cells. You can perform more PCRs at different cycles in the "test" phase and directly purify the best product for your library. Keeping some cDNA to amplify with the best number of cycles is useful when more final library material is needed for sequencing.
32. There may be small reads that can't be mapped with confidence. Setting a minimum length of 18 or 20 nucleotides will filter those out.

    1>> processing.log will generate a file with the processing stats from cutadapt.
33. Your favorite aligner and softwares can be used—for example, Hisat2 combined with Stringtie have also been used for previous analyses [7, 9].
34. If you use STAR to map your reads, you can also generate output alignments that are translated into transcript coordinates (—quantMode TranscriptomeSAM option as given in the command) that can be used as input for Salmon. The advantage is that you can get bam files needed for some softwares such as the Integrative Genomics Viewer, and still quantify with Salmon.
35. In our example, gencode.transcript.canonical.fa is generated in **step 2** of Subheading 3.6. and gencode.assembly.genome.file.fa is GRCm39.primary_assembly.genome.fa.
36. Determining half-lives requires comparing the abundance of each transcript only to itself between different time-point samples, which bypasses the need to correct for transcript length bias. Furthermore, because exogenous RNAs are spiked in for normalization to account for pull-down efficiency and sequencing depth bias, the number of counts/reads can be used to estimate the decay rates of transcripts (assuming a good quality of spike-ins) [18, 34]. We ran a comparison using both TPM or counts levels and observed a high

correlation of the calculated half-lives or CSCs between the two methods. Salmon outputs both the number of TPM or counts (NumReads).

37. Note that if the timescale of the experiment is longer than the doubling time of the cells, the equation may have to be adapted to include the basal dilution rate due to cell division, where $T_{1/2} = \log(2)/(k - k_{\text{growth}})$, with k_{growth} determined by the growth rate of the cells.
38. The threshold value is set arbitrarily to filter out transcripts with really low expression, which is close to noise either at time-point 0 h or across the time-points, and can be determined based on the sequencing depth.
39. The function *nlsList* allows the process to continue when the fitting fails on given transcripts, for which there will be a "warning" statement. The start values are defined from the linear modeling on the restricted set of transcripts. A_0 should be close to 1 as the data was normalized to time-point 0 h. The parameters in *nls.control*, such as the maximum number of iterations during fitting *maxiter*, can be modified from defaults to control the optimization algorithm depending on your experiment.
40. Check the number of transcripts excluded by those filtering steps; if high, it may reveal some issues with the experiment setup or the processing steps. You might choose to keep negative half-life values and substitute them by a maximum "$T_{1/2}$ threshold" that would be arbitrarily assigned to transcripts that are highly stable. Furthermore, transcripts with a half-life that is quite higher than the timescale of the experiment might be discarded as they are inferred with less confidence (this will depend on the length of the chase and the number of transcripts to exclude), or again assigned a "$T_{1/2}$ threshold" such as the last time-point value and considered "highly stable." Ultimately, for this method aimed at calculating CSCs, it is more important to estimate with confidence the differences in stability between transcripts rather than the absolute half-life values for each mRNA.

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Chapter 12

Measurement of rRNA Synthesis and Degradation Rates by ^{3}H-Uracil Labeling in Yeast

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Abstract

In order to measure the actual synthesis and degradation rates (SR, DR) for rRNA in yeast, we developed a method based on the pulse labeling and quantification of newly synthesized large rRNA molecules by a known mass of cells. The SR is calculated as the ratio of new rRNA molecules (synthesized after a short [5,6-^{3}H]-uracil pulse) to total rRNA (a proxy of cell mass), calculated by northern blotting after hybridization with a ^{32}P-labeled rRNA probe. Then to measure the DR we perform a chase of the existing ^{3}H-labeled rRNA for several hours during yeast culture growth. We have used this method in control experiments where the yeast cell volume varies as a way to check if the SR and DR are constant with the cell volume.

Key words Transcription rate, Synthesis rate, Degradation rate, rRNA, RNA polymerase I, Yeast, *Saccharomyces cerevisiae*

1 Introduction

For any transient molecule, such as RNAs and proteins, their cellular concentrations are determined by an equilibrium between synthesis rates (SR) and degradation rates (DR). Additionally, in growing cells, dilution caused by the increase in cell mass (and volume) also contributes to the disappearance of molecules [1]. Therefore, the calculation of synthesis and degradation rates is very important to describe the behavior of the current expression status of a given cell [2, 3].

With transcription, which is the main regulatory step for most genes (*see* [4] for a detailed review), the calculation of RNA synthesis rates in eukaryotes is based on two types of techniques that conceptually measure different magnitudes. One group of them detect and quantify elongating RNA polymerases (RNA pols) and measure the so-called nascent transcription rate (nTR) [5]. These techniques detect either RNA pols bound to the gene

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transcribed region or the nascent RNAs bound to them in the nucleus [5–9]. Another group of techniques focuses on the detection of new born mature RNA molecules, most of which are located in the cytoplasm. These techniques provide a mature transcription rate (TR). Finally, when dealing with chemical equilibriums or chemical reactions, it is necessary to employ the concentration of molecules instead of the number of molecules. This is especially important when studying or comparing cells with different volumes. When using correct techniques that detect newly synthesized mature RNA molecules in concentration terms, we can finally talk about true RNA SRs [5].

For the determination of DRs, there are many different methods based on either a transcription shutoff followed by the quantification of the remaining amount of RNA or the simultaneous quantification of the RNA concentration and the SR to derive the DR by assuming steady-state conditions (*see* [10] for a detailed review).

We have previously studied both the SR and DR for mRNAs synthesized by RNA pol II in the yeast *Saccharomyces cerevisiae* as a eukaryotic model cell. Our results support the importance of knowing both the SR and DR in describing the regulatory strategies of protein-coding genes in eukaryotes [1, 4]. Although RNA pol II (the enzyme that transcribes most genes in eukaryotes, including those coding for proteins) is the most studied RNA pol, the other two eukaryotic RNA pols are also very important for cell physiology [11, 12]. RNA pol I and III regulation is particularly relevant for cell homeostasis because they transcribe the most abundant RNA molecules in the cell, rRNAs, and tRNAs, which are fundamental elements of translation machinery [13, 14].

As translation is the most energy-consuming step during cell growth [4], RNA pol I and III regulation is paramount in the control of growth and its changes during all cell physiological transitions [15, 16]. RNA pol I is the most important one as regards energy consumption during cell transcription [4]. Therefore, a good method to measure RNA pol I activity is needed to understand global changes in cell physiology. There are several important differences in determining both the SR and DR of the products of this RNA pol. First, RNA pol I only transcribes a single gene called rDNA, which is, however, repeated as many times as clusters of identical units [16, 17]. This means that genome-wide methods are not needed for its study. Second, rRNA molecules are extremely abundant and can be easily detected in agarose gels because their size is known. Finally, rRNA is much more stable than mRNAs [4], which makes its DR determination quite challenging. This is especially hard in growing cells like yeast, where the dilution rate (equivalent to growth rate, GR) is quantitatively much more important than the DR.

We have developed a simple experimental protocol that quantifies mature, but newly synthesized rRNA, in growing yeast cells by a short ^{3}H-uracil pulse and the posterior detection and quantification of mature 18S and 25S in a denaturing agarose gel. The ^{3}H signal reports the number of tritiated rRNA molecules (newly synthesized), which is then converted into the real SR by dividing it by the quantification of all the rRNA molecules (newly synthesized and old ones). The quantification of all the rRNA molecules is done by transferring the same agarose gel to a nylon membrane and performing northern hybridization with specific 18S and 25S ^{32}P-labeled rRNA probes. Here the ^{32}P signal is considered a proxy of the yeast cell mass used in each sample because it is well known that the rRNA concentration remains constant in most cells, including yeast cells when growing at a constant GR [18].

The DR can also be measured by applying the same principle used to quantify the rRNA concentration. During active growth, preexisting ^{3}H-labeled rRNA disappears as a function of its DR and the known dilution rate (= GR). Thus we can perform a chase experiment for several hours after the short [5,6-^{3}H]-uracil pulse to follow the disappearance of the newly synthesized molecules and to calculate their DR [18]. We have used this technique with different sized cells to compare their SR and DR. The true SR can diverge from their RNA pol I nTR given that, as explained above, the nTR is conceptually distinct from the SR and probably conditioned by cell size variation.

2 Materials

2.1 Media and Solutions

Prepare all the culture media using distilled water and autoclave them at 120 °C for 20 min to avoid cross-contamination. Employ ultrapure water for analytical grade reagents. Use RNase-free ultrapure water for the solutions needed when preparing solutions to work with RNA (*see* **Note 1**) and autoclave at 134 °C for 60 min to degrade the remaining RNases (unless otherwise indicated). Prepare and store all the reagents at room temperature (unless otherwise indicated). Diligently follow all the waste disposal regulations when disposing waste materials and pay special attention to radioactive substances, contaminated material and waste.

1. Synthetic minimal medium without uracil (SC-URA dropout): 0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, and 2% glucose. Supplemented with the essential amino acids: lysine, adenine, methionine, tryptophan, leucine, and histidine (*see* **Note 2**).
2. SC + SuperURA medium: dissolve 1 mg of solid uracil in 1 mL of SC-URA medium.

3. 70% and 96% EtOH.
4. 1% NaCl.
5. [5,6-^{3}H]-uracil (1 mCi/mL).
6. RNase-free ultrapure water.
7. TES Buffer: 10 mM Tris-HCl pH 7.5, 10 mM EDTA pH 8, 0.5% SDS.
8. ≥ 99% Acid phenol-chlorform (5:1).
9. ≥ 99.8% Chloroform stabilized with ethanol.
10. 3 M sodium acetate pH 5.3.
11. RNase cleaning agent.
12. Agarose low EEO.
13. MOPS 10X Buffer: 0.23 M MOPS, 0.04 M sodium acetate pH 5.3, 0.01 M EDTA pH 8, pH 7 (*see* **Note 3**).
14. MOPS 1X Buffer: Dilute 80 mL of MOPS 10X buffer with RNase-free ultrapure water to a total of 800 mL.
15. ≥ 99.5% formamide.
16. Commercial RNA Loading 6X Buffer mixed with ethidium bromide at the 9:1 proportion.
17. SSC 20X Buffer: 3 M NaCl, 0.3 sodium citrate, pH 7 (*see* **Note 4**).
18. SSC 6X Buffer: Dilute 450 mL of SSC 20X buffer with RNase-free ultrapure water to a total of 1.5 L.
19. Commercial T4 Polynucleotide Kinase (T4-PNK) and Kinase 10X Buffer.
20. [γ-^{32}P]-ATP (10 mCi/mL).
21. Hybridization Buffer: 0.3 M Na_2HPO_4, 0.2 M $NaHPO_4$ H_2O, 7% SDS, 1 mM EDTA pH 8, 1% BSA (*see* **Note 5**).
22. Washing Solution I: 0.5% SDS, SSC 2X.
23. Washing Solution II: 0.5% SDS, SSC 0.1X.
24. Stripping Solution: 0.1% SDS, RNase-free ultrapure water.

2.2 Oligonucleotide Sequences

Oligonucleotide stocks for [γ-^{32}P]-ATP labeling are at 100μM and working stocks at 10μM (dilute 10 μL of the oligonucleotide stock with RNase-free ultrapure water to a total of 100 μL).

1. 18S rRNA probe (+734 from rDNA TSS): CATGGCTTAATC TTTGAGAC.
2. 25S rRNA probe (+3292 from rDNA TSS): CTCCGCTTA TTGATATGC.

2.3 Ware and Accessories

1. Sterile 5 mL glass tubes for pre-inocula.
2. Sterile 100 and 250 mL glass flasks for liquid cell cultures.
3. Sterile and RNase-free 1.5 and 2 mL safe-lock microtubes.
4. Sterile and RNase-free 15 and 50 mL conical centrifuge tubes.
5. Disposable spectrophotometer cuvettes.
6. Large ice bucket.
7. Liquid nitrogen.
8. Plastic and glass trays.
9. UV-transparent trays, caster and 15-well comb for 11 × 15 cm agarose gel casting.
10. Tweezers.
11. Spatula.
12. Gloves with thermal protection.
13. Protective goggles.
14. Positively charged nylon hybridization membranes.
15. Paper towel (or filter paper) stack.
16. Whatman paper.
17. Glass plate.
18. 1–1.5 kg flat weight.
19. Phosphor imaging plates and cassettes suitable for ^{3}H-uracil and for ^{32}P-ATP detection.
20. 1.5 mL microtubes lid locks.
21. Large hybridization glass tubes with silicon rings for hermetic sealing.
22. Saran wrap.
23. Lighter.
24. Small needle with a diameter of 0.6 or 0.8 mm.

2.4 Apparatus

1. Thermostatic incubator at 30 °C with agitation at 180 rpm.
2. Spectrophotometer to measure cell mass at a wavelength of 600 nm and to measure the RNA concentration at a wavelength of 260 nm with disposable cuvettes or with a microvolume UV-spectrophotometer (as a NanoDrop instrument).
3. Thermomixer for microtubes at 30 °C and 65 °C with agitation at 650 rpm.
4. Vortex.
5. Refrigerated and non-refrigerated centrifuges for microtubes with a centrifugal force of 16,000× *g*.
6. Refrigerated and non-refrigerated centrifuges for 15 mL conical tubes with a centrifugal force of 1000× *g*.

7. Heat blocks at 37 °C, 65 °C and 100 °C.
8. Fridge.
9. −20 °C and −80 °C freezers.
10. Fume hood.
11. Microwave.
12. Horizontal electrophoresis cell system with a generator to apply up to 200 constant voltages.
13. UV crosslinker.
14. Phosphor imaging plate scanner.
15. Phosphor imaging plate eraser.
16. Hybridization incubators with tube rotators at 38 °C and 42 °C.
17. Geiger counter.
18. Tray shaker.

3 Methods

Carry out all the procedures at room temperature unless otherwise specified.

3.1 Cell Culture, Sample Collection, and [5,6-^{3}H]-Uracil Pulse for rRNA Labeling and Synthesis Rate Determination

Due to the characteristics of the experiment, the utilized yeast strains must be capable of growing in the complete absence of uracil (*see* **Note 6**).

1. Plate yeast cells on SC-URA and grow in a thermostatic incubator at 30 °C.
2. Pick up a colony and inoculate it in as a 5 mL SC-URA preinoculum. Let it grow in a glass tube overnight in a thermostatic shaker at 30 °C and 180 rpm.
3. For each studied condition, prepare a glass flask with fresh SC-URA as indicated in **step 3** and dilute the preculture to an initial OD_{600} of less than 0.1. Grow the new cell cultures in a thermostatic shaker at 30 °C and 180 rpm, and collect a sample every hour to measure the OD_{600} of cultures. This allows the calculation of the GR (*see* **Note 7**).
4. Preheat an aliquot of 1 mL of SC-URA medium and another of SC + SuperURA in a thermomixer at 30 °C (keep it running until the end of the experiment).
5. When the OD_{600} of cultures is around 0.3, collect in 15 mL conical tubes two independent aliquots of 10 mL of each cell culture condition, centrifuge for 4 min at 1000× *g* and eliminate the supernatant (repeat the process to completely

dry the cell pellet). Flash freeze the samples in liquid nitrogen and store at −80 °C until RNA extraction and quantification (*see* Subheading 3.2).

6. When the OD_{600} of cultures is around 0.4, collect in 2 mL safe-lock microtubes the same mass of cells of each cell culture condition. Take a 2 mL sample that would correspond to an exact OD_{600} of 0.4 as a reference (*see* **Note 8**). Centrifuge for 3 min at 16,000× *g* and eliminate the supernatant (repeat the process to completely dry the cell pellet). Leave the cell cultures so they can continue to grow in a thermostatic incubator at 30 °C and 180 rpm.
7. Resuspend the cell pellets in 19.5 μL of pre SC-URA medium and keep the samples in a thermomixer at 30 °C and 650 rpm.
8. For tritium labeling, add 2 μL of [5,6-^{3}H]-uracil to each sample and incubate in a thermomixer for 5 min at 30 °C and 650 rpm. Add the tritium with a 1 min lag between samples so the pulse lasts the same for all the conditions. The same principle applies to the next two steps.
9. To stop tritium incorporation, add 189 μL of preheated SC + SuperURA medium and incubate in a thermomixer for 20 min at 30 °C and 650 rpm.
10. Flash freeze cell pellets in liquid nitrogen (*see* **Note 9**) and store at −80 °C until RNA extraction and quantification (*see* Subheading 3.2).

3.2 RNA Extraction and Quantification

There are two types of samples stored at −80 °C for RNA extraction: non-tritiated and tritiated (*see* **steps 6** and **11** of Subheading 3.1). The RNA extraction protocol is the same for both types except for waste disposal, the method employed for RNA quantification and their final use. Follow the protocol under a fume hood.

1. Defrost the sample pellets on ice and wash by adding 1 mL of cold RNase-free ultrapure water and vortexing for some seconds. Transfer the content to 1.5 mL safe-lock microtubes, centrifuge for 3 min at 4 °C and 16,000× *g*, and eliminate the supernatant.
2. Add 400 μL of TES Buffer and 400 μL of acid phenol-chloroform to each sample. Vortex and incubate in a thermomixer for 45–60 min at 65 °C and 650 rpm.
3. Cool the samples on ice for 5 min and centrifuge them for 5 min at 4 °C and 16,000× *g*.
4. Transfer 360 μL of the supernatant to a new 1.5 mL safe-lock microtube and add 400 μL of acid phenol-chloroform to each sample. Vortex and centrifuge for 5 min at 4 °C and 16,000× *g*.

5. Transfer 340 μL of the supernatant to a new 1.5 mL safe-lock microtube and add 400 μL of chloroform to each sample. Vortex and centrifuge for 5 min at 4 °C and 16,000× *g*.
6. Transfer 320 μL of the supernatant to a new 1.5 mL safe-lock microtube and add 40 μL of 3 M sodium acetate, pH 5.3, and 1 mL of 96% EtOH to each sample.
7. Incubate at least 2 h or overnight at −20 °C to precipitate RNA.
8. Centrifuge for 15 min at 4 °C and 16,000× *g*. Eliminate the supernatant and wash the white RNA pellets with 1 mL of 70% EtOH.
9. Centrifuge for 3 min at 4 °C and 16,000× *g*. Eliminate the supernatant and dry the RNA pellets on a heat block at 65 °C by leaving microtube lids open. Dry RNA pellets become translucent.
10. Dissolve the tritiated RNA samples in 50 μL of RNase-free ultrapure water and the non-tritiated samples in 150–200 μL.
11. For each tritiated sample, dilute 2 μL with 98 μL of RNase-free ultrapure water in a disposable cuvette and measure in a spectrophotometer to know the RNA sample concentration (*see* **Note 10**). These data will be used to calculate the volume to load of each sample on the gel for northern blotting (*see* Subheading 3.3). For each non-tritiated sample, dilute 1 μL with 99 μL of RNase-free ultrapure water in a new 1.5 mL microtube and measure in a microvolume UV-spectrophotometer to know the RNA sample concentration. These data will be used to calculate the RNA/cell mass ratio of each sample (*see* Subheading 3.5).
12. Store the rest of the samples at −80 °C.

3.3 Northern Blotting of the [5,6-^{3}H]-Uracil-Labeled rRNA Samples

The SR is calculated as the ratio of the newly synthesized rRNA molecules ([5,6-^{3}H]-uracil signal) to total rRNA ([γ-^{32}P]-ATP signal), calculated by northern blotting (Fig. 1).

3.3.1 Agarose-Formaldehyde Gel Electrophoresis

The mature 18S and 25S tritiated rRNAs samples (*see* Subheading 3.2) are separated by electrophoresis in 1% agarose-formaldehyde gel using an horizontal cell system. Follow the protocol under a fume hood. Clean all the necessary materials with an RNase cleaning agent, wash with RNase-free ultrapure water, and allow to dry before use.

1. Dissolve 1 gr of agarose in 72 mL of RNase-free ultrapure water in a 250 mL glass flask (*see* **Note 11**). Add 10 mL of MOPS 10X Buffer and 18 mL of 37% formaldehyde. Cast the gel on an 11 × 15 cm UV-transparent tray with the caster, place the 15-well comb and leave it to cool down and solidify for at least 40 min.

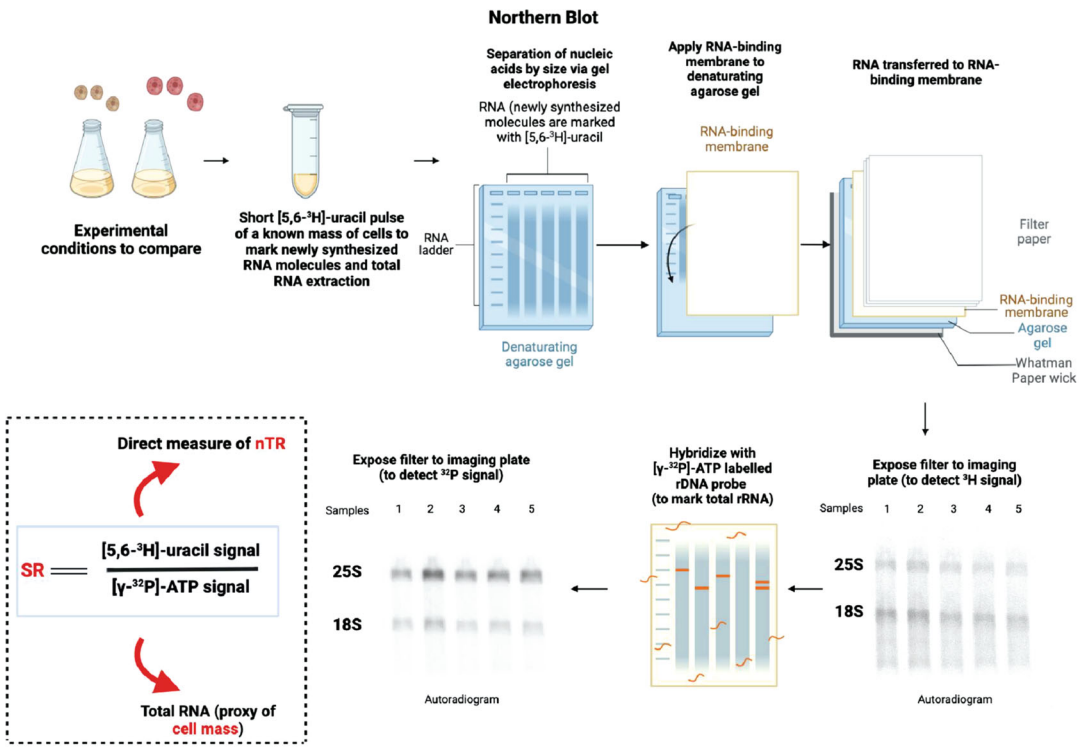

Fig. 1 Measurement of rRNA synthesis rate (SR) by [5,6-^{3}H]-uracil labeling. The process involves several key steps: culturing and harvesting of cells under specific experimental conditions; short-term pulsing of these cells with [5,6-^{3}H]-uracil to label nascent RNA molecules; RNA northern blotting. The latter comprises main stages: RNA is separated according to size by denaturing agarose gel electrophoresis followed by transfer to an RNA binding nylon membrane. After transfer, the membrane is dried and fixed by crosslinking both sides in a 365 nm UV transilluminator. Then, the blot is exposed to a specific ^{3}H phosphor imaging plate to detect ^{3}H signal. The same membrane undergoes hybridization with a mix of a couple of [γ-^{32}P]-ATP labeled rDNA probes, which are complementary to both the 18S and 25S rRNAs. After drying off the membrane, the RNA of interest, now specifically bound to the probe, is visualized and analyzed after exposure to a regular ^{32}P phosphor imaging plate designed for ^{32}P detection. Finally, the quantification of ^{3}H and ^{32}P signals is used for SR calculation. Created in BioRender.com

2. Remove the comb, place the gel in the electrophoresis cell, cover with 800 mL of MOPS 1X buffer and connect the power supply to the system. Pre-run the gel for 5 min at 70 V.
3. Prepare a loading mix for each sample. Add 1.3 µL of MOPS 10X buffer, 2.7 µL of 37% formaldehyde, 6.7 µL of formamide, 3 µL of the loading buffer with ethidium bromide, and the volume of sample needed to load 1µg of tritiated RNA (*see* **Note 12**).
4. Submit the samples to a centrifuge pulse, warm them up on a heat block at 65 °C for 15 min, and load one sample in each gel well (*see* **Note 13**).

5. Run for 1 min at 110 V and then run for 3 h at 70 V to separate the mature 18S and 25S rRNAs.
6. When gel electrophoresis ends, turn off the power supply and disconnect it from the system.
7. Take the transparent tray with the gel carefully out of the electrophoresis cell and drain any excess buffer (*see* **Note 14**).

3.3.2 Capillary Transfer to a Membrane and Crosslinking

Once separated by electrophoresis, the mature 18S and 25S tritiated rRNAs samples are transferred by capillarity from the gel to a positively charged nylon membrane (Fig. 1).

1. Place a gel tray upside down in the center of another larger plastic tray and fill it with approximately 1.5 L of SSC 6X buffer (*see* **Note 15**).
2. Make an 11 × 21 cm Whatman paper bridge, place it on the upside-down tray and bend both ends so they come into contact with the buffer. Completely wet the paper bridge with the same buffer.
3. Excise the gel from the transparent tray by placing the palm of your hand on top of the gel and flipping the tray. Place the gel in this direction (upside down) over the Whatman paper bridge and wet the surface with the buffer (*see* **Note 16**).
4. Cut the nylon membrane to the size of the gel (11 × 15 cm), wet it with the buffer, and gently lay it on top of the gel with the help of tweezers. Make a mark on the membrane to remember its orientation.
5. Cut three pieces of Whatman paper to the size of the gel (11 × 15 cm), wet them with the buffer, and stack them on top of the membrane. Remove any air bubbles by gently rolling a glass tube from the center outwardly in all directions.
6. Lay a paper towel (or filter paper) stack on top of the Whatman papers (*see* **Note 17**).
7. Place a glass plate on top of the structure and a flat weight of 1–1.5 kg on the plate so that the exerted pressure keeps the different layers in contact and so that the transfer of the buffer from the tray to the paper stack takes place. This will cause rRNAs to migrate from the gel and bind to the nylon membrane upon contact with it.
8. Leave overnight to transfer.
9. Carefully disassemble the transfer system and rescue the membrane. Leave it to dry for a couple of hours in the dark between filter papers and without putting pressure on it.
10. Expose both membrane sides to UV light of a crosslinker to permanently fix the RNAs that bind to the membrane. Follow your UV crosslinker instructions to crosslink RNAs to the membrane (usually 50 mJoule; *see* **Note 18**).

3.3.3 Exposing Imaging Plate for ^{3}H Detection and Digitization

1. Erase any previous signals from the ^{3}H phosphor imaging plate before exposing it to the new membrane. For this purpose, expose it to the white light of a phosphor imaging plate eraser for 20 min.
2. Place the membrane face upward on the bottom of a cassette and the erased ^{3}H imaging plate on top of it with the phosphor-sensitive side facing the membrane. Close the cassette and maintain exposure for 2–4 days.
3. Reveal it using a phosphor imaging plate scanner and save the digitized image (*see* Subheading 3.5 for the image analysis). Follow your scanner instructions for digitization (usually 650 nm red laser, [IP] filter, and 50μm pixel size).
4. If the protocol is not to be continued immediately after this step, store the membrane in a dry place and away from light to avoid fungal contamination.

3.3.4 Hybridization with the [γ-^{32}P]-ATP Nucleic Acid Probe

1. Defrost both the rRNA working oligonucleotide stocks and the Kinase 10X buffer on ice. Preheat three heat blocks at 37 °C, 65 °C and 100 °C, respectively.
2. Prepare the following mix to label the rRNA probes with [γ-^{32}P]-ATP: 14 μL of RNase-free ultrapure water, 0.5 μL of the 18S rRNA working oligonucleotide stock, 0.5 μL of the 25S rRNA working oligonucleotide stock, 2 μL of Kinase 10X buffer, 1 μL of T4-PNK enzyme, and 2 μL of [γ-^{32}P]-ATP.
3. Secure the microtube with a lid lock and incubate the mix in a heat block for 45–60 min at 37 °C.
4. Incubate the mix on a heat block for 25 min at 65 °C to inactivate the enzyme.
5. Boil the mix on a heat block for 3 min at 100 °C, cool on ice, and use or store at −20 °C.
6. Preheat a hybridization incubator with a tube rotator at 42 °C to prehybridize the membrane. Place the membrane, with the surface of the sample facing inwardly, inside a hybridization glass tube. Add 20 mL of the hybridization buffer and incubate for at least 30 min at 42 °C in the rolling hybridization incubator (*see* **Note 19**).
7. To hybridize the membrane with the radioactive probe, add 5–10 μL of the radioactive mix to the 20 mL of the hybridization buffer used to prehybridize the membrane. Close the glass tube well to avoid any leakage. Shake it gently and incubate overnight at 42 °C in a rolling hybridization incubator (*see* **Note 20**).
8. Recover all the 20 mL of the hybridization buffer with the radioactive probe in a 50 mL conical centrifuge tube and store at −20 °C (*see* **Note 21**).

9. Preheat a hybridization incubator with a rotator at 38 °C to wash any excess radioactive probe from the membrane. Add 20 mL of Washing Solution I and incubate for 5 min at 38 °C with agitation. Discard the wash and repeat once more with Washing Solution I and twice with Washing Solution II.
10. Remove the membrane from the hybridization tube with tweezers, drain it, and wrap it in Saran Wrap. Try to avoid bubbles between the membrane and Saran Wrap (*see* **Note 22**).
11. Check the intensity of the radioactive signal with a Geiger counter to verify the hybridization. The CPS (counts per second) value may vary depending on the amount of hybridized probe and the distance from the Geiger to the membrane. The measurement provides an approximate idea of the optimal exposure time of the membrane to the ^{32}P imaging plate.

3.3.5 Exposing Imaging Plate for ^{32}P Detection and Digitization

1. Follow the instructions given for the detection of [5,6-^{3}H]-uracil (*see* Subheading 3.3.3), but using a ^{32}P phosphor imaging plate and exposing it to the membrane for approximately 2 h (*see* **Note 23** and Subheading 3.5 for the image analysis).

3.3.6 Stripping Hybridization Probes

1. Place the membrane in a glass tray and add 200 mL of boiling stripping solution.
2. Wash the membrane by shaking it for 10 min in a tray shaker. Discard the wash.
3. Repeat washing 2–3 times until the Geiger counter no longer detects a radioactive signal.
4. Dry the membrane (*see* **step 9** of Subheading 3.3.2) and store it in a dry place away from light to avoid fungal contamination (*see* **Note 24**).

3.4 Cell Culture, Sample Collection, and [5,6-^{3}H]-Uracil Pulse for rRNA Labeling and Chase of the ^{3}H-rRNA for Degradation Rate Determination

The DR is calculated from the disappearance of new rRNA molecules ([5,6-^{3}H]-uracil signal) during active growth. After a short ^{3}H-uracil pulse, the newly synthesized ^{3}H-rRNA molecules are chased for several hours to follow their disappearance by northern blotting (as previously explained), but by taking into account the dilution associated with the culture's growth rate (GR; Fig. 2). In fact, DR for a first order reaction is the product of the degradation rate constant (k_d) by the [rRNA]. k_d is the relevant feature of an unstable molecule. Therefore, what we calculate here is the k_d.

1. Grow cells as previously explained (*see* **steps 1–3** of Subheading 3.1). For each studied condition, inoculate 20 mL of SC-URA in a 100 mL glass flask with the necessary volume of the pre-inoculum to obtain a culture with an OD_{600} of approximately 0.7 after being grown overnight in a thermostatic incubator at 30 °C and 180 rpm.

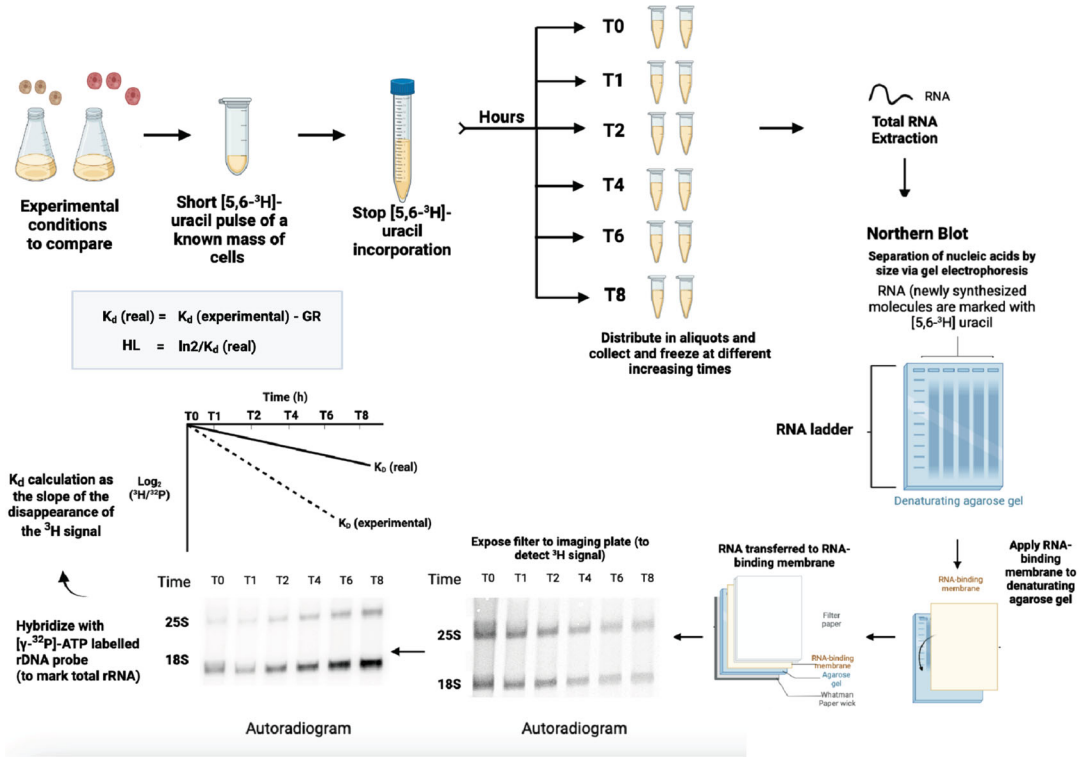

Fig. 2 Measurement of rRNA degradation rate (DR) by [5,6-^{3}H]-uracil labeling and chase. This method is similar to the one used for synthesis rate (SR) determination showed in Fig. 1. The three first steps are alike but, after stopping the [5,6-^{3}H]-uracil incorporation, the mixture is divided into different aliquots in order to let cells grow for several hours and collect them at different sampling times. Each one is processed as described for SR determination. Then, the ^{3}H/^{32}P signal is relativized to the "T0" sample and plotted versus the sampling time (hours after pulse). The slope of the regression line gives the "experimental k_d" (in hours^{-1}, dashed line) that should be corrected by subtracting the growth rate (GR, in hours^{-1}) of the culture to obtain the "real k_d" (solid line). The rRNA half-life (HL) can be calculated from the real k_d. Remember that DR = k_d [rRNA]. However, for most purposes k_d is more useful because it uses the same units as GR, it is easily converted into HL, and it is the variable that informs us about the variation in rRNA stability. Created in BioRender.com

2. Preheat an aliquot of 1–2 mL of SC-URA medium in a thermomixer at 30 °C (keep it running until the end of the experiment) and, per condition, another aliquot of 6.86 mL of SC + SuperURA in a 15 mL conical tube using a thermostatic incubator at 30 °C.
3. When the OD_{600} of cultures is around 0.7, collect in 2 mL safe-lock microtubes the same mass of cells of each cell culture condition. Take a 2 mL sample that would correspond to an exact OD_{600} of 0.7 as a reference (*see* **Note 8**). Centrifuge for 3 min at 16,000× *g* and eliminate the supernatant (repeat the process to completely dry the cell pellet).
4. Resuspend cell pellets in 140 µL of the preheated SC-URA medium and keep samples in a thermomixer at 30 °C and 650 rpm.

5. Add 14 μL of [5,6-^{3}H]-uracil to each sample for tritium labeling. Incubate in a thermomixer for 5 min at 30 °C and 650 rpm. Add the [5,6-^{3}H]-uracil with a 1-min lag between samples so that the pulse lasts the same for all the conditions. The same principle applies to the next two steps.
6. To stop tritium incorporation, carefully collect all the tritiated mixture and add it to the 6.86 mL of the preheated SC+Super-URA medium in a 15 mL conical tube. Mix it by vortexing and distribute the total volume into safe-lock microtubes with 500 μL each (*see* **Note 25**). Incubate all the aliquots in a thermomixer at 30 °C and 650 rpm.
7. After a 20-min incubation, collect together two of the 500 μL aliquots in a single new 1.5 mL safe-lock microtube with no hole in the lid (total volume of 1 mL), centrifuge for 3 min at 16,000× *g* and eliminate the supernatant. Flash freeze the pellet in liquid nitrogen (*see* **Note 9**) and store at −80 °C until RNA extraction and quantification (*see* Subheading 3.2). This sample will be considered to be "Time 0" or "T0."
8. Repeat **step 7** after 1, 2, 4, 6, and 8 h from "T0". The sampling time can be shortened or prolonged to suit other experimental approaches. These samples (two aliquots collected together per time point) will be taken as T1, T2, and so on.
9. Extract and quantify the RNA of samples (*see* Subheading 3.2) and perform the northern blotting as previously explained (*see* Subheading 3.3). Save the digitized images of the ^{3}H and ^{32}P phosphor imaging plates for the statistical data analysis (*see* Subheading 3.5).

3.5 Statistical Data Analysis

3.5.1 RNA/Cell Mass Ratio Calculation

In order to calculate the SR in the next section, the ^{3}H signal (newly synthesized rRNA) must be corrected taking into account the total amount of rRNA in each sample (^{32}P signal). For this correction to be valid, it is necessary to verify that the changes in the RNA concentration are proportional to the changes in cell mass. For this reason, the rRNA/cell mass ratio is calculated for each sample. It is verified that there are no significant differences between them.

1. Following the protocol described in Subheading 3.2, extract and quantify all the non-tritiated samples (two independent aliquots per condition).
2. Calculate the RNA/cell mass ratio for all the samples following the example in Table 1. The "harvest OD" is the OD_{600} of **step 6** in Subheading 3.1 and the "harvest (mL)" is the volume collected per independent aliquot. [E1] and [E2] are the concentrations of the independent aliquots quantified in Subheading 3.2. The "resuspension volume" is the volume of the RNase-free ultrapure water used in **step 14** to dissolve the non-tritiated RNA pellets.

Table 1
Example of data used for RNA/cell mass ratio calculation

REPLICA	SAMPLE	HARVEST OD	HARVEST VOLUME (mL)	OD UNITS	[E1] (ng/μL)	[E2] (ng/μL)	[AVERAGE] (ng/μL)	RESUSPENSION VOLUME (mL)	TOTAL RNA (μg)	RNA/CELL MASS RATIO	RELATIVE RNA/CELL MASS
	Example	X	Y	$Z = X \times Y$	W_1	W_2	$W = (W_1 + W_2)/2$	Q	$U = W \times Q$	$S = U/Z$	Ratio = S_n/S_A
R1	A	0.412	10	4.12	1001	949	975	0.1	97.5	23.67	1
	B	0.44	10	4.4	1269	1323	1296	0.1	129.6	29.45	1.24
	C	0.42	10	4.2	1309	1237	1273	0.1	127.3	30.30	1.28
	D	0.348	10	3.48	1088	1095	1091	0.1	109.1	31.35	1.32
	E	0.372	10	3.72	1062	1091	1076	0.1	107.6	28.93	1.22
R2	A	0.347	10	3.47	1087	1059	1073	0.1	107.3	30.91	1
	B	0.364	10	3.64	1368	1353	1360	0.1	136.0	37.37	1.21
	C	0.311	10	3.11	1191	1321	1256	0.1	125.6	40.40	1.31
	D	0.32	10	3.2	1345	1271	1308	0.1	130.8	40.88	1.32
	E	0.332	10	3.32	1278	1287	1282	0.1	128.2	38.63	1.25
R3	A	0.379	10	3.79	1135	1148	1141	0.1	114.1	30.11	1
	B	0.365	10	3.65	1239	1208	1223	0.1	122.3	33.52	1.11
	C	0.346	10	3.46	1989	1708	1848	0.1	184.8	53.41	1.77
	D	0.371	10	3.71	1406	1421	1413	0.1	141.3	38.10	1.27
	E	0.36	10	3.6	1389	1370	1379	0.1	137.9	38.32	1.27

3. Calculate the relative RNA/cell mass ratio to the control sample of each replicate (sample A in the example) as shown in the last column of Table 1.
4. Perform a one-way ANOVA statistical data analysis.

3.5.2 SR Calculation as the ^{3}H Signal/^{32}P Signal Ratio

The SR is calculated as the ratio of new rRNA molecules (synthesized after a short [5,6-^{3}H]-uracil pulse) to total rRNA (a proxy of cell mass), calculated by northern blotting after hybridization with a ^{32}P-labeled rRNA probe.

1. Following the protocols described in Subheadings 3.2 and 3.3, extract and quantify all the tritiated samples, and perform a northern blot assay.
2. Calculate the SR for all the samples following the example of Table 2. "X" and "Z" are the ^{3}H signals obtained for the mature 18S and 25S rRNA in **step 3** of Subheading 3.3.3. "Q" and "R" are the ^{32}P signals obtained for the mature 18S and 25S rRNA in Subheading 3.3.5. Both signals ^{3}H and ^{32}P are quantified (arbitrary units) using the GelQuant.NET computer program and relativized to the control sample of each replica (sample A in the example). The relative values are shown in columns "Y," "W," "U," and "S," respectively. Finally, the 25S and 18S rRNA SRs are calculated as the relative ^{3}H signals to relative ^{32}P signals ratios.
3. Represent the $^{3}H/^{32}P$ ratio of the samples of the different replicates and perform a two-way ANOVA statistical data analysis.

3.5.3 DR Calculation as the Disappearance Rate of the ^{3}H Signal

In order to measure the DR during active growth, the preexisting ^{3}H-labeled rRNA is chased for several hours after the [5,6-^{3}H]-uracil pulse to follow its disappearance. To measure the DR during active growth, the preexisting ^{3}H-labeled rRNA is chased for several hours after the [5,6-^{3}H]-uracil pulse to follow its disappearance.

1. Following the protocol described in Subheading 3.4 and the statistical analysis in Subheading 3.5.2 (*see* Table 2), calculate the $^{3}H/^{32}P$ ratios for all the samples (different time points in this case).
2. For each time sample, calculate the relative $^{3}H/^{32}P$ ratio to the "T0" sample and represent them versus the sampling time (hours after pulse).
3. Determine the slope of the regression line fitted to the point cloud (*see* Fig. 2). This value represents the sum of the DR (k_d), plus the dilution rate (equivalent to the GR). It is mentioned as the "*experimental* k_d" in Fig. 2.

Table 2
Example of data used for synthesis rate (SR) calculation

		^{3}H SIGNAL				^{32}P SIGNAL				^{3}H SIGNAL/^{32}P SIGNAL	
REPLICA	SAMPLE	25S	RELATIVE 25S	18S	RELATIVE 18S	25S	RELATIVE 25S	18S	RELATIVE 18S	25S	18S
	Example	X	$Y = X_n/X_A$	Z	$W = Z_n/Z_A$	Q	$U = Q_n/Q_A$	R	$S = R_n/R_A$	$Ratio_{25S} = Y/U$	$Ratio_{18S} = W/S$
R1	A	5163704	1	7290383	1	16907364	1	9458058	1	1	1
	B	7316575	1.417	11053230	1.516	29514993	1.746	14776337	1.562	0.812	0.970
	C	4986154	0.966	6962848	0.955	37079681	2.193	14022928	1.483	0.440	0.644
	D	5024959	0.973	7549783	1.036	35325136	2.089	13804797	1.460	0.466	0.710
	E	3441183	0.666	6718732	0.922	24789561	1.466	12256266	1.296	0.455	0.711
R2	A	7138395	1	14962875	1	21483387	1	11194571	1	1	1
	B	8459232	1.185	16102001	1.076	44770503	2.084	18190965	1.625	0.569	0.662
	C	5468957	0.766	11068032	0.740	26826263	1.249	9795644	0.875	0.614	0.845
	D	4982820	0.698	11001811	0.735	30085825	1.400	12159950	1.086	0.498	0.677
	E	5195201	0.728	10147850	0.678	31371846	1.460	11353195	1.014	0.498	0.669
R3	A	8755811	1	18384333	1	25527881	1	10705871	1	1	1
	B	6076620	0.694	13919081	0.757	24146470	0.946	11738441	1.096	0.734	0.691
	C	4444946	0.508	10212756	0.556	26738920	1.047	12707436	1.187	0.485	0.468
	D	6247777	0.714	12119807	0.659	34558307	1.354	15247166	1.424	0.527	0.463
	E	4652821	0.531	9500825	0.517	24118997	0.945	11214947	1.048	0.562	0.493

4. Calculate the real k_d following the equation described in Fig. 2. That is to say:

$$\text{real } k_d = \text{experimental } k_d - \text{GR}.$$

The GR can be calculated from the OD_{600} of the successive sampling points measured in Subheading 3.1 (*see* **Notes 7** and **26**).

5. Calculate the rRNA half-life (HL) following the equation described in Fig. 2:

$$\text{HL} = \ln 2 / \text{real } k_d.$$

4 Notes

1. RNase-free water is obtained by autoclaving distilled water at 134 °C for 1 h.
2. Add sterilized glucose after autoclaving to prevent caramelization. Add 2% agar before autoclaving for the agar plates of the same media.
3. Adjust pH with NaOH. All the solutions used to prepare 10X MOPS are autoclaved. Do not autoclave the final one because MOPS is temperature-sensitive. Store well away from light (wrapped with aluminum foil). The solution is damaged if it turns yellow.
4. Adjust pH with 37% HCl (or a dilution from it) if necessary.
5. To facilitate the dissolution of compounds, it is necessary to stir the mixture and apply heat. Adding BSA after autoclaving is recommended to avoid denaturing.
6. For ^{3}H-uracil labeling to correctly work, there must be no other source of uracil in the medium, otherwise cells are less efficient when incorporating the non-tritiated uracil instead of the tritiated one. In fact, the medium with excess non-tritiated uracil is administered to stop the incorporation of ^{3}H-uracil by cells after 5 min, which is what the pulse lasts. This is why cells must be able to grow in the absence of uracil.
7. Under these growth conditions, a culture is considered to be in the exponential phase when its OD_{600} is between 0.3 and 0.8. Nevertheless, this may vary from one yeast strain to another, and also depending on the growing conditions. So before carrying out the experiment, performing growth curves of strains is recommended to know their doubling time and to be able to predict how they will grow. Moreover, GR determination is needed for the DR calculation (*see* Subheading 3.5).
8. This calculation is based on the formula: $OD_1 \times Vol_1 = OD_2 \times Vol_2$.

9. For example, if the OD_{600} of the culture is 0.42 (not 0.4), 1.9 mL should be collected (instead of 2 mL) to obtain the same cell mass. When the OD_{600} is slightly lower than 0.4, more than 2 mL should be collected. If so, collect half the volume, centrifuge for 3 min at 16000× *g*, eliminate the supernatant, and collect the other half.
10. Immerse the microtubes in liquid nitrogen by holding them upright for a few seconds so that the sample freezes at the bottom and the radioactive contents do not touch the lid. Always use microtubes with screw caps to prevent them from opening unexpectedly when freezing in liquid nitrogen.
11. Measure the RNA stock concentration. To do so, a dispensable spectrophotomter UV-cuvette is used because samples are radioactive and cuvettes can be safely disposed after use. It is also possible to reduce radioactive waste by employing a single cuvette and washing it with RNase-free ultrapure water between measurements.
12. For the complete dissolution, warm the mixture by microwaving the flask (small pulses of no more than 30 s) and carefully shake between pulses. Wear gloves with thermal protection and protective goggles.
13. The amount of RNA loaded to gel is small because the tritium radioactive signal is weak. If too much RNA is loaded, the non-tritiated RNA molecules can shield the signal emitted by the tritiated molecules.
14. Place a black background under the electrophoresis cell to better see wells when loading the samples.
15. When removing excess buffer, hold the tray containing the gel with your fingers in the center to prevent the gel from sliding out of the tray when tilting it.
16. The upside-down gel tray serves as a platform on which to place the other layers needed for the transfer (Fig. 1). The amount of buffer added to the larger plastic tray will depend on the tray size. The buffer should cover at least half the platform (upside-down gel tray), but must never reach the top.
17. After electrophoresis, as samples run more closely to the bottom of the gel, we turn the gel upside down before placing the nylon membrane on top so the transfer distance is shorter. It is necessary to remember this to not confuse the order of samples (the first gel well corresponds to the last one in the membrane).
18. The stack of paper towels should be thick enough to suction sufficient buffer to perform the transfer. However, it should not be much larger than the gel (11 × 15 cm) because the ends will fall into the tray with the buffer when wet and folded.

19. As the membrane has a positive charge and RNA has a negative charge, they are bound by electrostatic forces. To create more stable covalent bonds, it is irradiated with UV light. However, UV irradiation should not be excessive because, otherwise, RNA would fragment and remain attached to the membrane at many different sites. If this occurs, there will be no free sites accessible for the binding of the probes labeled with [γ-^{32}P]-ATP.
20. Place an empty hybridization glass tube on the opposite side of the tube with the membrane to balance the rotator so that it can rotate normally.
21. If the radioactive probe has been frozen, repeat **step 5** before adding it to the hybridization buffer.
22. This hybridization buffer and radioactive probe mix can be reused several times. It can be stored frozen at −20 °C. If so, discard the hybridization buffer used for the pre-hybridization in **step 6** and add this old mix in **step 7** (after repeating **step 5**).
23. Wrapping the membrane, which is still wet, with plastic prevents damaging the ^{32}P imaging plate during exposure to the membrane.
24. The exposure time increases if the employed radioactive probe is old. This depends on the CPS value obtained by the Geiger counter (*see* **step 11** of Subheading 3.3.4) and the time that has elapsed since labeling.
25. If properly preserved, the membrane can be reused to check, for example, the presence of a certain mRNA under those experimental conditions. To do so, the protocol would have to be resumed from the prehybridization of the membrane (*see* **step 6** of Subheading 3.3.4) and it would have to be hybridized with the new radioactive probe.
26. By distributing the total volume into 500 μL aliquots, it is possible to obtain up to 14 samples with a starting OD_{600} of approximately 0.2 (a series of no more than 12–13 microtubes is recommended because, for the last aliquot, there could be shortage of volume due to pipetting errors). This step is necessary for three reasons. First, to follow the disappearance of the ^{3}H-rRNA signal, the original pulse must be divided into different aliquots that can be collected after distinct increasing incubation times. Second, as cells keep growing in microtubes instead of glass flasks, the aliquot volume and its initial OD_{600} must be low enough for cells to still grow in such a small space and with limited oxygen. To allow better culture oxygenation, making a little hole in microtube lids is recommended. This can be done by heating the tip of a small needle with a lighter and drilling the lid's plastic with it. Third, as RNA must be extracted from those aliquots and the first ones

are collected when the OD_{600} is still very low, we need to collect together a pair of microtube aliquots per incubation time point to obtain enough cell mass for correct RNA extraction.

27. To calculate the GR, represent the $\log_2(OD_{600})$ of the samples collected in Subheading 3.1 versus the sampling time (hours) and determine the slope of the regression line fitted to the point cloud. Calculate the duplication time (DT) following this equation: DT = 1/slope. Calculate the GR as follows: GR = $\ln_2$/DT (hours).

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Part V

Method for RNA Decay Induction

Chapter 13

A Method for Rapid Inducible RNA Decay

Lauren A. Blake, Leslie Watkins, and Bin Wu

Abstract

Modulating RNA decay is a powerful tool to investigate RNA degradation dynamics. Here, we describe a protocol to inducibly recruit protein factors to regulate target RNA metabolism, called Rapid Inducible Decay of RNA (RIDR). RIDR induces fast and synchronous decay of target mRNAs within minutes and enables direct visualization of mRNA decay dynamics and subcellular kinetics in living cells. Here, we provide detailed procedures to make stable cell lines, conduct fixed- and live-cell measurements, and perform data analysis. We discuss the potential pitfalls and make RIDR applicable to a general biology lab.

Key words RNA decay, Fluorescent in situ hybridization, Immunofluorescence, Single molecule imaging, Kinetics

1 Introduction

RNA degradation, a crucial aspect of cellular homeostasis, plays a pivotal role in maintaining transcript abundance and quality. However, conventional methods for studying RNA decay lack the spatiotemporal precision necessary to dissect its dynamics within specific subcellular compartments [1]. Membrane-less organelles like P-bodies, implicated in RNA metabolism, present a particular challenge due to their transient nature and diverse functions [2]. While traditionally viewed as sites of RNA storage, recent research suggests a more nuanced role in RNA decay, necessitating advanced methodologies to elucidate their function [3, 4].

Fluorescence imaging offers a promising avenue for real-time tracking of RNA dynamics with subcellular resolution [5]. However, challenges such as imaging artifacts and the rapid diffusion of mRNA molecules within cells hinder accurate measurements of decay kinetics. To address these limitations, we developed a novel

Authors Lauren A. Blake and Leslie Watkins have equally contributed to this chapter.

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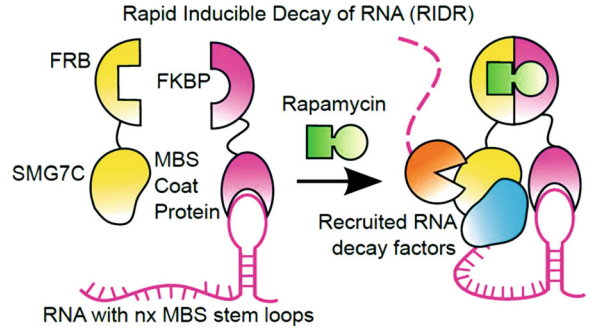

Fig. 1 Rapid Inducible Decay of RNA (RIDR) system uses the FRB/FKBP/Rapamycin chemically inducible dimerization system to instigate RNA decay on demand for MS2-tagged transcripts

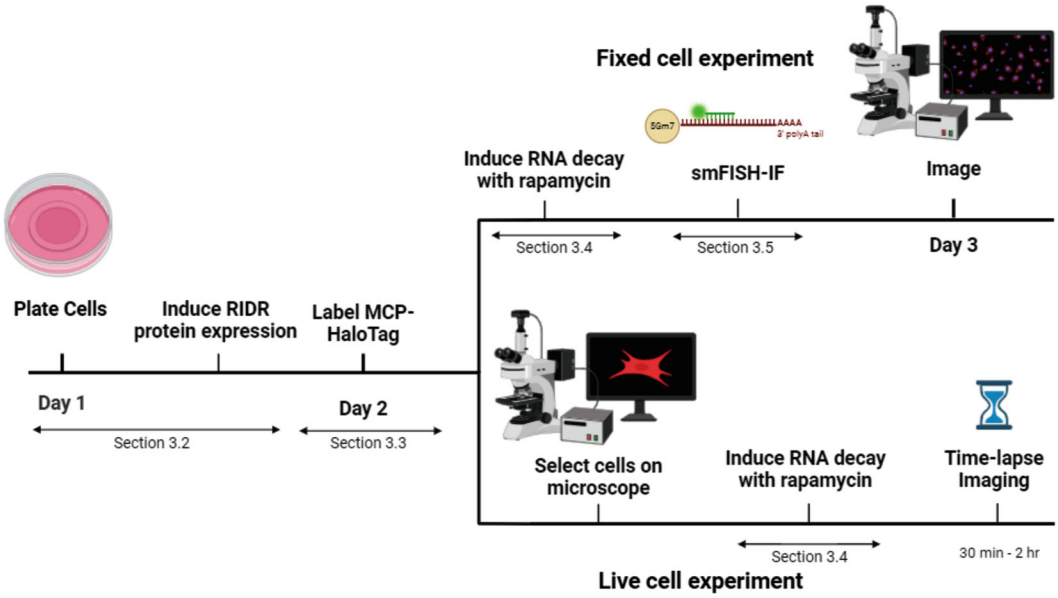

Fig. 2 Timeline of procedures for fixed-cell and live-cell imaging to study RNA decay using RIDR

approach termed Rapid Inducible Decay of RNA (RIDR) [6] which employs a chemically inducible dimerization system [7] to synchronize RNA decay for specific transcripts (Fig. 1). The synchronization enables precise modulation of RNA degradation on demand. By harnessing RIDR, we achieved rapid and synchronized decay of target mRNAs, facilitating the investigation of RNA dynamics within P-bodies and other cellular compartments.

The RIDR method can unravel the spatiotemporal intricacies of RNA metabolism. By combining the innovative techniques of live-cell imaging and single molecule fluorescent in situ hybridization (FISH) imaging with immunofluorescence (smFISH-IF) (Fig. 2), we uncovered the functional significance of P-bodies in

RNA decay processes [8, 9]. Through this high-resolution imaging, we provide unprecedented insights into the regulation of RNA decay dynamics spatiotemporally, paving the way for a deeper understanding of subcellular RNA metabolism.

2 Materials

Prepare all solutions using molecular biology grade water unless noted otherwise.

2.1 Making a Cell Line Stably Expressing RIDR

1. Mouse embryonic fibroblasts (MEF) are derived from a mouse line in which the endogenous ACTB gene was labeled with 24 MS2 Binding Sites (24xMBS) at the 3′ untranslated region (UTR). These were a gift from Robert Singer's Lab [10]. We call this cell line ACTB-MBS MEF. Cells should be at low confluency (~20–30%) for lentiviral infection.
2. HEK293T cells, younger than passage 15 at 90% confluent in a 10 cm dish.
3. Generation 2 plasmids for lentiviral production: tet2, gag, pol, vsvg [11].
4. Tet3G-FRB-SMG7C-IRES-FKBP-Halo-tdMCP (also referred to as tet3G-RIDR) and eGFP-DDX6 plasmids, at least 20 μg of each (*see* **Note 1**).
5. PEI stock: 1 mg/mL Polyethylenimine (PEI) linear MW = 25 kDa in deionized (DI) water. Store at −80 °C.
6. Low serum media (e.g. Opti-MEM). Warm to 37 °C when in use, otherwise store at 4 °C.
7. Complete DMEM: DMEM supplemented with 10% fetal bovine serum (FBS) (v/v), 100 U/mL penicillin, and 100 μg/mL streptomycin. Warm to 37 °C when in use, otherwise store at 4 °C.
8. JF549 HaloTag ligand stock: 10 μM Janelia Fluor 549 HaloTag ligand in DMSO. Store in a black 1.5 mL tube to protect from light at −20 °C.
9. PVDF membrane syringe filter, pore size 0.45 μm and syringes.
10. Amicon Ultra-15 centrifugal filter unit, 100 kDa NMWL.
11. Serum-free DMEM: DMEM without FBS or antibiotics. Warm to 37 °C when in use, otherwise store at 4 °C.
12. Doxycycline stock: 1 mg/mL doxycycline in 100% ethanol. Store at −20 °C.

2.2 Plating Cells for Imaging and Inducing RIDR Protein Expression

1. Cell line expressing MBS-tagged mRNA: ACTB-MBS MEF + tet3G-RIDR cells are used for fixed-cell experiments, while ACTB-MBS MEF + tet3G-RIDR + eGFP-DDX6 cells are used for live-cell experiments. Both cell lines are made using the protocol described in Subheading 3.1.
2. Diluted fibronectin solution: fibronectin bovine plasma solution diluted 1:400 in 1X Dulbecco's Phosphate-Buffered Saline (DPBS) warmed to 37 °C. Make fresh the day it will be used.
3. Complete DMEM.
4. 1X DPBS. Warm to 37 °C when in use, otherwise store at 4 °C.
5. Trypsin EDTA: 0.25% Trypsin, 2.21 mM EDTA. Warm to 37 °C when in use, otherwise store at 4 °C.
6. Trypan blue.
7. Doxycycline stock.
8. #1 12 mm German glass coverslips.
9. 35 mm Glass bottom dish with 20 mm micro-well #1.5 cover glass.

2.3 Labeling MCP-HaloTag with HaloTag Ligand

1. Complete DMEM.
2. Serum-free DMEM.
3. JF549 HaloTag ligand stock.

2.4 Induction of RNA Decay with Rapamycin

1. Rapamycin stock solution: 10 mM rapamycin in DMSO. Store at −20 °C.
2. Rapamycin working stock: 50 μM rapamycin in DMSO. Dilute rapamycin stock to 50 μM in DMSO. Pre-warm to 37 °C right before use, otherwise store at −20 °C. Avoid multiple freeze–thaw cycles.
3. DRB working stock: 50 mM 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) transcription inhibitor in DMSO. Store at −20 °C.

2.5 smFISH-IF to Measure Knock Down of RNA

1. PBSM: 1X PBS, 5 mM $MgCl_2$. Store at room temperature.
2. Fixation buffer: 4% paraformaldehyde in PBSM. Dilute 20% paraformaldehyde to 4% in PBSM. Make this solution on the day it will be used.
3. 50 mg/mL BSA stock: dissolve powdered Bovine Serum Albumin (BSA) in molecular biology grade water. Store at −20 °C (*see* **Note 2**).
4. 10% Triton-X 100 stock: dissolve powdered Triton-X 100 in molecular biology grade water. Store at room temperature.

5. Permeabilization buffer: 0.1% Triton-X 100, 5 mg/mL BSA, 10 U/mL Superase.In. Dilute 10% Triton-X 100 stock, 50 mg/mL BSA stock, and Superase.In in molecular biology grade water. Make this buffer the day it will be used and keep it on ice until use.
6. Pre-hybridization buffer: 10% deionized formamide, 5 mg/mL BSA, 10 U/mL Superase.In, 2X SSC. Dilute 100% deionized formamide, 50 mg/mL BSA stock, Superase.In, and 20X SSC in molecular biology grade water. Make this buffer the day it will be used and keep it on ice until ready to use (*see* **Note 3**).
7. FISH-probes: 48 3′ end-labeled DNA oligonucleotides complementary to a unique position on the RNA target. These can be synthesized in-house using the method described by Gaspar and colleagues [12] or purchased commercially from Stellaris (*see* **Note 4**).
8. Hybridization buffer: 10% deionized formamide, 1 mg/mL salmon sperm DNA, 10% dextran sulfate, 0.2 mg/mL Ambion Ultrapure BSA, 2X SSC, 2 mM RVC, 10 U/mL Superase.In, 20 nM MBSv1 FISH probes, 40–100 nM control FISH probes, 1:1000 Rabbit anti-DCP1a primary antibody in molecular biology grade water. Make this solution in a black 1.5 mL tube the day it will be used to prevent photobleaching of the fluorescent FISH probes (*see* **Note 5**).
9. 2X SSC, 10% deionized formamide pre-warmed to 37 °C. Dilute 100% deionized formamide and 20X SSC in molecular biology grade water. Make this buffer the day it will be used.
10. Secondary antibody buffer: dilute goat anti-rabbit IgG Alexa Fluor 750 conjugated antibody 1:1000 in 2X SSC, 10% deionized formamide pre-warmed to 37 °C. Protect from light by wrapping the tube in foil.
11. Hybridization chamber: place two paper towels in the bottom of a 20 cm cell culture dish and wet them with MilliQ water to humidify the chamber. Place the **lid** of a 10 cm cell culture dish on top of the paper towels so that the flat side is facing up. Cover the 10 cm lid with parafilm so that the clean side of the parafilm (the side that was protected with the wrapper) is facing up. Cover with the 15 cm lid and wrap the whole chamber in foil. The set-up is depicted in Fig. 3.
12. 2X SSC: dilute 20X SSC in molecular biology water. Store at room temperature.
13. Mounting media. We use Prolong Diamond + DAPI.
14. Microscope slides.
15. Nail polish.

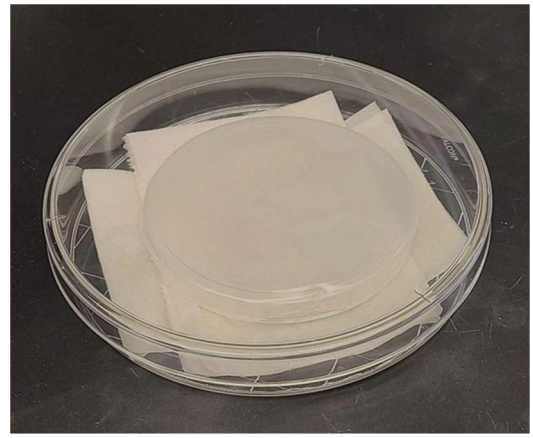

Fig. 3 Hybridization chamber set-up

3 Methods

3.1 Making a Cell Line Stably Expressing RIDR

Dispose of all solid and liquid waste from day 2 until 5 days post lentiviral infection in 10% bleach for at least 24 h before discarding. Proper personal protective equipment (PPE) (gloves, lab coat, safety goggles, etc.) should be used when handling lentiviruses. Culture ACTB-MBS MEF and HEK293T cells in complete DMEM and maintain at 37 °C and 5% CO_2.

1. On the evening of day 1, dilute 75 μL of PEI stock in 500 μL of low serum media and incubate at room temperature for 5 min.
2. Dilute 20 μg of the Tet3G-FRB-SMG7C-IRES-FKBP-Halo-tdMCP plasmid along with 1 μg of gag, pol, tat and 2 μg of vsv-g generation II viral packaging accessory plasmids in 500 μL of low serum media.
3. Vortex diluted DNA to mix and spin down briefly in a microfuge.
4. Add the entire volume of diluted PEI to the diluted DNA and mix gently by pipetting up and down three times. Incubate at room temperature for 15 min.
5. Add the PEI + DNA mixture to the 10 cm dish of HEK293T cells dropwise.
6. Incubate transfected HEK293T cells at 37 °C and 5% CO_2 overnight.
7. On the morning of day 2, replace the media with 15 mL of complete DMEM.
8. On day 3, collect media containing tet3G-RIDR lentivirus 48 h post transfection and pass through a 0.45 μm pore size filter.
9. Spin down for 5 min at 1000 rpm to remove cellular contents.

10. Add the filtered supernatant to the Amicon Ultra-15 centrifugal filter unit and spin down at maximum speed until it is concentrated to ~200 μL.
11. Combine 5–20 μL of concentrated virus with 600 μL of low serum media.
12. Remove cell culture media from the low-confluency ACTB-MBS MEF cells in a 6-well dish then add the diluted virus and let incubate at 37 °C and 5% CO_2 for 2 h.
13. Flash freeze the remaining concentrated supernatant, then store in −80 °C.
14. After 2 h, add 1.5 mL of complete DMEM to each well of the 6-well dish and let the cells recover overnight.
15. On day 4, replace the media on the lentiviral infected cells with complete DMEM.
16. Grow to confluency, then add doxycycline stock to a final concentration of 1 μg/mL to induce expression of the RIDR construct.
17. On the morning of the next day, remove media from the dish so that half of the volume is remaining. Add JF549 HaloTag ligand stock to a final concentration of 10 nM to label the HaloTag protein.
18. Incubate for 30 min at 37 °C and 5% CO_2.
19. Aspirate the media and replace it with 10 mL of serum-free DMEM.
20. Repeat **step 19** twice for a total of three washes of serum-free DMEM, then culture in complete DMEM until ready to sort.
21. Sort cells with the top 10% highest HaloTag protein expression. This is the ACTB-MBS MEF + tet3G-RIDR cell line that will be used for fixed-cell experiments.
22. For live-cell imaging experiments, repeat the above process with the eGFP-DDX6 plasmid to make the eGFP-DDX6 lentivirus and infect the ACTB-MBS MEF + tet3G-RIDR cell line with it as described above.
23. Grow to confluency, label HaloTag protein, and sort for the top 10% highest HaloTag protein expression and bottom 50% eGFP-DDX6 protein expression. This is the ACTB-MBS MEF + tet3G-RIDR +eGFP-DDX6 cell line that will be used for live-cell experiments.
24. Passage cells every 2–3 days once they reach ~75% confluency. Test cells monthly for mycoplasma infection to ensure they are negative.

3.2 Plating Cells for Imaging and Inducing RIDR Protein Expression

This section of the protocol should be started in the morning of a new day.

3.2.1 smFISH-IF Fixed-cell Imaging Experiment

1. Place one 12 mm #1 German glass coverslip into each well of a 24-well cell culture plate.
2. Cover each coverslip in the 24-well plate with 500 µL of diluted fibronectin solution and incubate in 37 °C for 30 min (*see* **Note 6**).
3. Aspirate the fibronectin solution from the 24-well plate, rinse the wells with 1X DPBS, and replace with 500 µL of complete DMEM.
4. Aspirate the cell culture media on the ACTB-MBS MEF + tet3G-RIDR cells and immediately rinse with 1X DPBS.
5. Aspirate the 1X DPBS then add enough trypsin to cover the bottom of the cell culture dish and incubate at 37 °C for ~5 min or until the cells start to visibly lift off the plate.
6. Rinse the plate with 10 mL of complete DMEM and pipette gently up and down to make a single cell suspension. Avoid introducing air bubbles to minimize disturbance to the cells.
7. Immediately mix 10 µL of the single cell suspension with 10 µL of trypan blue and count the concentration of live cells.
8. Plate 25-50 k live cells per well on top of the fibronectin-coated coverslip and shake the plate forwards to backwards and side to side to evenly disperse the cells over the coverslip (*see* **Note 7**).
9. Add 0.5 µL of doxycycline stock to each well of the 24-well plate at a final concentration of 1 µg/µL to induce transcription of the RIDR construct.
10. Incubate at 37 °C in 5% CO_2 overnight to prepare for experiment the next day.

3.2.2 Live-cell Imaging Experiment

1. Aspirate the cell culture media on the ACTB-MBS MEF + tet3G-RIDR + eGFP-DDX6 cells and immediately rinse with 1X DPBS.
2. Aspirate the 1X DPBS then add trypsin as described in **step 5** of Subheading 3.2.1.
3. Make a single cell suspension as described in **step 6** of Subheading 3.2.1.
4. Count the concentration of live cells as described in **step 7** of Subheading 3.2.1.
5. Plate 100,000 ACTB MBSMEF +tet3G-RIDR + eGFP-DDX6 cells on an uncoated 35 mm glass bottom dish and shake the plate forwards to backwards, and side to side to evenly disperse

the cells over the dish. For live-cell imaging, we do not recommend coating the glass with fibronectin (*see* **Note 8**).

6. Add 2 μL of doxycycline stock to each 35 mm glass bottom dish at a final concentration of 1 μg/μL.
7. Incubate at 37 °C in 5% CO_2 overnight to prepare for experiment the next day.

3.3 Labeling MCP-HaloTag with HaloTag Ligand

This part of the protocol should be started on the morning of the next day.

3.3.1 Live-cell Imaging Experiments

1. Add 2 μL of JF549 HaloTag ligand [13] stock to a final concentration of 10 nM to each 35 mm glass bottom dish. This will dye the HaloTag protein expressed in the RIDR construct. The dish should be lightly wrapped in foil to protect it from light for the rest of the protocol.
2. Incubate at 37 °C in 5% CO_2 for 30 min.
3. Gently aspirate the media and replace it with serum-free DMEM to remove unbound HaloTag ligand. Do this only once to minimize disturbance to the cells prior to live-cell imaging.
4. Replace the serum-free DMEM with 2 mL of complete DMEM.
5. Transfer to a 37 °C and 5% CO_2 incubator for at least 30 more minutes to equilibrate prior to imaging.

3.3.2 smFISH-IF Fixed-cell Imaging Experiments

1. Add 0.5 μL of JF549 HaloTag ligand [13] stock to a final concentration of 10 nM to each well of the 24-well plate. The plate should be lightly wrapped in foil to protect it from light for the rest of the protocol.
2. Incubate at 37 °C in 5% CO_2 for 30 min.
3. Gently aspirate the media and replace with serum-free DMEM to remove unbound HaloTag ligand.
4. Repeat **step 3** twice for a total of three serum-free DMEM washes.
5. Replace the serum-free DMEM with 500 μL of complete DMEM.

3.4 Induction of RNA Decay with Rapamycin

For live-cell experiments, proceed to Subheading 3.4.2 after selecting cells on the microscope with high RIDR protein expression, right before capturing images or timelapses.

3.4.1 smFISH-IF Fixed-cell Imaging Experiment

1. Add 1 μL of rapamycin working stock and 1 μL of DRB working stock warmed to 37 °C into a 1.5 mL tube at a final concentration of 100 nM rapamycin and 100 μM DRB.

2. Gently remove ~200 μL of media from the well that the rapamycin and DRB will be added to, and pipette to the tube containing rapamycin and DRB.
3. Pipette vigorously up and down ~10 times in the 1.5 mL tube to suspend the rapamycin and DRB in the cell culture media and immediately add back the entire volume to the well dropwise (*see* **Note 9**).
4. Incubate the cell culture dish at 37 °C and 5% CO_2 for desired length of time. We see efficient knockdown of the β-actin mRNA after 30 min of rapamycin addition.

3.4.2 Live-cell Imaging Experiment

1. Add 4 μL of rapamycin working stock and 4 μL of DRB working stock warmed to 37 °C into a 1.5 mL tube at a final concentration of 100 nM rapamycin and 100 μM DRB.
2. Remove media from the well and dilute rapamycin and DRB as in **step 2** of Subheading 3.4.1.
3. Mix the diluted rapamycin and DRB solution well and add dropwise back to the dish as in **step 3** of Subheading 3.4.1.
4. Capture still images or timelapse for desired length of time (*see* Subheading 3.7).

3.5 smFISH-IF to Measure Knock Down of RNA

1. Quickly and gently aspirate the media from the well and add 500 μL of PBSM to the well using the double-handed wash method. All buffer exchanges should be done with this method. This is one PBSM wash (*see* **Note 10**).
2. Repeat the PBSM wash.
3. Aspirate the media from the well then add 500 μL of fixation buffer and incubate at room temperature for 10 min.
4. Wash each well three times with PBSM letting the PBSM sit in the well for 5 min before removing during each wash. This is a safe stopping point. Keep the plate at 4 °C with PBSM in the wells for up to a few days or continue following the last PBSM wash with.
5. Aspirate the PBSM and add 350 μL of permeabilization buffer then incubate at room temperature for 10 min.
6. Repeat **step 4** for three more PBSM washes, 5 min each.
7. Aspirate the PBSM and add 350 μL of pre-hybridization buffer then incubate at room temperature for 30 min.
8. Pipette 25 μL droplets of the hybridization buffer on top of the Parafilm in the hybridization chamber.
9. Gently remove the coverslip from the well using forceps and place cell-side down on the drop of hybridization buffer (*see* **Note 11**).

10. Place the lid of the 20 cm cell culture dish on top of the hybridization chamber and cover the whole chamber with tin foil to protect the samples from light. Incubate at 37 °C for 3 h (*see* **Note 12**).
11. Fill each well of the 24-well plate with 500 μL of 2X SSC, 10% deionized formamide.
12. Gently place the coverslips back into the wells cell side up. Ensure the coverslips are submerged.
13. Aspirate the media and replace with 500 μL of 2X SSC, 10% deionized formamide three more times.
14. Aspirate the media and add 500 μL of secondary antibody buffer. Incubate at 37 °C for 20 min.
15. Repeat **step 14**.
16. Aspirate the media and replace it with 500 μL of 2X SSC. Repeat this two more times quickly for a total of three quick washes in 2X SSC.
17. Incubate at room temperature for 5 min.
18. Place a drop of mounting media on a microscope slide and then place the coverslip cell side down onto the mounting media using forceps. Press down on the coverslip gently with forceps to remove air bubbles.
19. Let the mounting media cure at room temperature in the dark for 24 h.
20. Seal the edges of the coverslip with clear nail polish.

3.6 smFISH-IF Imaging of RNA decay

1. Image slides using a wide-field fluorescence microscope. *See* Fig. 4 for representative images of cells in steady state versus those that were treated with rapamycin to induce RNA decay of *ACTB-MBS* RNA.
2. Select cells with high expression of the RIDR construct. We have found that a high expression of the construct is necessary to achieve knockdown of the MS2-tagged β-actin transcripts (*see* **Note 13**).

3.7 Live-cell Imaging of RNA Decay

1. During live-cell imaging, keep the cells at 37 °C with humidity control on a Tokai Hit stage top incubator. Do not begin the experiment until the temperature of the media in the imaging dish has reached 37 °C after placement in the incubator.
2. Select cells with high RIDR protein expression on the microscope (*see* **Note 14**).
3. Add 100 nM Rapa and 100 μM DRB final concentration as in Subheading 3.4.2.
4. For data collection, image each cell every 5 min for 2 h with 100 ms exposure time (*see* **Note 15**).

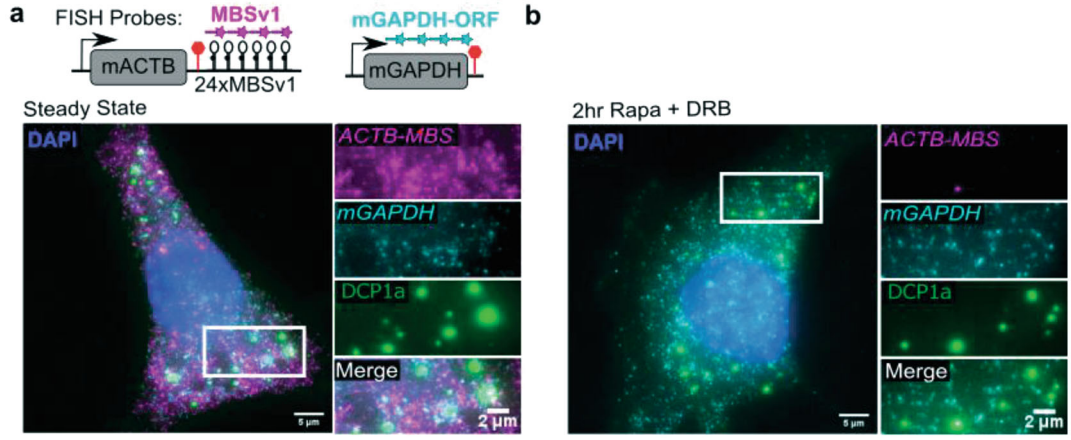

Fig. 4 Knockdown of MBSv1 tagged *ACTB* mRNA. ACTB-MBS MEF + tet3G-RIDR cells were given no treatment (**a**) or treated with 100 nM rapamycin and 100 μM DRB for 2 h (**b**) and then smFISH-IF was performed as described in Subheading 3.5. Magenta = *ACTB-MBS* mRNA, Cyan = *mGAPDH* mRNA, Green = anti-DCP1a antibody for immunofluorescence. Scale bar is 5 μm for cell image and 2 μm for inset

3.8 Image Analysis for smFISH-IF

1. Use an RNA detection platform, such as our in-house platform called uLocalize to count the compartmentalized P-body and cytoplasmic mRNAs separately. All custom codes for smFISH-IF analysis and theoretical modeling can be found at https://doi.org/10.5281/zenodo.7922686 [14].
2. To detect P-bodies in the DCP1a IF channel, filter the image with Laplacian of Gaussian filter.
3. Segment the P-body area using an intensity threshold.
4. Detect single RNAs using a Local Maximum detection algorithm, where the single RNA intensity is determined by fitting the spot to a 3D Gaussian function to extract the center and the amplitude.
5. To quantify the number of RNAs in P-bodies, we measure the integrated intensity of the max projected RNA channel in the segmented P-body area.
6. Normalize the integrated intensities of the RNA granules to RNA counts by dividing the median max projected single RNA intensity.
7. Sum the RNA counts of all the P-bodies in single cells to obtain the total P-body RNA per cell.
8. Count the cytoplasmic RNA per cell as all the detected RNA in the cytoplasm excluded from the segmented P-bodies.
9. Sum these RNA counts in the cytoplasm and P-bodies to obtain the total RNA amounts in single cells.

3.9 Analysis of Live-cell Imaging Data

1. Produce max projections of the live-cell z-stacks at each time point, which will be used for analysis. To produce movies for visualization, the max projections of the FKBP-Halo-tdMCP and eGFP-DDX6 channels can be background subtracted using the rolling ball algorithm.
2. Detect P-bodies in the eGFP-DDX6 channel using the Point Source Detection algorithm of uTrack-v2 [15].
3. Measure the intensity of the HaloTag channel over time in the regions segmented by the detected P-bodies.
4. Sum the intensity at each time point for total RNA signal in P-bodies. Normalize the summed intensity values by the maximum to control for different levels of intensity due to some conditions being imaged in different excitation conditions. Average the normalized intensities across multiple traces.

4 Notes

1. The Tet3G backbone allows for the RIDR construct expression to be induced with doxycycline right before an experiment, instead of being expressed continuously. Continuous expression of RIDR in the ACTB-MBS MEF cells results in loss of RIDR expression after a few weeks of passaging and lower expression levels overall. This is likely due to overexpression of SMG7C causing mild toxicity in the cell, therefore, it is selected out during rounds of passaging. Inducible expression via the Tet3G system prevents this loss.
2. It is important to use this BSA and to keep both the powder and the 50 mg/mL stock RNase free. If the BSA becomes contaminated or expired, the RNA FISH signal will be weak and not as clear.
3. The formamide will denature secondary structure in the RNA, allowing the FISH probes to bind. Bring the 100% deionized formamide stock to room temperature before opening the bottle to prevent condensation from accumulating on the lid of the bottle. It is important to add the formamide to the solution the day it will be used.
4. Listed in Table 1 are the sequences of the RNA-FISH probes targeting MBSv1. The control probe sequences for *mPol2RA* or *mGAPDH* can be found in the original publication describing the RIDR method [6]. MBSv1-targeting probes should be labeled on the 5′ and 3′ ends with the dye of choice and pooled together. We usually use Cy3 or Atto590. Because these probes bind repetitive regions within the MBSv1 sequence, we use 20 nM final concentration of these probes in the hybridization buffer instead of 40–100 nM. For non-repetitive probes, 48 unique probes binding the same transcript should be used.

Table 1
MBSv1 RNA FISH probe sequences

Probe Name	Sequence
MS2_LK20	TTT CTA GAG TCG ACC TGC AG
MS2_LK51-1	CTA GGC AAT TAG GTA CCT TAG
MS2_LK51-2	CTA ATG AAC CCG GGA ATA CTG

5. We have found that using ultrapure BSA in this step enhances the FISH signal. Always vortex and spin down the FISH probe stock before adding it to the hybridization buffer to ensure an accurate concentration of FISH probe is added.
6. Ensure coverslips are not floating on top of the coating solution. The glass should be completely covered in diluted fibronectin.
7. Typically, 35 k cells are plated 2 days before rapamycin addition. Cells should be ~50% confluent the day they are fixed so the cells do not overlap in the dish. Do not swirl the media in a circle. This will disperse the cells only on the edges of the well. An even distribution of cells is required for optimal imaging.
8. Live-cell imaging did not require coating with fibronectin, as to prevent cells from moving too much during imaging, and because they are not subjected to as many washes as smFISH preparation.
9. Vigorously mixing the rapamycin is required because it has limited solubility in water. Limit the amount of time the cell culture dish is outside of the incubator, because extended exposure to room temperature can affect the rate of mRNA decay. The volumes given in the method result in 100 nM final concentration of rapamycin treatment; however, 10 nM also sufficiently induced knockdown. A concentration lower than 10 nM reduces the efficiency of knockdown.
10. The double-handed wash method prevents the coverslip from drying out. In this method, hold the aspirator in one hand and a serological pipette filled with the next buffer in the other. Aspirate media with one hand and immediately fill the well with the next buffer, then repeat on the next well. Avoid touching the coverslip with the aspirator and use a low vacuum speed to prevent damage to the cells.
11. Do this part quickly so as not to bleach the fluorescent FISH probes. The probe hybridization step is done this way to ensure the entire coverslip is covered in the buffer while also minimizing the amount of buffer needed for each sample.

12. Make sure the lid of the hybridization chamber does not touch the coverslips. This can cause the coverslips to stick to the lid and lift off when the lid is removed.
13. RIDR works fastest when high levels of the RIDR construct are present, evident by high intensity of FKBP-HaloTag-tdMCP-NLS nuclear signal. When selecting cells for fixed-cell data analysis, cells are thresholded for high intensity of HaloTag signal in the nucleus. The nucleus was segmented using the DAPI signal.
14. When choosing cells to image, choose cells that have visible P-bodies via the eGFP-DDX6 signal, and that are also in the top 50% of FKBP-HaloTag-tdMCP-NLS nuclear signal. Selecting cells expressing higher expression of the RIDR construct will maximize response time.
15. Light stress can inhibit decay kinetics, so low excitation conditions should be used during live-cell imaging. On our microscope, this is 2% 488 and 2% 640 laser stimulation for 100 μs every 5 min. On the other hand, high excitation conditions simulate light stress. On our microscope, this is excitation with 2% 488 and 10% 640 laser stimulation for 100 μs every 5 min. More frequent stimulation and longer exposure time will also affect the degree of light stress.

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Part VI

Methods for the Functional Characterization of RNA-Proteins Interactions

Chapter 14

reCRAC: A Stringent Method for Precise Mapping of Protein–RNA Interactions in Yeast

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Abstract

Intricate interactions between RNA-binding proteins (RBPs) and RNA play pivotal roles in cellular homeostasis, impacting a spectrum of biological processes vital for survival. UV crosslinking methods to study protein–RNA interactions have been instrumental in elucidating their interactions but can be limited by degradation of target proteins during the process, low signal-to-noise ratios, and nonspecific interactions. Addressing these limitations, we describe reCRAC (reverse CRAC), a novel adaptation of the CRAC (crosslinking and analysis of cDNA) technique, optimized for yeast *Saccharomyces cerevisiae*. Like CRAC, reCRAC applies tandem affinity purification to yield highly enriched protein preparations. However, reCRAC is redesigned to work with unstable proteins. This is achieved by lysing the cells directly into highly denaturing buffer conditions, followed by stringent purification steps. The reCRAC method was successfully applied to the easily degraded yeast protein Pin4, allowing identification of precise binding sites at base-pair resolution with greatly reduced target protein degradation and improved signal-to-noise ratios.

Key words RNA-binding proteins, Protein–RNA interactions, UV crosslinking, reCRAC (reverse CRAC), CRAC (crosslinking and analysis of cDNA), Reduced protein degradation, Denaturing pulldown and purification

1 Introduction

RNA-binding proteins (RBPs) are critical to cellular function, playing crucial roles across a vast array of biological processes. These include transcription, as well as a host of posttranscriptional activities such as RNA processing, splicing, nuclear export, subcellular localization, and the precise control of mRNA stability, translation, and eventual decay [1, 2]. By adeptly facilitating necessary changes in the transcriptome in response to environmental stimuli, RBPs rapidly modulate gene expression, making them instrumental in maintaining cellular homeostasis, and ultimately ensuring the

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survival of the organism. In recent years, methodological advancements have transformed our ability to explore protein–RNA interactions within their native cellular environment, offering novel insights into the dynamic and often transient encounters between RNAs and protein partners. UV crosslinking lies at the heart of most of these methods [3]. This zero-distance crosslinking initiates covalent bonds between directly interacting RNA and proteins, preserving the interactions between protein and RNAs even under very stringent extraction conditions [4].

This represents a notable advance over techniques based on formaldehyde crosslinking, which trigger undesired protein–protein crosslinks, are less penetrative for large complexes, and the protein–RNA interactions can only be purified under semi-denaturing conditions, potentially generating false positive interactors [5–7].

Several protein-centric techniques to enrich the target proteins with the crosslinked RNAs have been reported, which differ in the method of library preparation [5]. Commonly used techniques, eCLIP, iCLIP, irCLIP, easy CLIP, and CRAC utilize 254 nm UVC light to induce crosslinking [8]. In contrast, PAR-CLIP employs 365 nm UVB light to exclusively crosslink RNA incorporating the uracil analogue 4-thioUracil (4sU), achieving a higher specificity and allowing for the study of dynamic processes such as RNA synthesis and turnover [9]. Nonradioactive, fluorescence-based CLIP versions are also available [10]. Subsequent protein purification is performed under varying conditions, from semi-denaturing (CLIP) to fully denaturing (CRAC). RNA-centric techniques recover bound proteins and include silica-based methods such as 2C, TRAPP, and PAR-TRAPP, or phase-separation techniques like PTex, OOPS, and XRNAX [11– 13]. All of these techniques are reviewed by Esteban-Serna et al. [14].

This chapter presents reCRAC (reverse CRAC), a novel modification of the tandem affinity-based CRAC (crosslinking and analysis of cDNA) method applied in yeast [15, 16]. reCRAC offers a more robust approach for the isolation of protein–RNA complexes by decreasing target protein degradation, and therefore, enhancing the detection of authentic RNA targets. It achieves this by implementing more stringent and denaturing conditions, particularly during cell lysis and the first round of affinity purification, relative to CRAC and CLIP protocols. This protects the target protein from proteases degradation, decreases nonspecific interactions, and minimizes background noise. The cell lysis buffer includes 6 M Guanidine-HCl and 300 mM salt, and is followed by denaturing purification on the Nickel (Ni-NTA) beads. The subsequent secondary purification is on anti-Flag beads, reversing the order of tandem purification steps in the CRAC method.

Briefly, reCRAC starts by engineering yeast strains to express the protein of interest tagged with a Flag-4xAla-8xHis N-terminal sequence (N-FH), or 8xHis-4xAla-Flag C-terminal sequence (HF-C) from the endogenous gene locus. This ensures that the fusion protein, regulated by its natural promoter, is the only variant produced. Strains in growth medium are irradiated with UVC light at 254 nm to facilitate formation of covalent bonds between the target proteins and interacting RNAs. After denaturing cell lysis, complexes are bound to Nickel Ni-NTA affinity resin. Enzymatic processing is then carried out on the Ni-NTA column including the partial Benzonase digestion, radioactive labelling with [^{32}P], and linker ligation on the 3′ and 5′ ends of RNA. Following elution from the Nickel column, a second purification on anti-Flag beads is performed under more native conditions that are permissive for antibodies. Protein–RNA complexes are eluted, run on denaturing SDS-PAGE, and the RNA is visualized by exposing the gel to film. The target protein–RNA complexes are excised from the gel and treated with Proteinase K to release an RNA fragment including the protein binding site. This RNA is recovered, converted to cDNA through reverse transcription, PCR amplified, and sequenced. The outline of the experimental steps in reCRAC and their approximate time duration is given in Fig. 1.

Like all UV-crosslinking-based methods, reCRAC is not without its limitations. The efficiency of crosslinking is expected to be variable, potentially displaying a bias for preferential binding of pyrimidines to specific amino acids [13]. The efficiency of crosslinking can also fluctuate depending on the medium used, the cell type being studied, and the target protein itself [6]. Additionally, the process of epitope tagging, essential for reCRAC method, can potentially influence protein stability, expression level, or RNA-binding efficiency. We therefore recommend confirmation of tagged protein synthesis and, if possible, function prior to reCRAC. Notably, reCRAC has currently been applied only in yeast cells and requires a significant quantity of starting material. However, the technique should be applicable to other organisms, with suitable adjustments to cell lysis conditions.

In our hands, reCRAC has demonstrated its efficacy in precisely mapping RNA binding sites for the yeast protein Pin4. This largely uncharacterized RNA-binding protein was previously implicated in the response to different stresses, particularly glucose withdrawal [17]. In standard CRAC procedure, Pin4 suffered a high level of protein degradation (Fig. 2a, b), which was greatly reduced by reCRAC (Fig. 2a, b). We also observed improved cDNA library signal (Fig. 2c) and improved signal-to-noise ratios (Fig. 2d). We believe that reCRAC is a valuable tool for identification of biologically relevant protein–RNA interaction sites.

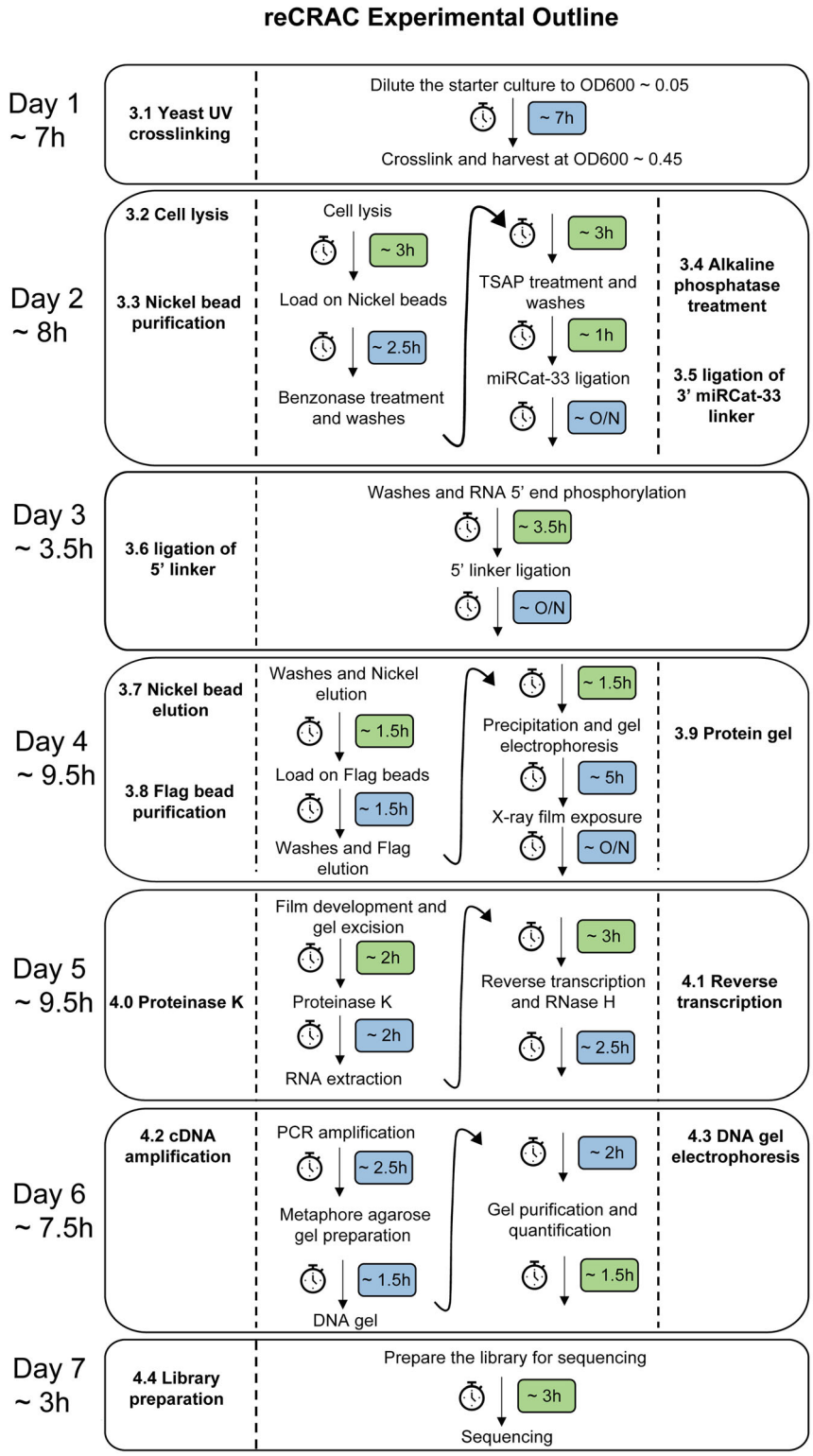

Fig. 1 Outline of the reCRAC experiment. Each bubble represents a separate day with key experimental steps, numbered based on the description in the protocol. The duration of each part is indicated in color-coded boxes: green for active hands-on time and blue for mainly incubation hands-off steps. It is important to note that these durations are approximate and can vary depending on the experience and the quantity of samples being processed. The time duration given here should be approximately for six samples

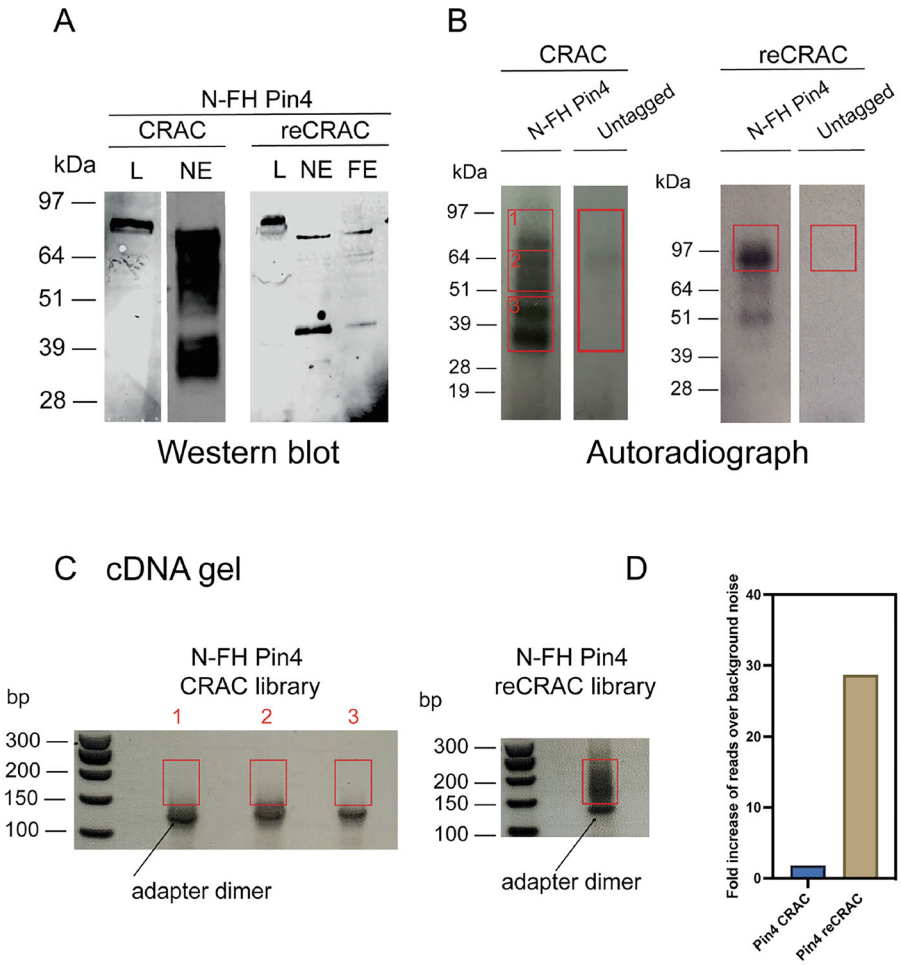

Fig. 2 CRAC and reCRAC analysis of Pin4. (**a**) Western blot analysis showing the N-FH (Flag-4xAla-8xHis) tagged Pin4 protein in the lysate (L) and after the elution from Nickel purification step (NE), and Flag elution step (FE) in CRAC and reCRAC. The protein is visibly degraded to a very large extent during CRAC, while in reCRAC, the degradation is reduced to two bands. (**b**) Autoradiograph indicating the presence of covalently crosslinked N-FH Pin4 - RNA complexes in both CRAC and reCRAC. The red boxes indicate the regions excised for further steps. Three distinct regions (1, 2, 3) have been marked for excision in CRAC. The untagged strain was used as a negative control. (**c**) Metaphore agarose gel electrophoresis of the resulting cDNA library from the CRAC and reCRAC method. The regions: 1, 2, 3; in CRAC cDNA gel correspond to the bands excised from the autoradiograph. The smear indicating the presence of cDNA products is readily visible in reCRAC and shows a good length distribution. Regions excised for sequencing are indicated in red. The lower strong band represents adapter dimer artifacts. (**d**) Fold increase of reads over background (untagged strain) in CRAC (left bar) versus reCRAC (right bar)

2 Materials

In preparation for the experiment, care should be taken to ensure that all materials, pipettes, and work surfaces are free of DNases and RNases. We recommend using RNase-free filter pipette tips throughout the experiment. The use of disposable gloves is mandatory throughout the process, and all laboratory practices should prioritize safety. When handling live cells, ensure a sterile environment to avoid contamination, and wash the bench and your gloves with 70% ethanol. All buffers should be prepared using DNase/RNase-free water. Once stock solutions are prepared, they should be filter-sterilized and stored at 4 °C. Buffer A and Buffer B should be made fresh on the day of the experiment. Additives such as β-mercaptoethanol and protease inhibitors should be mixed into buffers immediately before use; these solutions are not suitable for overnight storage. Stock β-mercaptoethanol handling and phenol-chloroform extraction should be done in a fume hood. Follow all waste disposal regulations. Additionally, it is essential to carefully plan the experiment due to its length and labor-intensive nature, with some days requiring more than 8 h of laboratory work.

2.1 Yeast Strains and Culture Media

For the successful isolation of protein–RNA complexes, the protein of interest is tagged with either an N-terminal tag Flag-4xAla-8xHis (N-FH-protein) or a C-terminal tag 8xHis-4xAla-Flag (protein-HF-C). To explore the RNA partners of the uncharacterized *S. cerevisiae* RNA-binding protein Pin4, we engineered a yeast strain that expresses the N-FH tagged version of Pin4 from the endogenous *PIN4* gene locus. The tagged protein is therefore the only form of Pin4 in the cells, which showed no visible growth defects. The untagged parental yeast strain BY4741 was used as a negative control in our experiments.

Our yeast cultures were grown in a medium composed of 2% glucose (or other carbon source as required) and 2% yeast nitrogen base, supplemented with 2% amino acids excluding tryptophan (SD-Trp). The omission of tryptophan is critical as it absorbs light at 254 nm, the wavelength used for UVC crosslinking, potentially leading to reduced crosslinking efficiency.

2.2 Buffers and Solutions

1. DNase/RNase free water.
2. Buffer A: 50 mM Tris-HCl pH 7.5, 300 mM NaCl, 0.1% NP-40, 4 mM imidazole pH 8.0, 6 M Guanidine-HCl, 5 mM β-mercaptoethanol.
3. Digestion buffer: 50 mM Tris pH 7.8, 20 mM NaCl, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol.

4. 1× PNK buffer: 50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 0.5% NP-40, 5 mM β-mercaptoethanol. It can be stored at 4 °C long-term (several months) without β-mercaptoethanol.
5. 5× PNK buffer: 250 mM Tris-HCl pH 7.8, 50 mM $MgCl_2$, 25 mM β-mercaptoethanol
6. Elution buffer: 50 mM Tris-HCl pH 7.8, 500 mM NaCl, 0.3% SDS, 250 mM imidazole pH 8.0, 5 mM β-mercaptoethanol.
7. TN150: 50 mM Tris-HCl pH 7.8, 150 mM NaCl, 0.1% NP-40, 5 mM β-mercaptoethanol. It can be stored at 4 °C long-term (several months) without β-mercaptoethanol.
8. Buffer B: 50 mM Tris-HCl pH 7.8, 50 mM NaCl, 0.1% NP-40, 4 mM imidazole pH 8.0, 5 mM β-mercaptoethanol.
9. Proteinase K buffer: 50 mM Tris-HCl pH 7.8, 50 mM NaCl, 0.1% NP-40, 4 mM imidazole pH 8.0, 5 mM β-mercaptoethanol, 1% SDS, 5 mM EDTA.
10. 0.5 M EDTA-NaOH pH 8.0.
11. 1 M Tris-HCl pH 7.5.
12. 1 M Tris-HCl pH 7.8.
13. 10% NP-40.
14. 10% Triton X100.
15. 100% and 70% ethanol.
16. 10× TBE buffer: 890 mM Tris base, 890 mM boric acid, 20 mM EDTA.
17. 14.3 M β-mercaptoethanol.
18. 2.5 M imidazole-HCl pH 8.0.
19. 20% SDS.
20. 25:24:1 phenol-chloroform-isoamyl alcohol mixture.
21. 3 M NaOAc pH 5.2.
22. 5 M NaCl.
23. Acetone.
24. Guanidine HCl powder.

2.3 Enzymes and Other Consumables

1. 100 mM ATP aliquots stored at −20 °C.
2. 100 mM DTT.
3. 2.5 mM dNTP mix.
4. ^{32}P-γATP (6000 Ci/mmol, Hartmann Analytic).
5. Benzonase: working stock prepared by diluting 25 U/μL of Benzonase (Merck) in 1:250 of Digestion buffer with 50% glycerol, store long term at −20 °C.
6. DNA gel loading dye (6×), purple, no SDS.
7. D (+)-Glucose Anhydrous.

8. EDTA-free cOmplete protease inhibitor cocktail.
9. Flag peptide (Sigma-Aldrich).
10. GlycoBlue.
11. GeneRuler 50 bp DNA ladder.
12. Kodak BioMax MS Autoradiography X-ray film.
13. La Taq polymerase with 10× La Taq buffer (Takara).
14. Magnetic anti-Flag beads (Sigma-Aldrich).
15. Metaphore Agarose (Lonza).
16. MinElute Gel extraction kit.
17. MOPS running buffer 20×.
18. Ni-NTA Agarose beads (Qiagen).
19. Nitrocellulose membrane (Thermo Scientific).
20. NuPAGE bis-Tris 4–12% precast gradient gels.
21. NuPAGE LDS Sample Buffer 4×.
22. NuPAGE transfer buffer 20×.
23. Proteinase K (Roche).
24. Qubit dsDNA HS Assay Kit.
25. RNase H 5 U/μL (NEB).
26. RNasin, recombinant 40 U/μL.
27. SeeBlue Plus2 Pre-stained Protein Standard (Invitrogen).
28. Single dropout (SD)-Trp.
29. Pierce Spin-columns.
30. SuperScript III with 5× first strand buffer (Invitrogen).
31. SYBR Safe.
32. T4 PNK 10 U/μL (NEB).
33. T4 RNA ligase I 10 U/μL (NEB).
34. TSAP (Promega).
35. Yeast Nitrogen Base.
36. Zirconia beads (Thistle Scientific).

2.4 Specific Equipment

1. UV crosslinker. We are using the Vari-X linker [18]. An alternative crosslinker such as the Megatron and Stratalinker device can be used [15, 19].
2. Qubit 3.0 Fluorometer.
3. Phosphoimager, with the option of printing gel scan in its original size. We are using the Typhoon FLA9500 laser scanner (GE Healthcare Life Sciences).
4. Radiation room and authorization to work with radioactivity.
5. Geiger counter.

Table 1
Oligonucleotides used in reCRAC

Illumina barcoded 5′ linker[a]	L5Aa	5′ - invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrN**rUrArArGrC-OH - 3′**
	L5Ab	5′ - invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrN**rArUrUr ArGrC-OH - 3′**
	L5Ac	5′ - invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrN**rGrCrGrCrA rGrC-OH - 3′**
	L5Ad	5′ - invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrN**rCrGrCrUr UrArGrC-OH - 3′**
	L5Ba	5′ - invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrN**rArGrArGrC-OH - 3′**
	L5Bb	5′ - invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrN**rGrUrGrAr GrC-OH - 3′**
	L5Bc	5′ - invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrN**rCrArCrUr ArGrC-OH - 3′**
	L5Bd	5′ - invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrN**rUrCrUrCr UrArGrC-OH - 3′**
	L5Ca	5′ - invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrN**rCrUrArGr CrN-OH - 3′**
	L5Cb	5′ - invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrN**rUrGrGrAr GrCrN-OH - 3′**
	L5Cc	5′ - invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrN**rArCrUrCrArGrCrN-OH - 3′**
	L5Cd	5′ - invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrN**rGrArCr UrUrArGrCrN-OH -3′**
Illumina 3′ linker	miRCat-33	5′ AppTGGAATTCTCGGGTGCCAAG/ddC/3′
RT primer	miRCat RT	5′ - CCTTGGCACCCGAGAATT - 3′
PCR primers	P5_forward	5′ - AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCT CTTCCGATCT - 3′
	PE_reverse	5′ - CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGGCCTT GGCACCCGAGAATTCC - 3′

[a]Barcode marked in bold, random nucleotides as N

2.5 Oligonucleotides

Integrated DNA Technologies (IDT) supplied all oligonucleotides used in these experiments. Details of each are provided in Table 1. reCRAC requires Illumina-compatible 5′ and 3′ adapters and both RT (reverse transcription) and PCR forward and reverse primers. The 3′ adapter is a pre-adenylated miRCat-33 linker, the 5′ linker is barcoded and includes a sequence of three random nucleotides that helps in the identification of PCR duplicates and quantification during data analysis. The forward and reverse PCR primers contain specific sequences that are required for the binding of the amplified PCR products onto an Illumina flow cell. For stability and to prevent degradation, these adapters are aliquoted and stored at −80 °C.

3 Methods

3.1 Yeast Cell Culture and UV Crosslinking

1. Begin by streaking the FH-tagged yeast strains and the corresponding negative controls (e.g.. untagged strain) from their glycerol stocks onto YPD agar plates (*see* **Note 1**). Incubate these at 30 °C for approximately 2 days to allow colony growth.
2. Inoculate individual colonies into 70 mL of 2% single dropout (SD)-Trp medium, supplemented with 2% yeast nitrogen base and 2% glucose. Incubate the cultures overnight at 30 °C with constant shaking at 200 rpm.
3. The following morning, dilute the overnight cultures to an optical density (OD_{600}) of 0.05 in 800 mL of pre-warmed SD-Trp medium supplemented with 2% yeast nitrogen base and 2% glucose. Continue to incubate with shaking at 200 rpm until the culture reaches an OD600 of 0.45, which indicates a mid-log phase of growth. This depends on the strain and the sugar source; for BY4741, the time to reach the mid-log phase takes around 7 h.
4. Prior to crosslinking, thoroughly cleanse the Vari-X crosslinker with water and prewarm the UV lamps for 5 min to ensure optimal performance.
5. Crosslink using the Vari-X crosslinker set at 254 nm for 8 s at room temperature with a dose of ~150 mJ/cm^2. This induces the formation of covalent bonds between proteins and the interacting RNA molecules.
6. Immediately following crosslinking, harvest the cells swiftly by filtration onto a nitrocellulose membrane. Put the filter into 50 mL of ice-cold water prepared in a 50 mL falcon tube and resuspend the cells by shaking the tube vigorously.
7. Carefully remove the membrane from the falcon tube and centrifuge the cells down at 4500× *g* for 2 min at 4 °C.
8. Remove the supernatant, collect any remaining water droplets with a vacuum pump, freeze the pellets, and store them at − 80 °C.

3.2 Cell Lysis

The cell lysis process is conducted **on ice**, to prevent degradation of the cellular components.

1. Resuspend the frozen yeast cell pellets in 800 µL of Buffer A supplemented with 20 mM β-mercaptoethanol and EDTA-free cOmplete protease-inhibitor cocktail (1 tablet per 50 mL of Buffer A).

2. Put 1.5 mL of Zirconia beads to the cell suspension.
3. Lyse the cells with six pulses of 1 min on a benchtop vortexer. Between each pulse, allow samples to cool on ice for 1 min to prevent heat build-up.
4. Add 2.4 mL of Buffer A without β-mercaptoethanol containing protease inhibitors. To completely mix the sample, vortex the tube vigorously for 10 s using the benchtop vortexer, and then centrifuge at 4500× *g* for 5 min at 4 °C to separate the lysate from the cell debris.
5. Transfer the supernatant carefully (~ 4.5 mL) to three 1.5 mL locking cap tubes. Care is needed, as the supernatant will be foamy due to the presence of guanidium. Spin the lysate for 20 min at maximum speed in a microcentrifuge at 4 °C. Collect the three supernatants from each sample and combine to a single tube per sample, mix briefly.
6. Take 20 μL as an "Input" lysate for a western blot analysis (*see* **Note 2**). Guanidium cannot be loaded directly onto the gel, therefore, the sample is precipitated overnight. Dilute the input with 80 μL of TN150 buffer, then add 900 μL of 100% ethanol, and put to −20 °C overnight. The following day, centrifuge it at 13,000× *g* for 10 min at 4 °C. Wash the resulting pellet once with 70% ethanol to remove impurities, then resuspend in 1× LDS Sample buffer with 2% β-mercaptoethanol. Store at −20 °C until ready to be run with Nickel and Flag eluates.

3.3 Nickel Bead Purification

All steps are carried out at room temperature unless specified otherwise.

1. Pre-equilibrate the Ni-NTA beads with Buffer A including 5 mM β-mercaptoethanol by washing the beads twice in 10 volumes of buffer; centrifuging at 400× *g* to pellet the beads, and carefully discarding the supernatant after each wash. Make the pre-equilibrated Ni-NTA beads mixture by diluting the beads in 50:50 beads:buffer ratio.
2. Combine the lysate with 350 μL of the pre-equilibrated Ni-NTA beads mixture (containing 175 μL of beads) in a 5 mL Eppendorf tube. Use wide orifice pipette tips or cut the end of a tip with a sterile scalpel for more efficient pipetting of the beads.
3. Put the samples on the rotating wheel for 2 h at room temperature.
4. Transfer the bead-lysate mixture into a Pierce spin-column. Let it stand for a minute or until the filter is visibly saturated and wet. Centrifuge the column for approximately 5 s at a

maximum of 300× *g*. Using this speed is important, as higher speed spins can damage the beads.

5. Wash three times with 400 µL of Buffer A including 5 mM β-mercaptoethanol with gravity flow. Spin down the final wash at 300× *g* for 5 s (*see* **Note 3**).
6. Wash three times with 400 µL of Digestion buffer. During the first wash, carefully rinse the inner walls of the column to remove leftover denaturing buffer. Spin down the columns after the final wash for 5 s at 300× *g*.
7. Seal the bottom of the column with the cap provided by the manufacturer.
8. Place the columns in a 37 °C heat block.
9. Add 100 µL of Digestion buffer, pre-warmed to 37 °C and supplemented with 5 µL of Benzonase working stock to the column.
10. Incubate the column for 5 min at 37 °C with shaking at 900 rpm every 10 s to ensure adequate mixing of beads with the digestion solution.
11. After nuclease treatment, open the lid carefully and remove the cap. The cap can be reused for subsequent incubation steps with the same sample. Quickly centrifuge the column at 300× *g* for 30 s to remove the nuclease solution. Exercise caution to avoid contamination of gloves with the enzyme. Transfer the column to a new collection tube.
12. Immediately add 250 µL of Buffer A including 5 mM β-mercaptoethanol directly onto the beads at the bottom of the column to deactivate any residual nuclease.
13. Wash the column twice with 400 µL of Buffer A including 5 mM β-mercaptoethanol.
14. Wash the beads three times with 400 µL of cold 1× PNK buffer including 5 mM β-mercaptoethanol. From this point on, all steps should be carried out on ice. During the first wash, make sure to rinse the inner wall of the column to remove any traces of guanidium.

3.4 Alkaline Phosphatase Treatment of RNAs

1. Spin out the remaining buffer. Securely place a cap on the column and place into a clean Eppendorf tube. Prepare 80 µL of the following mix (set up at room temperature): 16 µL of 5× PNK buffer, 8 µL of TSAP, 2 µL of RNasin, and 54 µL of nuclease-free water (*see* **Note 4**).
2. Close the lid on the column and gently mix the contents by flicking the column with a finger. The beads should be visibly resuspended.

3. Place the column in a thermal mixer and incubate at 37 °C for 30 min. Set the mixing to 800 rpm for 10 s every 5 min.
4. Following the incubation, carefully open the column lid and remove the cap from the bottom. Wash the beads on ice once with 400 μL of Buffer A including 5 mM β-mercaptoethanol to inactivate TSAP.
5. Wash the beads three times on ice with 400 μL of 1× PNK buffer including 5 mM β-mercaptoethanol to get rid of guanidium.

3.5 On-Bead Ligation of miRCat-33 DNA Linker to 3′ End

1. Spin out the remaining buffer, securely place a cap on the column and place into a clean 1.5 mL tube. Prepare ligation mix (80 μL final volume), adding T4 RNA ligase I last. The mix includes 16 μL of 5× PNK buffer, 8 μL of adenylated 3′ miRCat-33 linker, 2 μL of RNasin, 50 μL of nuclease-free water, and 4 μL of T4 RNA ligase I (*see* **Note 5**).
2. Allow the ligation to proceed for 6 h or overnight at 16 °C (recommended). Set the mixer to agitate at 800 rpm for 10 s every 5 min.
3. Following the incubation, open the column-lid with care and remove the bottom cap. Wash the beads on ice once with 400 μL of Buffer A including 5 mM β-mercaptoethanol.
4. Wash the beads on ice three times with 400 μL of 1× PNK buffer including 5 mM β-mercaptoethanol to eliminate any traces of guanidium. Ensure that the first wash circulates around the rim of the column.

3.6 RNA 5′ End Phosphorylation and On-Bead Ligation of 5′ Linker

1. Spin out the remaining buffer, securely place a cap on the column, and transfer into a clean 1.5 mL tube. Add the following mix: 16 μL of 5× PNK buffer, 4 μL of T4 PNK, 3 μL of [^{32}P]-γATP (10 μCi/μl), and 56 μL of nuclease-free water (*see* **Note 6**). We recommend preparing the mix without radioactive ATP, and then adding the radioisotope in a designated lab area for radioactive work.
2. Incubate the reaction for 40 min at 37 °C with 800 rpm mixing every 5 min for 10 s.
3. Add 1 μL of unlabelled 100 mM ATP for an additional 20 min to ensure thorough phosphorylation of the RNA 5′ ends.
4. At room temperature, wash the beads three times with 400 μL of Buffer A with 5 mM β-mercaptoethanol. We recommend carefully changing the elution tube with each wash, to minimize radioactive contamination.
5. Wash the beads with 1× PNK buffer with 5 mM β-mercaptoethanol three to four times until radiation levels in the flow-through drop below ~30 counts per second.

6. Spin out the remaining buffer and add 80 μL of ligation mix: 16 μL of 5× PNK buffer, 0.8 μL of 100 mM unlabelled ATP, 2 μL of uniquely barcoded 100 μM 5′ linker, 2 μL of RNasin, 4 μL of T4 RNA ligase I, and 55.2 μL of nuclease-free water (*see* **Note 7**).
7. Incubate at 16 °C for 4 h or overnight (recommended) with mixing at 800 rpm for 10 s every 5 min to facilitate the ligation of the 5′ linker to the RNA's phosphorylated 5′ end.

3.7 Nickel Bead Elution

1. Wash the beads three times with 400 μL of Buffer A including 5 mM β-mercaptoethanol.
2. After spinning out the void volume, perform the elution twice using Elution buffer. For each elution, place a cap on the column, incubate the beads with 100 μL of Elution buffer for 10 min at room temperature with mixing at 750 rpm for 10 s every minute. Spin into a new Eppendorf tube, pool eluates.
3. Take 10 μL of the Nickel eluate, add 10 μL of 2× LDS Sample buffer with 4% β-mercaptoethanol, boil for 5 min, and store at −20 °C until ready to run on a gel. This is the "Nickel eluate" checkpoint for the western blot.
4. Dilute the eluate sixfold to make it compatible with Flag bead binding; use 50 mM Tris (pH 7.8) with 1% Triton X100, reducing SDS concentration to 0.05%, NaCl to 83.3 mM, and Imidazole to 41.7 mM.

3.8 Flag Bead Purification

Maintain **a cold environment** throughout this procedure.

1. Prepare 100 μL of magnetic anti-Flag beads suspension per sample (containing 50% of beads) prewashed twice with 5 mL of TN150 buffer. The washes are performed by first collecting the beads with a brief pulse spin to 1000 rpm in a microcentrifuge, and then using a magnetic rack to remove supernatant. After the final wash, resuspend 50 μL of the beads in TN150 buffer to 100 μL final.
2. Combine the diluted eluate from **step 4** of Subheading 3.7 with the prewashed beads and place on a rotating wheel for 1.5 h at 4 °C to bind (*see* **Note 8**).
3. After the incubation, pellet the beads by spinning them up to 1000 rpm in a microcentrifuge. Use a magnetic rack to remove the supernatant.
4. Resuspend the beads in 1 mL of TN150 buffer and transfer them to a new 1.5 mL tube. Rotate for 5 min at 4 °C for uniform washing.
5. Repeat the wash twice with 1 mL of TN150.

6. Elute twice with 150 μL of Flag peptide, prepared at 100 μg/mL in 1× TN150. For each elution, incubate the beads at 37 °C for 15 min with vigorous shaking at 1200 rpm.
7. Combine the eluates post-elution and immediately cool on ice for 1 min.
8. For the western blot, take 10 μL of the Flag eluate, add 2× LDS Sample buffer with 4% β-mercaptoethanol, boil for 5 min, and store until ready to run on a gel. This is the "Flag eluate" checkpoint for the western blot.
9. When the western blot analysis of Input, Nickel, and Flag eluates is ready to be run, heat the samples at 95 °C for 5 min, briefly centrifuge.
10. Load the samples onto a 1.5 mm thick, 10 well NuPAGE 4–12% gradient gel. Run the gel with 1× NuPAGE MOPS running buffer with SeeBlue2 pre-stained protein standard gel for 1 h at 150 V.
11. Transfer onto a nitrocellulose membrane using the 1× NuPAGE transfer buffer with the parameters optimized to the protein of interest.
12. Block the membrane, perform the primary and secondary antibody incubation, and visualize the membrane.

3.9 Gel Electrophoresis

1. Add 2 μL of GlycoBlue, 4 volumes of acetone (1.2 mL) to the combined eluate from **step 7** of Subheading 3.8, and incubate for at least 2 h or overnight, at −20 °C.
2. Centrifuge the samples at maximum speed in a microcentrifuge for 20 min at 4 °C. Carefully remove the supernatant ensuring that the small blue pellet remains undisturbed. No wash of the pellet is required unless the pellet appears large and white, in which case an additional acetone wash is needed. Allow the pellets to dry at room temperature with the lids open for a few minutes. Avoid over-drying, which can impede resuspension.
3. Gently resuspend the pellet in 30 μL of 1× LDS Sample buffer including 2% β-mercaptoethanol. Pipette up and down, checking resuspension progress with a Geiger counter (*see* **Note 9**).
4. Heat the samples at 65 °C for 10 min. Briefly centrifuge post-heating.
5. Load the samples onto a 1.5 mm thick, 10 well NuPAGE 4–12% gradient gel, using 1× NuPAGE MOPS running buffer. Add a protein ladder such as SeeBlue2 pre-stained protein standard.
6. Electrophorese at 150 V for 1 to 1.5 h, or until the dye reaches the foot of the gel.
7. After the run is finished, dismantle the gel cassette, retaining the gel on one of the plastic sides (*see* **Note 10**). Wrap in a saran

wrap, place in an X-ray film cassette, secure with tape (crucial to avoid movement!) and expose to X-ray film for 1 h or overnight at −80 °C, depending on the radioactive signal intensity. Include a chemiluminescent marker to aid in subsequent alignment of the film with the gel. The most sensitive film, MS film should normally be sufficient for detection using a 1-h exposure. Overnight X-ray film exposure might be necessary.

8. Develop the X-ray film and align it with the gel using the chemiluminescent markers as guides. Excise the region corresponding to the size of your target protein plus an additional 20 kDa, to account for RNA crosslinked to the protein of interest. Excise the equivalent region of the negative control lane. Put the excised fragments of the gel into a clean 1.5 mL tube (*see* **Note 11**).
9. Smash the excised gel sections into sub-millimeter pieces with a bent P200 pipette tip exercising caution to prevent gel fragments from scattering out of the tube. Crush the pieces against the tube walls with the tip until they have a paste-like consistency. Bending the tip beforehand helps to avoid accidental suction of gel pieces during this process.

3.10 Proteinase K Treatment

These steps are crucial to release RNA from protein–RNA complexes.

1. Incubate the gel slices in 600 μL of Buffer B including 1% SDS and 5 mM EDTA. Add 100 μg (5.0 μL) of Proteinase K from a frozen stock solution of 20 mg/mL in water and incubate at 55 °C for 2 h with vigorous shaking at 1100 rpm to ensure thorough enzymatic digestion.
2. To remove the gel fragments, transfer the digested mixture to a Pierce spin-column. If necessary, cut the end of a 1 mL pipette tip with a sterile scalpel to make the transfer of the gel chunks easier. Centrifuge the column at the maximum speed for 1 min. Check to ensure all the liquid passes through, leaving gel pieces trapped on the filter.
3. Carefully transfer the supernatant to a new tube. Add 75 μL of 3 M NaOAc pH 5.2, and 750 μL of phenol:chloroform:isoamyl alcohol (24:25:1) in the fume hood.
4. Vigorously vortex the mixture, then centrifuge at room temperature for 20 min at maximum speed. Carefully collect the aqueous top layer to a new tube, taking care to avoid the bottom layer or the interphase. Repeat the phase separation process using the same volumes.
5. Precipitate the RNA with 1.5 mL of ice-cold 100% ethanol and 2 μL of GlycoBlue. Incubate the samples at −80 °C for 30 min or at −20 °C overnight.
6. Centrifuge the samples at 4 °C for 20 min at maximum speed in a microcentrifuge. Wash the RNA pellet with 750 μL of 70%

ethanol, vortex, and centrifuge again for 5 min at 4 °C at maximum speed. Allow the pellet to air-dry for ~5 min.

3.11 Reverse Transcription of Purified RNAs

This will generate cDNA from the extracted RNAs.

1. Resuspend the RNA pellet in the following mix: 4 μL of 2.5 mM dNTP mix, 1 μL of 10 μM RT primer, and 8 μL of nuclease-free water.
2. Heat this mixture to 80 °C for 3 min. Immediately after, snap chill the samples **on ice** for 5 min (*see* **Note 12**).
3. Collect the contents by brief centrifugation and add 6 μL of the following mix: 4 μL of 5× first strand buffer, 1 μL of 100 mM DTT, and 1 μL of RNasin.
4. Incubate the mixture at 50 °C for 3 min to allow the RT primer to bind to the RNA. Subsequently add 1 μL of SuperScript III reverse transcriptase. Incubate the reaction for 1 h at 50 °C, which will synthesize the cDNA from the RNA template.
5. Following the reverse transcription, inactivate the SuperScript III enzyme by heating the sample to 65 °C for 15 min.
6. To remove the RNA template, add 2 μL of RNase H and incubate at 37 °C for 30 min.

3.12 PCR Amplification of cDNA

1. Set up three PCR reactions per sample (*see* **Note 13**), combining the following: 5 μL of 10× La Taq buffer, 1 μL of 10 μM P5_forward primer and 1 μL of 10 μM PE_reverse primer, 5 μL of 2.5 mM dNTPs, 0.5 μL of La Taq, 3 μL of reverse transcription reaction, and 34.5 μL of nuclease-free water.
2. Program the PCR thermocycler as follows: initial denaturation at 95 °C for 2 min; 20–25 cycles of 98 °C for 20 s, 52 °C for 20 s, 68 °C for 20 s; final extension at 72 °C for 5 min.
3. Pool and precipitate the three PCR products per sample: on ice add 1 μL of GlycoBlue, 0.1 volume of 3 M NaOAc, and 2.5 volumes of 100% ice-cold ethanol.
4. Put the mixture to −80 °C for 30 min, then spin for 20 min at 4 °C.
5. Wash the pellet with 70% ethanol, spin for 20 min at 4 °C, and air-dry for 5 min.
6. Resuspend the pellet in 15 μL of nuclease-free water.

3.13 DNA Gel Electrophoresis and Purification

1. Add 3 μL of 6× DNA loading dye to the sample.
2. Prepare a 3% Metaphore agarose gel by first soaking agarose in 1× TBE for 30 min. Heat it in a microwave, until all of the agarose is dissolved. Once it is cooled to approximately body temperature, add 1:10,000 SYBR safe DNA stain for

visualization, and then pour the gel. Allow the gel to solidify at room temperature, and then place at 4 °C for at least 30 min.

3. Load the entire volume of the samples alongside a 50 bp DNA ladder onto the prepared 3% Metaphore agarose gel. Run the gel on a metal block set in a container filled with ice to maintain a low temperature. Run the gel at 80 V until the dye front has migrated to the end of the gel. This usually requires ~2 h.
4. Scan the gel with a phosphoimager. The strong band at 120 bp are amplified linker-linker dimers, with the cDNA libraries forming a smear above this. Print the scanned image at 100% size.
5. Overlay the gel onto the printed image on a glass plate. With a sterile scalpel, excise the section of the gel containing the cDNA library, typically running from 150 to 250 bp, while avoiding the 120 bp primer dimers (*see* **Note 14**).
6. Place the excised gel slices in a clean tube. Re-scan the gel to confirm that the intended region has been accurately removed.
7. Add 6 volumes of Buffer QG from the MinElute Gel Extraction Kit to 1 volume gel (100 mg ~ 100 μL). Melt at 42 °C for 10–15 min or until the gel slice has completely dissolved.
8. Transfer the liquid gel mixture to a MinElute column placed in a provided 2 mL collection tube and centrifuge for 1 min at 16,000× *g*. Discard the flow-through and repeat the process with any remaining sample to ensure complete binding to the column.
9. Perform additional wash with 750 μL of Buffer QG. Spin for 1 min at 16,000× *g*, and then discard the flow-through.
10. To remove any remaining impurities, add 750 μL of Buffer PE from the MinElute Gel Extraction Kit and let it sit for 10 min at room temperature. Optionally, you can invert the column to ensure all of Buffer QG will be washed away. Centrifuge again for 1 min at 16,000× *g* and discard the flow-through.
11. Spin the empty columns at 16,000× *g* for 1 min.
12. Transfer the columns to new, clean 1.5 mL tubes for elution.
13. Elute the DNA by adding 10 μL of pre-warmed (65 °C) nuclease-free water to the column. Allow the water to penetrate the column matrix by letting it stand for a few minutes, then centrifuge for 1 min at 16,000× *g*. Repeat the elution step.
14. Use a Qubit dsDNA HS Assay Kit to quantify the cDNA library. Store the purified libraries at −20 °C until they are ready for sequencing.

3.14 Sequencing and Analysis

Libraries can be pooled and sequenced in a single run. We used an Illumina MiniSeq system, with single end sequencing and 75 bp read length, using the High Output Reagent Cartridge and followed the manufacturer's instructions. Paired-end sequencing can be also performed; however, modifications to the bioinformatics analysis would be necessary.

Analysis of Pin4 sequencing data obtained from reCRAC was performed using custom scripts and the pyCRAC software packages [15, 20]. Below, we describe the main steps in processing of raw reads and the most commonly used pyCRAC tools. The analysis can be adapted for specific proteins and to address specific biological questions. The analysis was performed on a server using a Bash terminal.

3.14.1 Debarcoding, Filtering, and Adapter Removal

The sequencing analysis starts by separating the reads originating from different samples/libraries into independent files, i.e. demultiplexing the raw output file, Output.fastq, by the 5′ linker barcodes, using the pyBarcodeFilter.py tool:

```
$ pyBarcodeFilter.py -f Output.fastq -b Barcodes.list
```

The Barcodes.list is a tab-delimited file that contains the list of barcodes and the names of the samples corresponding to the barcodes. Here is an example of the Barcodes.list used in reCRAC:

```
NNNTAAGC By4741_R1_AA
NNNAGAGC Pin4_R1_Ba
```

Where By4741_R1_Aa is the untagged control, and Pin4_R1_Ba is the target protein.

The pyBarcodeFilter.py will pool all the reads with the same 5′ linker barcode into the same file, named according to the Barcodes.list. In addition, the random nucleotides present in 5′ linker are trimmed at this step and incorporated into the headers of each sequence within the resulting fastq file, which will be crucial during the subsequent Collapsing step. These random nucleotides help distinguish PCR duplicates from independent ligation events.

Header example of the resulting fastq file from pyBarcodeFilter.py:

```
@MN00209:99:000H2WLCG:1:11102:7607:1095 1:N:0:0##CCA
TTATATTACTTNTTAAATACNTNTTACGATAAAAAATAAAANTTACATTATAA
GTTTTTGAAAANAATGGAATT
+
```

```
FFFFFFFFFFF#FFFFFFFF#F#FFFFFFFFFFFFFFFFFF#AFFFFFF
FFFFFAFFFFFFFAF#FFFFFFFFF
@MN00209:99:000H2WLCG:1:11102:14093:1097 1:N:0:0##ACT
AGTGTTCATGCNAGTAGATAATNTAATGATGATAAGTTCGANAATGCCCTTATAA
AAAAAAAAANAAAAAAAAA
+
F=FAFF=FF/F#FFFFFFFFFA#FFFFFFFFFFFFFFFFFF#FFFFFF
FFAFFFFFFFFFFFFF#FFFFFFFFF
@MN00209:99:000H2WLCG:1:11102:9562:1099 1:N:0:0##CAG
AAAATGATGAAAAAAGGACACGNTAATTGGATGCGTTAAAANAAAAAAAAA
GGGGGATTGGGGGNTTGCAAGGG
```

Quality of the sequencing data can be checked with FastQC:

```
$ fastqc Pin4_R1.fastq
```

Alternatively, FastQC can be run on all fastq files in parallel:

```
$ for i in *.fastq; do (fastqc $i &) ; done
```

Next, the Flexbar tool is used for the quality filtering and adapter trimming [21]:

```
$ flexbar –r Pin4_R1.fastq -a adapter.fa --removal-tags –
at RIGHT –ao 4 –u 3 –m 7 –n 16 –t
```

The file, adapter.fa, contains the sequence of the adapter to be trimmed (TGGAATTCTCGGGTGCCAAGGC). This is the sequence that was added on the 3′ end during cloning procedure and PCR amplification. The parameters are set to specify trimming process details. We advise performing a quality control with FastQC on the output fastq files to confirm adapter removal and ensure that the fastq files passed the quality controls.

3.14.2 Collapsing

This code removes reads that are predicted to be PCR duplicates, by collapsing reads with identical sequences including the three random nucleotides present in the 5′ linker, using the pyFastqDuplicateRemover.py tool. The following command iterates through all fastq files in the working directory and puts the output of each as collapsed.fasta file:

```
$ for i in *fastq; do (pyFastqDuplicateRemover.py -f $i
-o ${i}_collapsed.fasta &); done
```

The example lines of the resulting fasta file from pyFastqDuplicateRemover.py:

```
>458_CTC_10
TAAGCGATTTAATCTCTAATTATTAGTTAAAGTTTTATAAGCATTTTATGTAACG
AAAAATAAATTGGTTCATA
>459_TAC_33
ACCCATACGCGTAATGAAAGTGAACGTAGGTTGGGGCCTCGCAAGAGGTGCAC
AATCGACCGATCCTGATGTCT
>460_TAG_23
ATGAGAACTTTGAAGACTGAAGTGGGGAAAGGTTCCACGTCAACAGCAGTTGGAC
GTGGGTTAGTCGATCCTAA
>461_CCC_106
TGTCATTTTGCGTGGGGATAAATCATTTGTATACGACTTAGATGTACAACGGG
GTATTGTAAGCAGTAGAGTAG
```

3.14.3 Alignment

The collapsed reads are aligned to the reference genome of *Saccharomyces cerevisiae* (SGD v64) using the Novoalign tool (V2.07.00, Novocraft) with genome annotation from Ensembl (EF4.74) [22]:

```
$ for i in *collapsed.fasta ; do (novoalign -f $i -s 1 -r
Random -d Saccharomyces_cerevisiae.EF4.74.dna.toplevel.
shortChrNames_k10_s1.novoindex > `basename $i .fasta`.
novo &); done
```

The Saccharomyces_cerevisiae.EF4.74.dna.toplevel.shortChrNames_k10_s1.novoindex is the genome-specific index file generated by novoindex with parameters -k10 s-1, fasta.novo is the ouput file. The resulting novo file has the following header and structure:

```
# novoalign (2.07.00MT - Aug 5 2010 @ 18:45:42) - A short
read aligner with qualities.
# (C) 2008 NovoCraft
# Licensed for evaluation, educational, and not-for-profit
use only.
# novoalign -f Pin4_R1.fasta -s 1 -r Random -d /datas-
tore/home/s1507601/CRAC/CRAC_support_files/Saccharomy-
ces_cerevisiae.EF4.74.dna.toplevel.shortChrNames_k10_s1.
novoindex
# Interpreting input files as FASTA.
# Index Build Version: 2.7
# Hash length: 10
# Step size: 1
>5_CAC_Flexbar_removal_adapter_1                    S
```

```
AATTGCAATGTTATTTTGCGTGNGGATAAATCATTTGTATACGACTTAGATGTA-
CAACG . R 36 3 >chrXII 460994 R . . . 48G>A
>14_TTT_1 S CAAGCAGTAAAGATAGGAAAGATTAATATTATCTCACCCAAA-
TAGTTTCATACATGCACATATAGCTACTACAG . U 60 150 >chrV 29518
F .. . 45A>G 74T>G
```

3.14.4 Read Counting

All reads that align to each feature in the genome, such as introns, exons, CDS, are summed using the pyReadCounters.py from the pyCRAC package. It accepts input files in either .novo or .sam format, along with a gene transfer format (GTF) file that contains gene ID coordinates. For our analysis, we utilized the Ensembl GTF version EF4.74, which includes the mRNA annotations with UTRs, as well as different noncoding RNA categories such as CUTs and SUTs. Additionally, the pyReadCounters.py offers various options, including the type of normalization, read orientation, and alignment quality filtering. In the code below, `---rpkm` option was used to RPKM normalize the reads:

```
$ for f in *.novo; do (pyReadCounters.py -f $f --
gtf=Saccharomyces_cerevisiae.EF4.74_SGDv64_CUTandSUT_-
withUTRs_noEstimates_antisense_intergenic_4xlncRNAs_f-
inal.pyCheckGTFfile.output.gtf --rpkm &); done &
```

The script provides several outputs, including a read count file, with the RPKM normalized cDNA counts mapped to genomic features; GTF file containing additional information about the reads overlapping genomic features in a sample; and read statistics file providing the information about the complexity of the dataset.

3.14.5 Data Visualization

We can visualize the protein binding sites across the whole genome using the Integrative Genomics Viewer (IGV) [23]. Reads are aligned again using Novoalign with modified parameters to obtain the output in a different format (SAM):

```
$ for i in *.fasta ; do (novoalign -f $i -s 1 -o SAM -r
Random -d Saccharomyces_cerevisiae.EF4.74.dna.toplevel.
shortChrNames_k10_s1.novoindex > ‘basename $i .fasta‘_r-
andom.sam &); done
```

The SAM files are then converted into binary BAM files, which we sorted and indexed with “samtools”:

```
$ for f in *.sam ; do samtools view -bS $f > ${f%.*}'.bam'
; done
$ for f in *.bam ; do samtools sort $f > ${f%.*}'_sorted.
bam' ; done
```

To prepare the data for visualization, a scaling factor is calculated to normalize the reads to the size of the library. This normalization factor is based on the number of mapped reads, often normalized to a standard number such as one billion reads. To find out how many reads were mapped:

```
$ samtools stats Pin4_random.sam | grep "reads mapped:"
```

As an example of scaling factor calculation: Pin4_random.sam has 1,448,215 reads, dividing one billion by this number gives the scaling factor of 690.5. Using "bedtools," coverage is computed separately for each strand and scaled by the calculated factor, generating bedgraph files:

```
$ bedtools genomecov -split -strand + -ibam Pin4_ran-
dom_sorted.bam -bg -scale 690.5 > Pin4_random_plus_-
strand.bedgraph
$ bedtools genomecov -split -strand - -ibam Pin4_random_-
sorted.bam -bg -scale 690.5 > Pin4_random_minus_strand.
bedgraph
```

The bedgraphs files are then loaded into IGV.

Another useful data visualization step involves using 'pyPileup.py' tool to analyze the distribution of reads along specific genomic regions of interest. The pyPileup.py provides insights into the specific sites where crosslinks occur based on the number of deletions and substitutions at a nucleotide resolution. These result from nucleotide misincorporation or skipping during reverse transcription, at the site of the amino acid-nucleotide crosslink. The code needs the following inputs: a GTF output from pyReadCounters.py, and a text file containing the gene name(s) of interest. The code also contains the GTF annotation file with the standard genomic feature annotations, and the .tab file with the genomic reference sequence in a more concise format:

```
$ for f in *pyReadCounters.gtf; do (pyPileup.py --file_-
type=gtf -f $f --gtf=Saccharomyces_cerevisiae.
EF4.74_SGDv64_CUTandSUT_withUTRs_noEstimates_antisen-
se_intergenic_4xlncRNAs_final.pyCheckGTFfile.output.gtf --
```

```
tab=Saccharomyces_cerevisiae.EF4.74.dna.toplevel.fa.
shortChrNames.tab -g genes_of_interest.list &); done
```

The output of this code is a tab-delimited file containing the number of deletions and substitutions per each nucleotide of the target gene.

4 Notes

1. It is advisable to incorporate a control sample within the experimental design in addition to experimental replicates. Suitable controls may include an untagged strain or a no-UV control. These controls are anticipated to yield negligible signals during SDS-PAGE analysis, and any libraries generated from sequencing should display significantly fewer reads than target samples; yielding at least 100-fold fewer unique cDNAs.
2. We collect samples from both the lysate and the eluate at each step of purification for western blot analysis. This approach allows monitoring of the target protein throughout the experiment and identifies the specific step should any problem arise.
3. For wash steps, allow the solution to pass through the column by gravity for a minimum 30 s, before spinning them for approximately 5 s at no more than 300× *g* to avoid damaging the beads. Sometimes the columns exhibit slow or no flow by gravity, which we observed with certain batches. In this case, centrifuge the columns at 300× *g* until all the liquid pass through.
4. Treating the RNAs with alkaline phosphatase will remove any 5′ phosphates left behind by the Benzonase cleavage of RNA to prevent self-ligation of RNA molecules.
5. The ligation utilizes a specialized 3′ miRCat-33 linker, which is chemically blocked at its own 3′ end (ddC) to prevent self-ligation, and pre-activated by adenylation at the 5′ end (App) to enable the ligation reaction without the addition of ATP.
6. Phosphorylation using radiolabelled ATP is initially employed to facilitate the detection of protein bound RNA on the SDS-page gel in the subsequent steps. This is followed by addition of high concentration of nonradioactive ATP, allowing for efficient phosphorylation at all RNA 5′ ends, which is required for subsequent 5′ linker ligation.
7. The 5′ linkers are designed with a blocked 5′ end (invddT) to prevent self-ligation and incorporate unique barcodes for

accurate sample identification, allowing for pooling multiple samples into one sequencing run in later step (Subheading 3.9, **step 3**), and the identification of each original sample during the demultiplexing phase. It is crucial to use different barcodes for each sample. The 5′ linkers also include random nucleotides, enabling removal of PCR duplicates.

8. The samples will be still very radioactive at this point. We therefore recommend putting the rotating wheel into a fridge located in the radioactive lab if possible.
9. When analyzing identical proteins under different conditions or across multiple replicates, this is the step when multiple samples can be pooled together. Begin by resuspending one sample pellet in 30 μL of 1× LDS Sample buffer, and then transfer everything to the second sample pellet, which will be merged during resuspension. Combining the samples at this step can minimize background noise and technical variations, ensuring consistent handling and uniform gel extraction in later steps.
10. Accurate alignment of the X-ray film over the gel is essential for precise excision of protein–RNA complexes. To ensure proper alignment, firmly secure the gel within the cassette using tape to prevent it from moving. Aligning the film to the upper left or right corner of the gel also aids in achieving an accurate cut.
11. An alternative method is cutting out from a membrane. This involves transferring the protein–RNA complexes from a gel to a nitrocellulose membrane, exposing the membrane to an X-ray film, and excising the band(s) from the membrane. This offers a cleaner result as documented by Delan Forino et al. [16]. However, this approach comes with a risk of losing a large proportion of the protein–RNA complexes if transfer is inefficient.
12. Heating at 80 °C will denature any secondary structures within the RNA, while snap chilling the samples on ice immediately after locks the RNA strands in a linear form, facilitating better primer annealing.
13. PCR reactions should be prepared in a UV-sterilized environment to minimize the risk of contamination. The goal is to achieve a total cDNA yield of 5–50 ng, which necessitates adjusting the number of PCR cycles accordingly. Typically, 21 cycles are adequate to produce diverse libraries from cDNA synthesized from Pin4-associated RNAs. If the cDNA is of low abundance, it may be necessary to increase the cycle count to approximately 23–25. However, it is essential to avoid overamplification, which can skew the representation of the library.

14. At the stage of DNA gel electrophoresis, adjusting the size distribution of the DNA library is possible. The size selection should be guided by the intended sequencing length, the nature of the protein under study, and the specific biological questions being addressed by reCRAC. If sequencing will be limited to, e.g., 80 base pairs, it is practical to exclude overly long cDNAs as they may reduce the resolution of protein-binding site identification. Conversely, it is generally beneficial to limit the presence of very short sequences (under 20 nucleotides) in the library since they can be challenging to map with confidence, and they are preferentially amplified in the flow cell, introducing a bias.

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Chapter 15

Snapshot Moments of the Cell Revealed by UV-Crosslinking and RNA Co-immunoprecipitation of Transient RNA–Protein Complexes

Ivo Coban and Heike Krebber

Abstract

RNA co-immunoprecipitation serves as a powerful technique for elucidating the interactions between RNA and RNA-binding proteins, pivotal for understanding posttranscriptional regulation mechanisms. This method captures the dynamics of protein–RNA associations across various cellular processes, applicable to both coding and noncoding RNAs. Here we describe a convenient and compelling method using nanobody coupled beads and UV-crosslinking. Importantly, this method can also be utilized to isolate short-lived complexes, for instance in RNA-degradation. The obtained RNA can be used for many downstream applications, such as qPCR or RNA-sequencing.

Key words RNA, Protein, Immunoprecipitation, UV-crosslinking, Nanobody-coupled beads

1 Introduction

RNA co-immunoprecipitation (RIP) became an important technique used to investigate the interactions between RNA and RNA-binding proteins within cells. RIPs can be carried out from different cells types and tissues. We focus on the RIP protocol from *Saccharomyces cerevisiae* for which we will provide all details [1, 2]. RIP is especially valuable in studying the complexities of posttranscriptional regulation and the roles of ribonucleoprotein complexes (RNPs) in various cellular processes. This method coprecipitates the RNAs that are bound to a specific protein, allowing for their subsequent identification and analysis. It provides a snapshot of the dynamic interactions that occur between protein and RNAs during transcription, processing, transport, translation, and degradation of the RNA.

The principle behind RIP involves the use of specific antibodies to target and precipitate an RNA-binding protein of interest and its bound RNA from cultured cells or tissues. Recently, nanobodies

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coupled to beads have been used in preference, which promise more resistant bonding and a higher yield of RNA [3]. Here, the protein-recognizing nanobody is already coupled to the beads. Thus, these beads significantly reduce incubation time of the lysate by eliminating the need for prior incubation with a selected antibody. This can be particularly useful when isolating highly reactive complexes. Purchasable nanobody-coupled beads are mainly directed against known protein-tags, which means that tagging of the protein of interest is a requirement.

RIP can be conducted with various fixation methods that preserve the native conformation and interactions of RNPs, providing a physiologically relevant picture of RNA–protein interactions [4]. There are several fixation methods, two of which have proven to be effective tools for capturing RNAs in active RNP complexes: UV cross-linking and formaldehyde fixation. However, fixation with formaldehyde also forms crosslinks between various different interaction partners [5]. In contrast, UV-crosslinking at 254 nm is much more specific for interactions between nucleic acids and proteins as DNA and RNA absorb light of this wavelength efficiently [5]. The absorbed energy can cause formation of covalent bonds in areas of close proximity potentially at or near sites where hydrogen bonding occurred. The efficiency with which covalent bonds are formed depends heavily on the respective interaction partners. The formation of these stronger covalent bonds results in a significant stabilization of protein–RNA complexes, making them more resistant to conditions that would disrupt weaker hydrogen bonds. Furthermore, it leads to the inhibition of enzymatically active proteins on the RNA such as helicases and nucleases. Either they become covalently bound to the RNA or other covalently bound proteins block these enzymes as they cannot be displaced. In order to avoid interfering with downstream applications, a digestion of the covalent bound proteins after immunoprecipitation is necessary.

After immunoprecipitation, the coprecipitated RNAs can be purified via trizol or hot-phenol RNA extraction followed by precipitation or column-based kit isolation methods. Subsequently, identification and quantification of the RNA can be achieved by using various molecular biology techniques, such as qPCR, microarray analysis, or next-generation sequencing (NGS) [6]. These analyses allow the identification of specific RNAs that interact with the protein or complex of interest. Due to the previous UV-crosslinking and the efficient nanobody-coupled beads, these can also be short-lived complexes, such as degradation complexes, which gradually degrade their RNA [7] (*see* **Note 1**).

2 Materials

Any water used in this protocol must be RNase-free and demineralized. This can be achieved, for instance, with diethyldicarbonat (DEPC) treatment. For this purpose, demineralized water is incubated with DEPC in a ratio of 1:1000 for 24 h with constant stirring. Afterwards the water is then autoclaved at 121 °C for 20 min, in order to neutralize unreacted DEPC by hydrolyzation to ethanol and carbon dioxide.

2.1 RNA Co-immunoprecipitation (RIP)-Buffer

The RIP-buffer is designed to preserve the integrity of both proteins and RNAs and their interactions. For this purpose, the buffer has a low ionic strength. Moreover, the Triton-X-100 is a nonionic detergent used to lyse cells and solubilize cellular membranes without denaturing proteins. Phenylmethylsulfonyl fluoride (PMSF) and dithiothreitol (DTT) preserve the integrity of proteins by preventing degradation and disulfide bond formation. RNase inhibitor is essential for inhibiting RNase activity, thus protecting RNA from degradation during the procedure.

1. 1 M Tris-HCl, pH 7.5, in RNase-free water.
2. 100 mM PMSF, in isopropanol.
3. 1 M DTT, in RNase-free water.
4. RIP-buffer: 25 mM Tris-HCl, 2 mM $MgCl_2$, 150 mM NaCl. Prepare 1 L. This can be stored at room temperature. Before starting the experiment, take an appropriate amount (mostly 50 mL are enough) of the stock solution and add the following components freshly to achieve the specified concentrations: 0.2% Triton-X-100, 0.2 mM PMSF, 500 μM DTT, 1 U/mL Ribolock or comparable RNase inhibitor (*see* **Note 2**). Keep the freshly prepared RIP-buffer on ice (*see* **Note 3**).

2.2 Proteinase K (PK)-Buffer

The PK-buffer is designed to promote the activity of proteinase K. In contrast to the RIP buffer, it is important that protease inhibitors such as PMSF are omitted.

1. 1 M Tris-HCl, pH 7.5, in RNase-free water.
2. 1 M DTT, in RNase-free water.
3. PK-buffer: 50 mM Tris-HCl, pH 7.5, 50 mM NaCl. Prepare 200 mL of stock solution. This can be stored at room temperature. Before starting the experiment or preferably during the incubation period, take an appropriate amount (mostly 10 mL are enough) of the stock solution and add the following components freshly to achieve the specified concentrations: 0.2% Triton-X-100, 500 μM DTT, 1 U/mL Ribolock or comparable RNase inhibitor. Keep the freshly prepared PK-buffer on ice.

2.3 Other Components

1. Nanobody coupled beads, that are directed against the selected protein tag (e.g. trap or selector beads).
2. 10 mg/mL Proteinase K.
3. DNase I.
4. RNase-Inhibitor (Ribolock).
5. Protease-Inhibitor.
6. Glass beads (0.25–0.5 mm of diameter).
7. 0.5 M EDTA, pH 8.0.
8. 10% SDS.
9. TRIzol™.
10. 2-Propanol.
11. Ethanol.
12. Glycogen.
13. Chloroform.
14. Petri dishes (15 cm of diameter).
15. RNase-free 1.5 mL microcentrifuge tubes.
16. 15 mL centrifugation tubes.
17. 50 mL centrifugation tubes.
18. Refrigerated centrifuge.
19. Refrigerated microcentrifuge.
20. Fast Prep-24 machine or similar lysis equipment.
21. UV-chamber with 254 nm light bulb.

3 Methods

To minimize enzymatic activities that could degrade nucleic acids and proteins during the process, carry out all procedures on ice unless otherwise specified. Upon completion of this protocol, four specific samples are prepared for each strain, cell line, or treatment to facilitate a thorough evaluation of the experimental outcomes (*see* **Note 4**).

(a) RNA Reference Sample (Lysate): this serves as a control to assess the total RNA present before the co-immunoprecipitation process. It provides the baseline for the eluted RNA.

(b) Protein Sample (Lysate): this sample is analyzed to ensure that the protein of interest and other cellular proteins have remained intact throughout the procedure. It is crucial for validating the conditions used during the sample preparation.

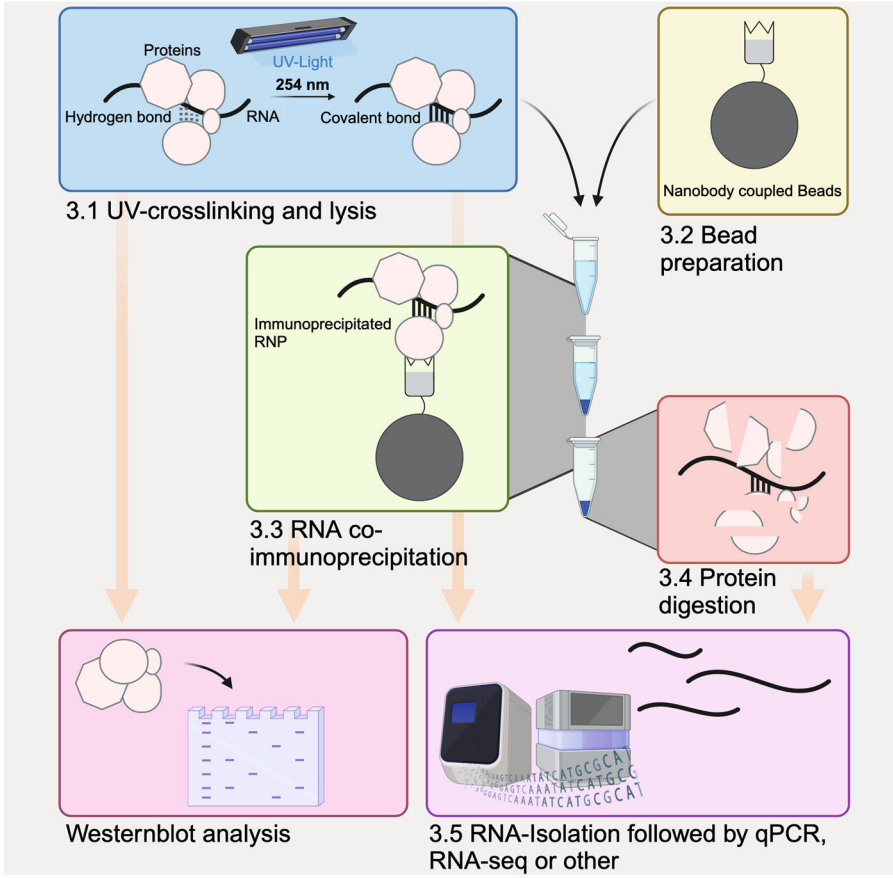

Fig. 1 Flow chart of the general experimental steps. The experiment can be divided into five parts. First, cells are UV-crosslinked and lysed (Subheading 3.1), while the beads are prepared (Subheading 3.2) for subsequent immunoprecipitation (Subheading 3.3). Afterwards proteins are digested (Subheading 3.4) and the RNA is isolated and purified for downstream analysis (Subheading 3.5). Created with BioRender.com

(c) Protein Pulldown Sample (Eluate): This is used to confirm the successful immunoprecipitation of the target protein–RNA complex. The presence of the target protein in this sample, as detected by western blotting, indicates that the pulldown was successful.

(d) Bound RNA Sample (Eluate): This sample contains the RNA that was specifically bound to the target protein. Bound RNA can be identified and analyzed by comparing to the reference.

Figure 1 provides a schematic representation of the steps that are detailed below.

3.1 UV-Crosslinking and Lysis of Saccharomyces cerevisiae

1. Grow a 400 mL culture of cells expressing the tagged protein of interest to log phase. Check by counting ($1–2 \times 10^7$ cells/mL) or use OD (0.8) at 600 nm.
2. Pellet the cells at 4 °C and 4000× *g* for 4 min.
3. Resuspend the cell pellet in 20 mL of media equivalent to that used for growth (preferably YPD, as long as no selection is necessary) and transfer the cells to a 15 cm Petri dish (*see* **Note 5**).
4. Place the Petri dish on a cold metal block to dissipate heat generated by the upcoming UV exposure.
5. Place them in the UV-chamber with a distance of 14 cm to the UV-source.
6. Treat the cells two times for 3.5 min at 0.6 J/cm with 254 nm UV light on the cold metal block with light shaking in between the two exposures.
7. Transfer the cell-suspension to a 50 mL centrifugation tube and centrifuge crosslinked cells at 4 °C and 4000× *g* for 4 min.
8. Discard the supernatant and resuspend the cell pellet in 5 mL of H_2O and transfer the suspension into a 15 mL centrifugation tube.
9. Centrifuge the suspension again at 4 °C and 4000× *g* for 4 min.
10. Discard the supernatant and remove as much as possible of the remaining with a pipette or vacuum pump.
11. Add two pellet volumes of RIP buffer, one pellet volume glass beads, additional protease inhibitor (5 μL per 100 μL of cell pellet) and additional RNase inhibitor (0.12 μL/100 μL of pellet volume). During lysis, the cells own proteases and RNases are released, which requires further addition of inhibitors for this step (*see* **Notes 6**, **7** and **8**).
12. To disrupt the cells, vortex three times for 30 s at 5–6 rpm/s in a Fast Prep-24 machine or similar. Cool down on ice in between each vortexing step for 5 min to prevent excessive heating of the cell lysate, which would lead to the degradation of proteins and RNA.
13. Centrifuge the lysate at 4 °C and 4000× *g* for 4 min.
14. Transfer the supernatant into a 1.5 mL tube and centrifuge at 4 °C and 12,000× *g* for 5 min to clear the lysate.
15. Keep 2% of the cleared lysate (e.g. 20 μL of 1 mL) as the protein lysate sample for western blot analysis and 5–10% lysate (e.g. 50 μL of 1 mL) as RNA lysate sample for RNA isolation.
16. Add DNase I to the RNA lysate sample (14 K units per 100 μL) and to the remaining RIP sample (6.5 K units per 100 μL).

This allows DNA digestion during the subsequent process and usually makes further digestion unnecessary.

3.2 Bead Preparation

1. For each RIP sample, take 20 μL of the bead slurry coupled with nanobodies according to the selected protein-tag into a fresh RNase-free 1.5 mL tube (*see* **Notes 9** and **10**).
2. Add 1 mL of RIP buffer and centrifuge at 4 °C and 2500× *g* for 2 min.
3. Remove the supernatant so that only the beads are left in the buffer.
4. Repeat these washing steps for a total of four times (*see* **Note 11**).
5. During the last washing step transfer the beads to a new tube.

3.3 RNA Co-immunoprecipitation

1. After the last removal of the washing buffer, add the remaining lysate (RIP sample) to the beads.
2. Incubate for 1 h at 4 °C while rotating end over end.
3. Wash the beads five times with RIP-buffer as described before. These washing steps are essential to remove any nonspecific components bound to the beads (*see* **Note 12**).
4. Wash the beads one time with 1 mL of PK-buffer.
5. Resuspend in 1 mL of PK-buffer and split bead suspension by taking 700 μL for RNA isolation in a fresh tube and leave 300 μL for protein control.
6. Centrifuge both samples at 4 °C and 2500× *g* for 2 min and take off the supernatant.
7. For the protein control samples: add 2× SDS loading buffer to the eluate (beads) and lysate samples, boil for 5 min at 95 °C, separate by SDS-PAGE, and detect by western blot (Fig. 2).

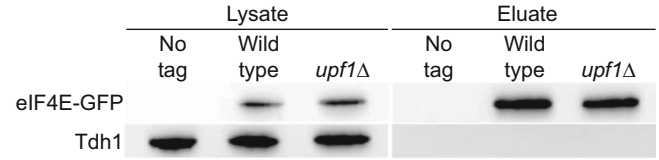

Fig. 2 Western blot to analyze the protein precipitation. Wild-type and a mutant strain of *S. cerevisiae* were transformed with plasmids harboring the GFP-tagged translation initiation factor *eIF4E* and a constructed nonsense mediated decay (NMD)-target ($CBP80^{PTC}$) [7]. eIF4E-GFP and its bound RNAs were precipitated via GFP-beads, and after intense washing, the protein eluates and lysates were analyzed in western blots. The protein of interest was equally precipitated from the different indicated strains and thus the isolated RNA was forwarded to qPCR. A noninteracting protein, in this case the mitochondrial protein Tdh1, is used as a washing control and thus should be absent from the eluates

3.4 Protein Digestion

1. Only for the samples for RNA isolation, resuspend the beads in 100 µL of Proteinase K buffer.
2. Add 0.5% SDS, 5 mM EDTA, and 80 µg (lysate sample) or 40 µg (eluate sample) of Proteinase K. For beads in 100 µL, this is 5 µL of 10% SDS, 1 µL of 0.5 M EDTA, and 4 µL of Proteinase K (10 mg/mL in H_2O). For 50 µL of lysate sample, which was set aside at the beginning, add 2.5 µL of 10% SDS, 0.5 µL of 0.5 M EDTA, and 8 µL of Proteinase K to the lysate for RNA isolation.
3. Incubate for 1 h at 55 °C and 1000 rpm (*see* **Note 13**).
4. Proceed with RNA isolation.

3.5 RNA Isolation

The RNA can also be isolated using a standard hot-phenol protocol or any commercially available RNA isolation kit. Below, RNA isolation using the TRIzol™ reagent is described. Carry out the next steps under a hood.

1. Add 1 mL of TRIzol™ to the beads and lysate.
2. Incubate at 65 °C and 1200 rpm for 10 min.
3. Add 200 µL of chloroform and invert the tube several times carefully.
4. Leave the tubes at room temperature for 3 min. The colorless aqueous phase should now slowly form at the top.
5. Centrifuge the samples at 12000× *g* for 15 min.
6. Prepare a new 1.5 mL tube for every sample by adding 500 µL of 2-propanol and 0.5 µL of glycogen as a precipitation help (*see* **Note 14**).
7. After centrifugation, two distinct phases have now formed. The top clear aqueous phase contains the RNA, and the bottom phenol-chloroform one contains proteins. The intermediate phase contains the remaining DNA, as it is still associated with histones.
8. Take 400 µL of the upper phase into the newly prepared tubes and vortex shortly.
9. Precipitate the RNA overnight at −20 °C.
10. Pellet the RNA at 12000× *g* and 4 °C for 30 min.
11. Take off the supernatant. Note that there may still be residual chloroform and phenol in the supernatant, which should be disposed of properly.
12. Add 1 mL of 70% ethanol and centrifuge again at 12000× *g* and 4 °C for 15 min.
13. Repeat the washing step with 70% ethanol.
14. Take off the ethanol and air-dry the RNA pellet.

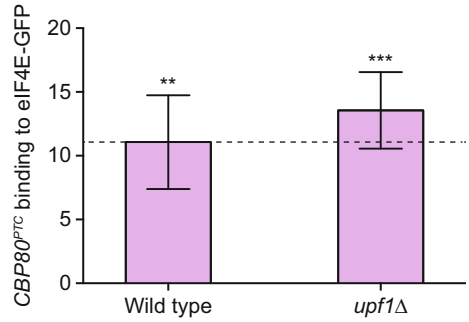

Fig. 3 qPCR detect the amount of specifically bound RNAs. Upon RNA isolation, the RNA was used in qPCRs. In the shown example, the binding of the NMD-reporter RNA, *CBP80*^{PTC} to the translation initiation factor eIF4E-GFP was measured in the indicated strains. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. In the wild type, *CBP80*^{PTC} is usually degraded due to its premature termination codon (PTC). In the *upf1*Δ strain, the recognition of the PTC is disturbed and therefore its amount increases, as it is not eliminated. In order to eliminate possible deviations in the procedure, it is common to normalize the analyzed RNA to nonspecific targets, such as the mitochondrial ribosomal *21S* rRNA

15. Resuspend the RNA in RNase-free H_2O (*see* **Note 15**).
16. The RNA can now be forwarded to downstream analysis, like qPCR (Fig. 3).

4 Notes

1. Although the described protocol is recommended in this form, it can certainly be used as a template for further variations. If the protein cannot be expressed as a tagged protein, G-Sepharose beads and direct antibodies can be used. This requires the lysate to be incubated with the antibody prior to addition to the beads. Despite protein digestion, UV cross-linking can leave protein residues on the RNA, which can be used for CLIP or CRAC analysis. But if these interfere with downstream analyses an approach without cross-linking could be used, omitting the UV and protein digestion steps.
2. 100% Triton-X is very viscous; thus a 10% Triton-X stock solution can be prepared for easier handling. Of this, 1 mL is then added to a 50 mL RIP buffer preparation.
3. The freshly prepared RIP buffer should not stand for longer than 3 h. If a longer incubation time is selected on the beads, it is recommended to prepare fresh RIP buffer again for washing after incubation.
4. It is more convenient for the experimental procedure if all tubes are prepared and labelled before the start. Five tubes

are required per sample: one for clearing the lysate, one for the protein sample of the lysate, one for the RNA sample of the lysate, one for the incubation with the beads, which will ultimately contain the protein eluate sample, and another to finally receive the beads for the RNA eluate sample.

5. To optimize the UV-irradiation, UV-crosslinking should not be done in small standard petri dishes of 10 cm of diameter. As a result, too many cells lie on top of each other and do not receive UV radiation evenly.
6. When physically disrupting cells via glass beads, it is advantageous to choose a tube whose capacity is close to the total volume of the contents. This increases the efficiency of the cell lysis. Thus, if the total volume is less than 2 mL, a 2 mL screw top tube can be preferentially be used.
7. For lowly expressed proteins, it may be helpful to concentrate the lysate higher and add only 0.5–1 volume of RIP-buffer to the cell pellet, instead of the mentioned double volume.
8. Lysing the cells in 0.5% Triton-X-100 instead of 0.2% can improve the yield of nuclear proteins or proteins of the nuclear envelope for some *S. cerevisiae* strains. After the lysis, the lysate should be diluted to 0.2% Triton-X-100 again, as unspecific bindings to the beads increase with a high concentration of triton. For instance, if you decide to carry out the cell disruption with a higher concentration of detergent, the amount of RIP buffer added should be equal to the same volume of the cell pellet and then be diluted with the same amount of RIP buffer without detergent after the disruption.
9. In our own experience, it is most time-efficient if the beads are washed during cell lysis and clearing of the lysate. It is not problematic if they are left in the cooled centrifuge or on ice for a longer period of time during this process.
10. To avoid unspecific protein binding, beads can additionally be blocked with BSA (10 mg/mL in PBS) prior to the RIP-experiment: after washing the beads two times in RIP-buffer, incubate the beads in BSA for 30 min at 4 °C (optionally in an end over end incubator). Afterwards, wash the beads again three times in RIP-buffer.
11. When washing the beads, remove as much of the supernatant as possible so that the remaining buffer just covers the pellet. When adding the fresh buffer, make sure that all the beads are whirled up and no pellet remains.
12. When GFP-tagged proteins are extracted, nearly saturated beads shimmer green and are therefore clearly visible. In other cases, the beads may be less visible, and good lighting will be very helpful.

13. RNA suffers a lot from the heating steps in this protocol (especially recognizable at the 5′ end). If your primers for qPCR are designed to bind close to the 5′ end of your cDNA, you will get lower amounts of amplification products. The amount of heating steps in this protocol can be reduced by using an RNA isolation Kit to isolate the RNA after the RIP or by reducing the temperature and incubation time during Proteinase K digestion. In this case, at least 37 °C and 10 min should be applied.
14. Adding glycogen for RNA precipitation can improve the precipitation and handling, and therefore the yield of RNA. The amount of RNA coprecipitated in the eluate can sometimes be very low. In this case, it may be worth using blue glycogen to improve visibility.
15. If the DNA contamination is still too high within the RNA sample, the dissolved RNA can be treated again with a selected DNase and precipitated a second time, e.g., with 1/10 volume of NaAc and 3 volumes of ethanol.

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Chapter 16

Using the Tether Function Assay to Identify Potential Regulators of mRNA Translation and mRNA Decay

Maïté Courel

Abstract

RNA binding proteins (RBPs) and their associated partners are key factors of posttranscriptional control of gene expression. To study and manipulate the functional consequences of binding of these regulators to their targets, several tethering assays have been developed, in which a protein of interest is brought to a reporter mRNA through heterologous RNA–protein interaction motifs. The effect of such constrained binding is then monitored by measuring the accumulation of the reporter protein and mRNA. This chapter describes a protocol for the λN-BoxB tether system in transiently transfected mammalian cells. Combining the luciferase reporter technology to quantify protein amounts by light measurement and RNA amounts by RT-qPCR, this assay provides a simple and robust way to analyze the consequences of any protein binding in a controlled and defined manner.

Key words Tethering approach, RNA-binding protein, mRNA decay, mRNA translation, Luciferase reporter

1 Introduction

The cytoplasmic fate of an mRNA is governed by the combination of the proteins bound to it. The interaction of these RNA binding proteins (RBPs) with their target mRNAs, either directly or within mRNP complexes, through specific RNA motifs or not, regulates mRNA subcellular localization and storage, and mRNA stability and translation rate [1–4]. With a number of identified RBPs into the four-digit range in mammalian cells, for example [5, 6], these interactions play a major role in the regulation of protein expression, and efforts are made to determine the specific roles of each RBP and to elucidate the molecular mechanisms by which they achieve such function.

As a first step to study the involvement of a protein in mRNA stability and translation, the following questions have to be answered: (i) Does the protein have an influence on protein

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https://doi.org/10.1007/978-1-0716-4176-7_16,

production when bound to the mRNA? (ii) If so, does the mRNA amount explains the change in protein levels? To address both questions, a classic *in cellulo* approach is to set up a series of tether function assays, in which the studied protein is bound artificially to a reporter mRNA through a specific and heterologous protein/RNA interaction system [7, 8]. For this purpose, the protein of interest is expressed in frame with a known RNA-binding domain to produce an RBP fusion protein, which has the added capacity to interact with a specific RNA sequence. This RNA sequence is introduced into the 3′ UTR of a reporter mRNA and the consequences of RBP binding to the reporter mRNA are analyzed in cells. With this tether approach, the function of the protein under investigation is independent of (i) the RNA-binding capacity of the studied protein and (ii) the transcriptional regulation of both the reporter target mRNA and the studied protein. The first point is particularly relevant when studying a protein (i) that is part of a complex without being itself the RBP, or (ii) with either unspecific or unknown RNA-binding sequence and/or (iii) with either a wide range of or unknown endogenous target mRNAs. It is also of interest when elucidating molecular mechanisms occurring once the protein is bound to its mRNA target, as it can be combined with drugs treatment and/or silencing of cofactors.

Classical heterologous protein/RNA interaction systems available for tether function experiments include the MS2 bacteriophage coat protein/MS2 RNA stem-loop, the λ bacteriophage antiterminator N protein/BoxB stem-loop, the eukaryotic iron response element binding protein IRP/IRE stem-loop, and the spliceosomal U1A protein/U1 snRNA stem-loop II [7, 8]. Here, we will describe the use of the λN-BoxB system [9] (Fig. 1), which has been proven to be useful to identify novel RBPs and members of their complexes in wide-screen analyses [10, 11], and to investigate RBP functions in translation (*e.g.* [12–17]) and mRNA turnover (*e.g.* [15, 18–20].). In this system, the 22 amino acids *trans*-acting RNA binding domain of the antiterminator N from bacteriophage λ (λN) binds specifically to the 19 nt, hairpin-forming *cis*-acting RNA sequence called BoxB [21]. The protein under investigation is fused or not to the λN domain in a so-called effector plasmid, while multiple copies of the BoxB sequence are cloned in the 3'UTR of the sea pansy *Renilla reniformis* luciferase reporter (RLuc). Each effector plasmid is transiently co-transfected with the reporter plasmid in mammalian cells (Fig. 2), along with an additional plasmid (control plasmid) to correct for transfection efficiency. We use for this purpose a second luciferase protein, originating from the firefly *Photinus pyralis* (FLuc). The next step is to measure the accumulation of protein and mRNA produced from the reporter construct, which we express as a ratio of RLuc to FLuc protein or mRNA amount. The protein amount is

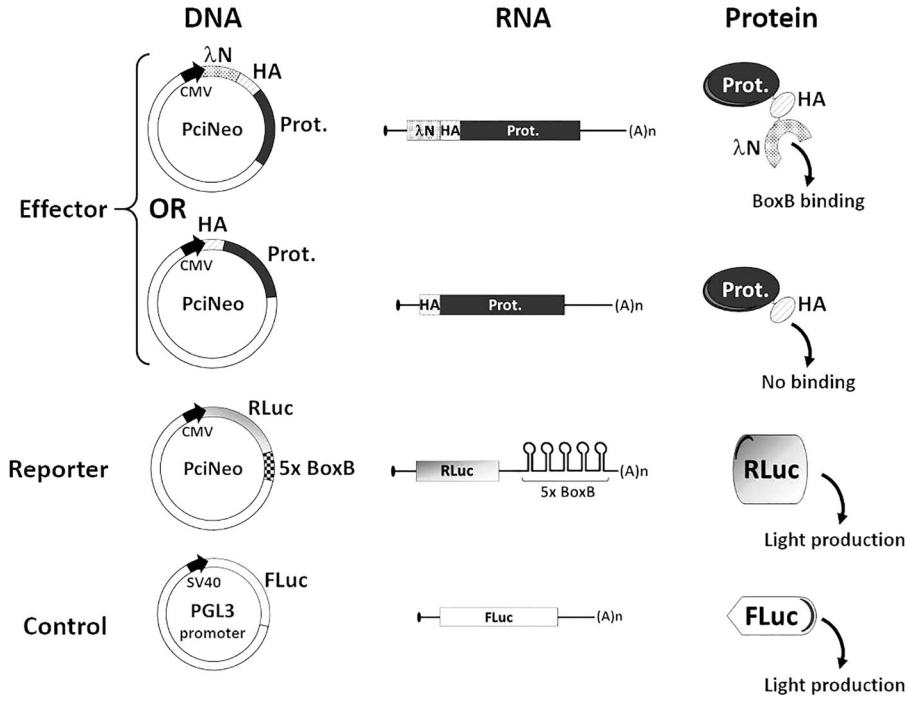

Fig. 1 Plasmids used in the λN-BoxB tether system. Schematic representation of the plasmids and their mRNA and protein products. The protein product of the effector plasmid (λN-HA-protein) will specifically bind to the mRNA product of the reporter plasmid (RLuc-5xBoxB), with possible consequences on RLuc-5xBoxB mRNA stability and translation, while the HA-protein will not. The mRNA and protein produced from the control plasmid (FLuc) will be used to correct for transfection efficiencies

determined with a dual luciferase assay system, in which FLuc and RLuc activities are measured sequentially in the same sample [22]. In parallel, mRNA extraction followed by reverse transcription and quantification by quantitative (q) PCR allows for determining RNA amounts. Finally, a western blot procedure serves as a control for consistent expression of the effector protein across all samples. The comparison of RLuc/FLuc ratios obtained with the protein under investigation fused or not to the λN domain provides a mean of assigning it a potential role in mRNA translation or stability (Fig. 3). Once such a role has been identified, several possible extensions of the tether function assay are possible. For example, the role of individual domain(s) of the protein of interest can be tested, or of its enzymatic function (*e.g.* the ATPase activity of an RNA helicase), by truncation and mutagenesis. Further downstream assays include characterization of proteins that interact with the studied protein, often leading to an appreciation of a larger complex that influences translation and/or mRNA stability.

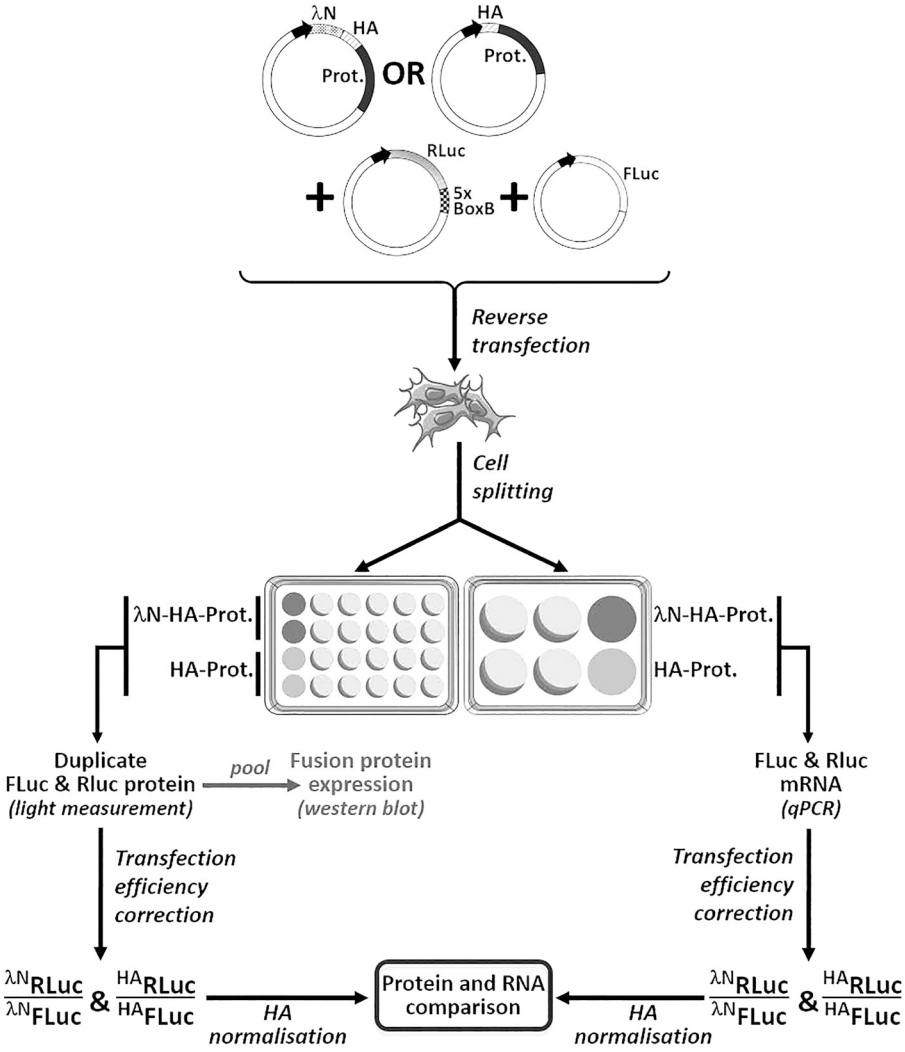

Fig. 2 Protocol overview of the tether function assay experiment. Cells are reverse transfected with the indicated plasmids and immediately split into the indicated culture plates. After plasmid expression, cells are harvested for either protein analysis (enzymatic activity measurement and western blot verification) or mRNA reverse transcription and qPCR analysis

2 Materials

2.1 Cell Culture and Transfection

1. Plasmids to be transfected (Fig. 1). These include (i) the effector plasmid coding for the protein of interest fused or not to the λN domain, (ii) the reporter plasmid coding for RLuc with or without the BoxB sequences, and (iii) the control plasmid encoding the FLuc. The effector fusion protein is expressed from a pCI-neo plasmid (Promega, CMV promoter) and includes the HA epitope between λN and the studied protein to allow the verification of RBP expression by western blot

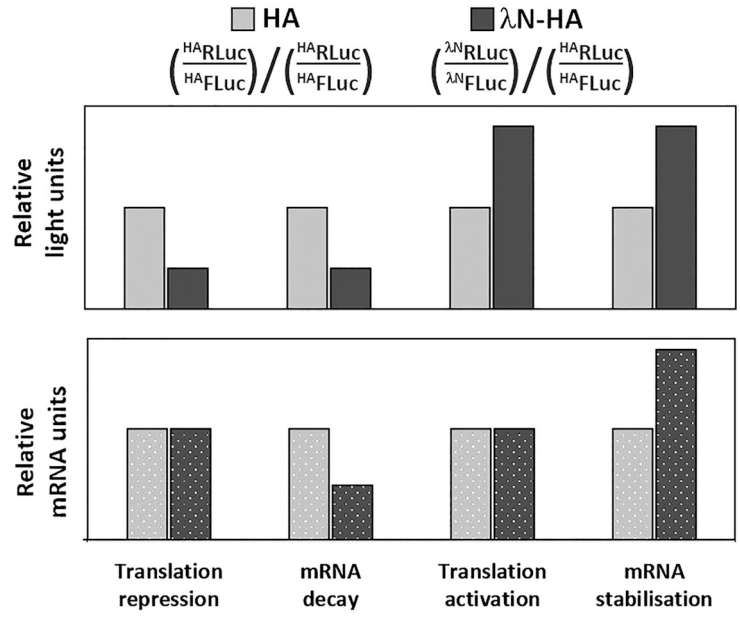

Fig. 3 Typical expected results of a tether function assay experiment. For easier comparison, all data are normalized so as to set RLuc/FLuc of the HA protein (without λN) at 100%. If a decrease or increase is observed only at the reporter protein level, then the tested protein inhibits (translation repression) or activates (translation activation) translation. If a decrease or increase is observed at both the reporter protein and mRNA levels, then the tested protein destabilizes (mRNA decay) or stabilizes (mRNA stabilization) mRNA

using an anti-HA antibody [12, 18]. The N-terminal sequence of all λN-HA fusion proteins is MDAQTRRRERRAEKQAQWKAANPPLEAAQAYTMA*YPYDVPDYA* where the underlined amino acids sequence represents the λN domain and the italic sequence the HA epitope. The reporter construct, also expressed from pCI-neo, contains an optimized version of RLuc coding sequence for expression in mammalian cells and five copies of the BoxB sequence [12]. The control construct, coding for an optimized version of FLuc, corresponds to the pGL3-promoter plasmid (Promega).

2. Appropriate cell culture media, plasticware, and transfection reagent. For studies in human cells, we routinely use HEK 293 or HeLa cells grown at 37 °C with 5% CO2 in DMEM high glucose supplemented with 5% Fetal Bovine Serum and 1% Pen/Strep antibiotics. We transfect these cells with the GenJet transfection reagent, which is suitable for reverse transfection (SignaGen Laboratories; *see* Subheading 3.3).

2.2 Protein Analyses

1. Dual luciferase assay commercial kit (Promega). The kit includes the 5X Passive Lysis buffer (PLB), the Luciferase Assay Buffer II and Lyophilised Luciferase Assay Substrate, the Stop & Glo® Buffer and the 50X Stop & Glo® Substrate. It is stored at −20 °C. The required amount of 1X Passive Lysis Buffer (PLB) is prepared extemporaneously by diluting the 5X PLB with water. Luciferase Assay Reagent II (LARII, substrate for FLuc) is reconstituted according to the manufacturer's instructions, aliquoted, and stored at −80 °C. The required amount of Stop & Glo® reagent (substrate for RLuc) is prepared extemporaneously according to the manufacturer's instructions. All three ready-to-use reagents are kept at room temperature during the experiment.
2. Luminometer (*e.g.* Lumat LB 9507, Berthold) and corresponding plasticware for luminescence measurement (*e.g.* 5 mL, 12 × 75 mm tubes).
3. SDS-PAGE (4–12%) and western blotting reagents.
4. Primary monoclonal anti-HA antibody.
5. Secondary anti-mouse antibody coupled to horseradish peroxidase.
6. ECL revelation kit.

2.3 RNA Analysis

1. Nuclease-free water.
2. TRIzol reagent. Keep at 4 °C.
3. Chloroform solution. Keep at 4 °C.
4. 0.3 M NaOAc. Prepare from a 3 M NaOAc, pH 5.2 stock solution by diluting in sterile water. To make a 3 M NaOAc, pH 5.2 stock solution: Add about 80 mL of H_2O in a beaker. Weigh 24.6 g of sodium acetate, mix, adjust pH to 5.2 using glacial acetic acid, and complete to 100 mL with H_2O. Filter-sterilize the solution. Keep at 4 °C.
5. Isopropanol. Keep at room temperature.
6. 20 mg/mL glycogen. Keep at −20 °C.
7. 75% Ethanol. To a 50 mL polypropylene centrifuge tube, add 37.5 mL of 100% analytical grade ethanol. Make up to 50 mL with sterile water. Invert to mix. Keep at room temperature.
8. TURBO DNA-free Kit. The kit includes the 10X TURBO DNase buffer, the TURBO DNase and the DNase Inactivation reagent. Store at −20 °C.
9. SuperScript II Reverse Transcriptase kit. It includes the 5X First Strand Buffer, 0.1 M DTT and the Superscript II RT. Store at −20 °C.
10. 500 ng/μL random primers. Store at −20 °C.

11. Specific luciferase reverse primers (10 μM, keep at −20 °C):
    Firefly: 5′-GTATTCAGCCCATATCGTTTCAT-3′.
    Renilla: 5′-TCGCCCTTCTCCTTGAATG-3′
12. 10 mM dNTP solution. Keep at −20 °C.
13. RNaseOUT (40 U/μL). Store at −20 °C.
14. qPCR Master Mix (*e.g.* GoTaq qPCR Master Mix). Store at −20 °C.
15. Primers for luciferase amplification (10 μM, stored at −20 °C):
    Firefly Forward: 5′-TGAGTACTTCGAAATGTCCGTTC-3′
    Firefly Reverse: 5′-GTATTCAGCCCATATCGTTTCAT-3′
    Renilla Forward: 5′-AGACAAGATCAAGGCCATCGTCCA-3′
    Renilla Reverse: 5′-TTTCTCGCCCTCTTCGCTCTTGAT-3′
16. qPCR equipment (*e.g.* Roche LightCycler 480 and appropriate 96-well plates).

3 Methods

3.1 Important Controls

First, FLuc expression serves as a control for transfection efficiency. Second, RLuc expression in presence of the protein of interest but without the λN domain allows for data normalization between experiments (*see* Subheading 3.9). Third, when setting up a tether assay, it is important to ensure that the results are indeed due to the direct interaction of the tethered protein to the reporter mRNA. To fulfill this requirement, we use a reporter plasmid without BoxB sequences. We only use this construct when initially working with a novel protein, in order to distinguish the consequences of specific BoxB binding from a nonspecific binding on all mRNAs (which would disturb the assay by also affecting control FLuc expression).

3.2 DNA Preparation for Cell Transfection

To ensure proper reproducibility of the results, it is important to pay specific attention to the starting DNA material.

1. A precise optimal ratio of reporter:control:effector plasmids (1:20:150 in our experiments) must be used (*see* **Note 1**). For each transfection (600,000 cells), we use 6 ng of reporter plasmid, 120 ng of control plasmid, and 900 ng of effector plasmid.
2. Determine DNA concentrations as precisely as possible using a spectrophotometer, and prepare DNA dilutions extemporaneously (*see* **Note 2**). We recommend as well working with premixes of the luciferase plasmids (*i.e.* reporter and control plasmids) before dispensing them to each transfection reaction.
3. Verify the absence of plasmid contamination (*e.g.* control plasmid within effector plasmid sample) by controlling RLuc and FLuc activity after individual transfections (*see* **Note 3**).

3.3 Cell Transfection

To improve consistency in transfection efficiency across replicates and conditions, we use the reverse transfection procedure, in which cells are mixed with the DNA in a 5 mL polypropylene culture tube prior to plating. This allows for immediate cells splitting into several wells (600,000 cells seeded in 2 wells of a 24-well plate and 1 well of a 6-well plate; *see* Fig. 2) for further specific harvesting and measurement of the same initial sample (*see* **Note 4**).

1. For one tested condition (*e.g.* λN-HA-protein + RLuc-BoxB + FLuc), first dilute 3 μL of transfection reagent in 50 μL of DMEM, then dilute appropriate amounts of plasmid DNA (*see* Subheadings 3.1 and 3.2) in 50 μL of DMEM, and finally mix both dilutions together and incubate at room temperature for 15 min.
2. Add 600,000 cells to the transfection mixture, let stand for 5 min (*see* **Note 5**).
3. Add enough medium to seed 2 wells of a 24-well plate (for duplicate enzymatic activity measurement followed by western blot analysis; *see* Fig. 2) and 1 well of a 6-well plate (for RNA analysis).
4. Replace the transfection mixture by fresh complete medium 4–6 h after seeding (*see* **Note 6**).
5. Incubate the cells during the appropriate time (24–72 h) before harvesting for downstream analyses (*see* **Note 7**).

3.4 Reporter Protein Expression: Enzymatic Activity Measurement

The RLuc and FLuc enzymes produced from the corresponding transfected DNA plasmids catalyze the luminescent conversion of their substrates (coelenterazine and luciferin, respectively) into their respective products (coelenteramide and oxyluciferin). Quantification of the emitted light during reactions performed with saturating amounts of substrates will therefore reflect the amount of enzyme in the sample, hence protein expression. For this quantification, we use the Dual Luciferase reporter assay system, which allows for sequential measurement of FLuc and RLuc activities within the same sample (*see* **Note 8**). The following procedure is given for 1 well of a 24-well culture plate and slightly differs from the original recommended manufacturer's protocol.

3.4.1 Sample Preparation

1. Replace the culture medium by 200 μL of 1X PLB (*see* **Note 9**).
2. Incubate at room temperature for 15 min on an orbital shaker.
3. Transfer the lysate in a microcentrifuge tube for clearing and centrifuge for 5 min at 2000× *g*, at 4 °C.

3.4.2 Enzymatic Activity Measurement (See Note 10)

1. Prepare an appropriate number of luminometer tubes with 40 μL of LAR II reagent (*see* **Note 11**).
2. Add 5 μL of sample (from **step 3** of Subheading 3.4.1), mix by pipetting twice (*see* **Note 12**).

3. Place the tube in the luminometer and record a 10 s light measurement for the FLuc.
4. Remove the tube from the luminometer.
5. Add 40 μL of Stop & Glo reagent to the luminometer tube, mix by pipetting twice.
6. Place again the tube in the luminometer and record a 10 s light measurement for the RLuc (*see* **Note 13**).

3.5 Western Blot Analysis of Effector Protein Expression

To assess the consistent expression of the effector fusion proteins across samples, a classic western blot analysis is performed using the previous lysates (*see* Subheading 3.4) as follows.

1. Pool the remaining lysates from technical duplicates.
2. Resolve 30 μL on a 4–12% SDS-polyacrylamide gel, and transfer proteins onto a nitrocellulose membrane (*see* **Note 14**).
3. After blocking for 15 min with 5% nonfat skimmed milk in PBS, incubate with anti-HA monoclonal antibody (1/1000 dilution) overnight at 4 °C on an orbital shaker.
4. Wash three times with PBS-Tween 0.1%.
5. Incubate with secondary anti-mouse antibody (1/10,000 dilution) for 45 min at room temperature on an orbital shaker.
6. Wash and subject to ECL detection (*see* **Note 15**).

3.6 RNA Extraction

RLuc and FLuc mRNA accumulation needs to be performed alongside the RLuc and FLuc protein quantification. The procedure for RNA extraction using TRIzol is detailed here for one well of a 6-well plate.

1. Replace the culture medium by 400 μL of TRIzol reagent, incubate for 2 min at room temperature with manual plate rocking and transfer into a 2 mL microcentrifuge tube (*see* **Note 16**).
2. Add 80 μL of chloroform, vortex for 10 s, and incubate for 2 min at room temperature.
3. Add 200 μL of 0.3 M NaOAc, vortex for 10 s (*see* **Note 17**).
4. Centrifuge for 10 min at 13,000× *g*, at 4 °C.
5. Transfer the aqueous upper phase (~400 μL) into a clean 1.5 mL microcentrifuge tube.
6. Add 1 μL of glycogen and 200 μL (0.5 volume) of isopropanol, vortex for 10 s, and incubate for 10 min at room temperature (*see* **Note 18**).
7. Collect the precipitated RNA by centrifugation at 13,000× *g* for 10 min at 4 °C.

8. Wash the RNA pellet with 1 mL of 75% ethanol (*see* **Note 19**).
9. Dry the RNA pellet and resuspend in 22.5 μL of RNase-free water (*see* **Note 20**).

3.7 DNase Treatment and RNA Quantification

When working with samples originating from transient transfection of plasmids, a substantial amount of contaminating DNA is inherently purified and precipitated together with RNA. It is crucial to remove efficiently these contaminants to avoid their amplification during the final qPCR step. The following procedure uses the TURBO DNA-free kit for this purpose.

1. Add 2.5 μL of 10X TURBO DNase buffer to the 22.5 μL of RNA sample and mix gently.
2. Add 1.5 μL of TURBO DNase, mix gently, and incubate for 45 min at 37 °C.
3. Add again 1.5 μL of TURBO DNase, mix gently, and incubate for another 45 min at 37 °C.
4. Add 5 μL of DNase Inactivation reagent and incubate for 5 min at room temperature with occasional shaking (*see* **Note 21**).
5. Centrifuge at room temperature for 2 min at 10,000× *g* to pellet the DNase Inactivation reagent, and transfer the supernatant into a clean microcentrifuge tube (*see* **Note 22**).
6. Measure RNA concentration using a spectrophotometer.

3.8 Reverse Transcription and qPCR

There are several commercial reagents and kits available to perform reverse transcription followed by qPCR. Although the components and individual steps may vary from one manufacturer to the other, the overall procedure is similar. The following steps apply to the SuperScript II Reverse Transcriptase and SYBR-based GoTaq qPCR Master Mix and are given for one RNA sample, and one PCR reaction respectively. For multiple reactions, prepare a master mix of common components.

1. In a 0.2 mL nuclease-free tube, mix 1 μg of TURBO DNase-treated RNA (*see* **Note 23**), 1 μL of random primers, and 1 μL of Renilla and Firefly reverse primers (*see* **Note 24**), in a 12 μL final volume.
2. Place the tube in a thermocycler for 5 min at 65 °C and then quickly transfer on ice.
3. Add 5 μL of 5X First Strand Buffer, 2.5 μL of 0.1 M DTT, 1.25 μL of 10 mM dNTP, 1 μL of RNaseOUT™, 1 μL of Superscript II™ RT (*see* **Note 25**), 2.25 μL of nuclease-free water.
4. Incubate the tube in the thermocycler for 10 min at 25 °C, for 50 min at 42 °C, and for 15 min at 70 °C.

5. Make a 4X dilution of the RT reaction sample using nuclease-free water (*see* **Note 26**).
6. In a 96-well qPCR plate, add 2 μL of 4X diluted RT reaction sample, 6 μL of GoTaq qPCR Master Mix, the desired volume of forward and reverse primers to satisfy the optimal final primers concentration (*see* **Note 27**), complete to 12 μL with nuclease-free water.
7. Run the following PCR program: 95 °C for 5 min (initial denaturation); 40 cycles [95 °C for 10 s; 60 °C for 15 s; 72 °C for 10 s] (PCR amplification); progressive temperature increase from 65 °C to 95 °C (PCR product Tm calculation for amplification specificity check).
8. Get the Ct (cycle threshold) value of the sample using the second derivative calculation method (*see* **Note 28**).

3.9 Analysis of the Results

1. For both protein and RNA quantification, first normalize the RLuc measurements to correct for transfection efficiency using FLuc measurements by calculating the RLuc to FLuc ratio. This is straightforward concerning the enzymatic activities measurements as light emission is directly proportional to protein quantity (*see* Subheading 3.4 and *see* **Note 29**). However, it requires a preliminary Ct to RNA quantity conversion step after the qPCR experiment, using the standards amplification results (*see* **Note 30**).
2. Normalize the RLuc/FLuc value obtained for the λN-HA-protein using the RLuc/FLuc value obtained for the HA-protein. This result is termed relative RLuc/FLuc protein or RNA amount.
3. Compare the relative protein amount with the relative RNA amount for the tested protein to characterize its effect when tethered to the RLuc reporter (*see* **Note 31** and Fig. 3).

4 Notes

1. The optimal ratio of reporter:control:effector plasmids may be determined for each specific protein of interest in order to optimize the observed effect of the tethered protein. For example, overexpression of the tethered protein may be deleterious for the cells requiring lower amount of transfected effector plasmid, which may imply scaling down the reporter plasmid as well.
2. Our plasmid stock solutions are at least at 500 ng/μL concentration and kept at −20 °C. We experienced a decrease in luciferase activity signals when working with stored DNA solutions at 120 ng/μL or less.

3. This verification is necessary, as invisible amounts of contaminating reporter or control plasmids on an agarose gel may result in important erroneous light signal during the enzymatic activity measurement. In order to keep similar transfection conditions for the verification, we use a pUC19 in the transfection mixes to complete the DNA quantity up to 1 μg.
4. The assay is compatible with any transfection reagent. We successfully used as well the Lipofectamine 2000 reagent, although it was more detrimental regarding cell survival. Depending on the transfection reagent used and the cell type, the cell number and the DNA:transfection reagent ratio need to be adapted.
5. Cells previously treated with siRNA (*e.g.* to remove the endogenous tested protein or a known partner) or any other relevant drug can be used. To avoid repeated trypsin treatment in the case of siRNA procedure, we use a classical siRNA transfection first (*i.e.* cell seeding 24 h before applying siRNA) and wait at least 12 h after siRNA transfection to start the reverse transfection procedure.
6. This step may not be necessary depending on the cell type and/or the transfection reagent used.
7. Although harvesting cells 48 h after transfection is a good starting point, it may be necessary to adjust the harvesting time in order to optimize the observed effect of the studied protein. When setting up an experiment in the lab, we include a 24 h time point.
8. Promega pioneered the dual luciferase assay system [22], which remains widely used, but other suppliers (*e.g.* Ozyme, BA0180) offer similar products.
9. We skip the cell wash with PBS step before addition of PLB indicated in the original protocol. Thus coating plates with poly-lysine when working with poorly adherent cells is not required, and does not change the subsequent measurements.
10. It is important to determine the linear range of the luminometer in order to get accurate light measurements. If light measurement falls above the linear detection range of the luminometer, we recommend diluting the samples in 1X PLB rather than scaling down the measurement volume.
11. The volume of both reagents has been reduced compared to the original protocol, without affecting the efficacy of either enzymatic reaction. Smaller volumes were not tested because of technical practicability (pipetting in narrow luminometer tubes).
12. Although the reagents are not prone to foaming, bubble formation, if any, during the different pipetting steps is not detrimental to the subsequent measurement.

13. To verify specific light emission, we include for each series of measurements two control samples, one containing only 5 μL of 1X PLB and the other containing 5 μL of untransfected cells (*i.e.* cells for which only the **step 1** of the cell transfection procedure (*see* Subheading 3.3) is omitted).
14. We do not perform a Bradford quantification of the total protein amount in the lysates. Loading equal volumes gives satisfactory results, as we transfect the same number of cells and hence obtain similar amounts of material for each experiment.
15. To verify the constant amount of loaded protein in each condition, we perform a total protein staining of the membrane or use a second antibody against a housekeeping protein.
16. TRIzol-treated samples can be stored at −80 °C before further processing (*see* **Note 31** as well).
17. NaOAc addition allows increasing the volume of aqueous phase to facilitate pipetting after phase separation by centrifugation. The volume can be adjusted to satisfy practical feasibility.
18. Glycogen acts as a carrier to facilitate RNA and salts precipitation, which allows for easy detection of the RNA pellet that will appear white instead of translucent.
19. RNA pellet does not stick well to the tube wall. It is critical to gently remove the supernatant with a pipette and not a vacuum pump system. Additional quick centrifugations may be necessary to achieve correct removal of all remaining supernatant.
20. RNA drying is achieved by a 2–3 min incubation in a 65 °C heat block. Subsequent pellet resuspension is facilitated by incubation at 65 °C and thorough vortexing.
21. DNase Inactivation reagent is cloudy and tends to settle, hence the need to shake at least twice during the incubation time.
22. Pelleted DNase Inactivation reagent does not stick well to the tube. Additional quick centrifugation may be necessary to get a maximum volume of DNase-treated RNA (~ 25 μL).
23. In order to calculate the primer efficiency for each qPCR experiment, we perform an additional RT reaction using 1 μg of an RNA template composed of equal amounts of at least four of the tested RNA samples. This standard sample is used to build a calibration curve of the primer efficiency.
24. The addition of specific reverse primers for reporter mRNAs increases by tenfold the detection threshold in the subsequent qPCR amplification. We noticed as well better reproducibility of the results when using random primers instead of oligo-dT primers.

25. For each series of RNA extraction followed by TURBO DNase treatment, we perform an additional reaction with the most concentrated sample in which the RT is replaced by nuclease-free water. This "negative RT" serves as a control for contaminating DNA.
26. If the obtained cDNA amount does not give sufficient qPCR amplification, we highly recommend increasing the starting RNA amount during the RT reaction (up to 5 μg) rather than using a less diluted RT reaction sample, as the RT components may interfere with the following qPCR amplification. To obtain a calibration curve covering the range of all the tested samples, the standard RNA (*see* **Note 23**) is first diluted 3X, and then it undergoes five twofold serial dilutions (*see* also **Note 30**).
27. Optimal primer concentration in the PCR reaction has to be determined for each primer pair. We typically test 300, 600, and 900 nM final concentrations on the standard and negative RT samples and choose the concentration at which the standards amplify with close to 100% efficiency, while the negative RT does not.
28. To collect reliable Ct values for each sample, we perform qPCR in duplicate runs, rather than duplicating the sample in the same qPCR run. We then calculate a mean Ct value when the difference between the duplicate Ct is <1 and use this mean Ct value for further analyses.
29. Since duplicate enzymatic activity measurements are made for each transfection experiment (2 wells in a 24-well plate; *see* Subheadings 3.2 and 3.3), the mean corrected value is then used for further normalization.
30. In order to convert Ct values for each tested sample into arbitrary RNA quantities, we plot a calibration curve $Ct = f(\log \text{quantity})$ using the mean Ct obtained from the serial dilution of the standard (*see* **Note 28**), to which we attribute an arbitrary RNA quantity (*e.g.* 100 for the initial 3X standard, and then 50, 25, 12.5, 6.25, and 3.12 for the subsequent twofold serial dilutions). This takes into account every primer pair efficiency for each qPCR run.
31. To perform a statistical evaluation of the results, the entire transfection and following analysis are repeated at least three times. While the protein quantification results are usually very robust, the RNA outcome may be less reproducible. To circumvent this caveat, we perform RNA analysis (*i.e.* RNA extraction, DNase treatment, reverse transcription, and qPCR) of all three replicates in a single experiment.

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Part VII

Methods for the Study of RNA Decay at the Subcellular Level

Chapter 17

Nascent and Mature RNA Profiling by Subcellular Fractionation in Human Cells

Marina Pinskaya, Julien Jarroux, Rocco Cipolla, and Antonin Morillon

Abstract

Transcription and RNA decay determine steady-state RNA levels in cells available for translation and RNA-mediated regulatory functions. Both processes can be assessed by various techniques, for majority, based on RNA labelling or chromatin immunoprecipitation, but require a high level of expertise. Here, we describe a cost-effective, fast, and simple protocol that enables the profiling of nascent and mature RNA in the cytoplasm, nucleoplasm, and chromatin through subcellular fractionation. The workflow can include α-amanitin inhibition of RNA Polymerase II to assess nascent RNAs as a proxy of transcriptional activity, or it can be used without this treatment to investigate distribution of partially processed or mature transcripts across distinct subcellular compartments. It is applicable for studying any of RNA biotypes, including small and long noncoding RNAs, mRNAs, and their splice variants, on both transcript-specific and transcriptome-wide scales. Nascent or mature RNAs isolated from each fraction can be further analyzed by any technique of choice (northern blot, reverse transcription, RNA sequencing).

Key words Subcellular fractionation, Nascent RNA, Transcription, Chromatin, α-amanitin, Long noncoding RNA, Protein

1 Introduction

The very first step in expression of genetic information is transcription. In eukaryotes, nascent RNAs are co-transcriptionally spliced prior to trafficking to other compartments, and ultimately degraded. The balance between transcription and decay determines steady-state RNA levels and the availability of transcripts for translation or RNA-mediated regulatory functions. It can change in response to external or internal stimuli and, consequently, defines cell identity and plasticity. Thus, assessing transcriptional activity and mature RNA levels in different subcellular compartments is important for our understanding of how cells change their properties over time and in response to stimuli.

Various techniques exist to measure transcription either based on RNA Polymerase II immunoprecipitation or nascent RNA

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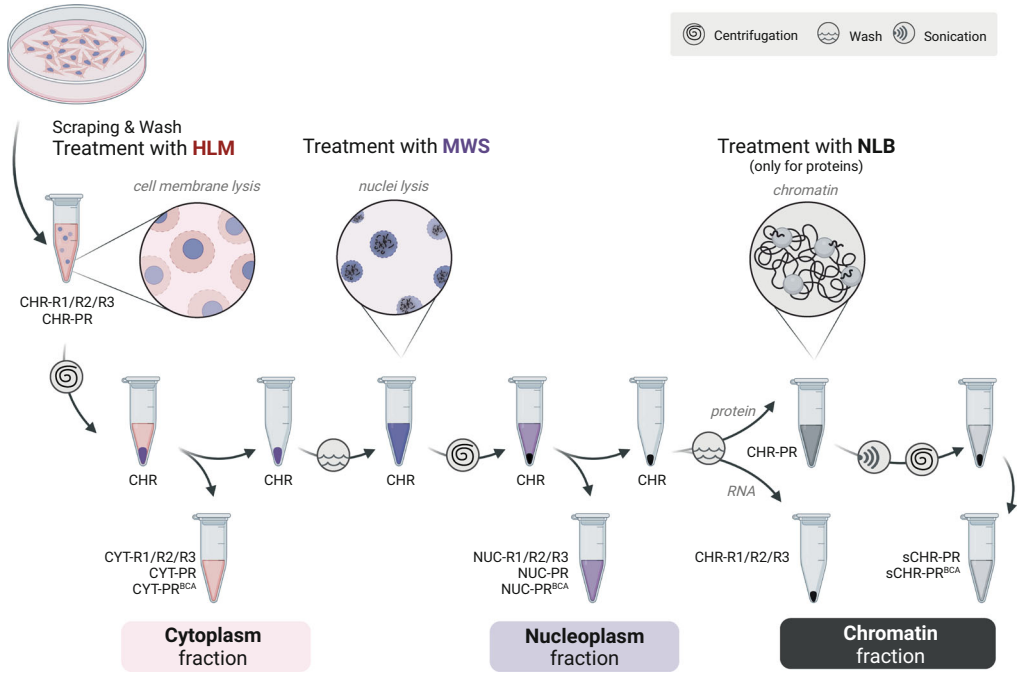

Fig. 1 Schematic view of the principle of subcellular fractionation steps and sample labelling. (Created with BioRender.com)

labelling [1–3], but they are often complex and time-consuming. We propose to approach this question by a method of subcellular fractionation and quantification of nascent RNAs by any of your favorite gene-specific or high-throughput RNA sequencing method (Fig. 1). The protocol is inspired by Gagnon et al. [4] but additionally includes an α-amanitin treatment. In mammalian cells, α-amanitin blocks the transcribing RNA polymerase II on chromatin with nascent transcripts still bound to it [5] (Fig. 2a). These yet unspliced RNAs can be further recovered from the insoluble chromatin fraction, reverse transcribed with random primers and measured by quantitative PCR with primer pairs complementary to intronic sequences of the gene of interest (Fig. 2b), or profiled transcriptome-wide through various total RNA sequencing methods.

On the other hand, a protocol variant without α-amanitin addition is suitable for profiling of transcripts at different stages of their lifespan within distinct subcellular compartments. It is informative for studies of RNA processing events, but also for functional studies of long noncoding (lnc)RNAs. Indeed, lncRNAs form the largest family of genes in mammalian genomes and can be found both in the nucleus and/or the cytoplasm, where they can exhibit various regulatory functions [6]. Thus, assessment of lncRNA subcellular localization is a prerequisite for functional studies.

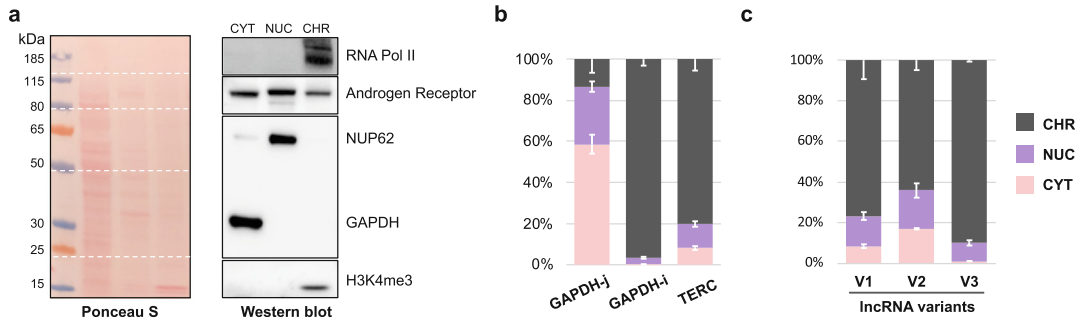

Fig. 2 Proteins, nascent and mature RNA distribution across chromatin (CHR), nucleoplasm (NUC) and cytoplasm (CYT) fractions. (**a**) Ponceau S staining of proteins after wet transfer of the 4–12% SDS-PAGE gel and western blot of H3K4me3, GAPDH, NUP62, Androgen Receptor, and RNA Polymerase II in LNCaP cells. White dashed lines correspond to membrane cuts made prior to incubation with individual primary antibodies. (**b**) Quantification of nascent (GAPDH-i) and mature GAPDH mRNA (GAPDH-j) and TERC by RT-qPCR in LNCaP cells fractionated with α-amanitin treatment, relative to GAPDH exon. (**c**) Quantification of three splice variants (V1, V2, and V3) of an lncRNA in LNCaP cells fractionated without α-amanitin treatment, relative to GAPDH exon

Moreover, when RNA imaging techniques, such as fluorescence in situ hybridization (FISH), fail in detecting lncRNAs or their specific isoforms, this protocol allows to assess subcellular distribution of distinct splice variants (Fig. 2c). In this case, random-primed reverse transcribed cDNA should be quantified with a primer set targeting an isoform-specific junction. Subcellular fractionation can be also used to profile nascent and mature transcripts transcriptome-wide using RNA sequencing of chromatin and/or cytoplasmic RNAs, allowing the definition of a gene's transcriptional boundaries on DNA and of its exon-intron organization through the sequencing of mature RNA transcripts. Hence, this approach is valuable for studying RNA processing events as splicing, miRNA maturation, or even RNA decay activities in different subcellular compartments.

Finally, recovery and analysis of proteins by western blot allow not only a quality control of fractionation but also assessment of proteins' levels, distribution, or even activity in different experimental conditions as originally proposed by Gagnon et al. [4].

Whether it is focused on RNAs or proteins, this method can be used as a semi-quantitative technique to reveal the relative abundance and distribution of mature or nascent transcripts and proteins across cytoplasm, nucleoplasm, and chromatin in time and under different experimental conditions (stress, gain- or loss-of-function studies). The protocol is cost-effective, simple, and fast. From subcellular fractionation to the detection and evaluation of RNA and protein levels, it takes 2–4 h to complete the fractionation and 1–2 days to complete the whole experiment.

2 Materials

Prepare all solutions using ultrapure nuclease-free water and analytical grade reagents. Store all reagents at room temperature (unless indicated otherwise).

2.1 Cell Culture

1. T150 flasks.
2. Cell scrapers (20–25 mm of blade width).
3. Conical 50 mL tubes, 2 mL and 1.5 mL microtubes.
4. Ice bucket.
5. Cell culture reagents for the cells of interest (medium, trypsin).
6. 1× Phosphate Buffer Saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4. Store at 4 °C. Keep on ice upon use.

2.2 Fractionation

1. α-amanitin: 1 mg/mL. Under a chemical hood, dissolve α-amanitin powder in water, aliquot, and use as 40× stock. Store at −20 °C. Keep on ice throughout use.
2. SUPERase-In (or equivalent) at 20 U/μL. Use as a 80× stock solution. Store at −20 °C. Keep on ice throughout use.
3. cOmplete™ Protease Inhibitor tablets or equivalent (PI). Dissolve one tablet in 2 mL of water and use as a 25× stock solution. Store at −20 °C. Keep on ice throughout use.
4. 10% IGEPAL CA-630™ or NP-40 nonionic detergent (prepared by dilution of a 100% stock solution in water).
5. 1 M urea. Pass through a 0.22 μm sterile filter.
6. 50% glycerol (prepared by diluting 100% glycerol in water).
7. 1 M Tris-HCl, pH 7.4.
8. 1 M Tris-HCl, pH 7.0.
9. 5 M sodium chloride (NaCl). Pass through a 0.22 μm sterile filter.
10. 0.1 M magnesium chloride ($MgCl_2$).
11. 1 M potassium chloride (KCl). Pass through a 0.22 μm sterile filter.
12. Hypotonic Lysis Buffer (HLB): 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.3% NP-40 (v/V), 10% glycerol (v/V) (*see* **Note 1**). Store at 4 °C. For resuspension of the cell pellet, HLB should be supplemented immediately prior to use with PI, SUPERase-In, and additionally with α-amanitin if used for the nascent transcriptome profiling. After supplementing, keep on ice for no more than 1 h.

13. Modified Wuarin-Schibler buffer (MWS): 10 mM Tris-HCl pH 7.0, 4 mM EDTA, 0.3 M NaCl, 1 M urea, and 1% NP-40 (v/V) (*see* **Note 1**). Store at 4 °C. For resuspension of the nuclei pellet, MWS should be supplemented immediately prior to use with PI and SUPERase-In and kept on ice for no more than 1 h.
14. Nuclear Lysis Buffer (NLB): 20 mM Tris-HCl pH 7.4, 150 mM KCl, 3 mM $MgCl_2$, 0.3% NP-40 (v/V), and 10% glycerol (v/V) (*see* **Note 1**). Store at 4 °C. For resuspension of chromatin pellets, NLB should be supplemented immediately prior to use with PI and kept on ice upon use for no more than 1 h.

2.3 RNA Isolation

1. 3 M sodium acetate (NaOAc), pH 5.5.
2. RNA precipitation solution (RPS): 150 mM NaOAc pH 5.5, 95% ethanol. Store at −20 °C.
3. 100% ethanol.
4. 0.5 M EDTA, pH 8.0.
5. Chloroform. Store at room temperature under a chemical hood.
6. miRNeasy Mini Kit (Qiagen) or equivalent (*see* **Note 2**) with the phenol-based reagent as Qiazol (Qiagen) or TRIzol or equivalent stored under a chemical hood.

2.4 Protein Quantification and Analysis

1. Pierce™ BCA protein Assay kit or equivalent.
2. NuPAGE™ 4 to 12% Bis-Tris 1.0 mm gel or equivalent.
3. 3× or 4× SDS sample loading buffer supplemented with 0.1 M DTT.
4. 20× NuPAGE™ MOPS SDS Running Buffer or equivalent.
5. Semi-dry or wet western blot transfer system with a nitrocellulose or PVDF membrane (*see* **Note 3**).
6. Antibodies for western blot: anti-H3K4me3; anti-GAPDH; anti-Nuclear Pore Complex; anti-Pol II; anti-Rabbit IgG; anti-Mouse IgG (*see* **Note 4**).
7. PageRuler Plus Prestained Protein ladder (MWL) or equivalent. Store at −20 °C.
8. PBS-Tween: 1× PBS, 0.1% Tween™-20.
9. 5% skimmed milk in PBS-Tween. Store at 4 °C for up to 4 days.
10. Ponceau S staining solution.
11. Chemiluminescent substrate (SuperSignal™ West Pico or Dura Chemiluminescent Substrate or equivalent).

Table 1
List of primers for qPCR quantification of human nascent or mature transcripts

Primer set	Strand	Sequence 5′-3′
GAPDH junction (GAPDH-j)	FRW	AAAGCCTGCCGGTGACTAAC
	REV	GTATTGGGCGCCTGGTCA
GAPDH intron (GAPDH-i)	FRW	GCTGCATTCGCCCTCTTAATG
	REV	GACAAGAGGCAAGAAGGCATGA
GAPDH exon	FRW	GTATTGGGCGCCTGGTCA
	REV	ATGAAGGGGTCATTGATGGCA
TERC	FRW	CTAACCCTAACTGAGAAGGGCGTA
	REV	GGCGAACGGGCCAGCAGCTGACATT
V1 (lncRNA splice variant 1)	FRW	TGGTCGTGTGACAAGAGCCT
	REV	CAAAACTCGTATTCCTTCCTC
V2 (lncRNA splice variant 2)	FRW	TGGTCGTGTGACAAGAGCCT
	REV	CTCTGTGAAACCCCTTCCTC
V3 (lncRNA splice variant 3)	FRW	TGGTCGTGTGACAAGAGCCT
	REV	ACAATTGAGAGTCACCTCAGG

2.5 RNA Analysis

1. SuperScript II Reverse Transcription kit or equivalent (RT). Store at −20 °C.
2. Random hexamer primers: 50 ng/μL. Store at −20 °C (*see* **Note 5**).
3. 10 mM dNTPs. Store at −20 °C.
4. SYBR Green qPCR Master kit or equivalent. Store at −20 °C.
5. qPCR primer sets at 100 mM stock concentration (*see* Table 1 and **Note 6**). Store at −20 °C.

3 Methods

The procedure is described for one sample prepared from 10^7 of adherent mammalian cells collected by scraping of the T150 flask at 60–80% of confluency. All manipulations are performed on ice. Centrifuges and sonicator must be set to 4 °C, and tubes and buffers should be kept on ice.

3.1 Cell Culture

1. Grow cells under appropriate conditions until they reach up to 80% confluency in four T150 flasks: one flask for proteins isolation and three for RNA isolation in three technical replicates (*see* **Note 7**).

2. Discard cell media completely.
3. Either in a cold room or with the T150 flask on ice, add 15 mL of ice-cold PBS, and scrape the cells. Transfer the cell suspension into a conical 50 mL tube kept on ice.
4. Repeat scraping with an additional 10 mL of ice-cold 1× PBS and transfer into the same conical 50 mL tube.
5. Centrifuge for 3 min at 800× *g* at 4 °C.
6. Discard the supernatant, resuspend the cells in 0.5 mL of ice-cold PBS by pipetting or vortexing, and transfer the cell suspension to pre-chilled 1.5 mL microtubes. Tubes labelling: three tubes CHR-R1/R2/R3 for RNA isolation; one tube CHR-PR for protein analysis.
7. Centrifuge for 3 min at 800× *g* at 4 °C, and then completely discard the supernatant.

3.2 Cell Lysis and Fractionation

A schematic view of fractionation steps is presented in Fig. 1. All manipulations are performed on ice with prechilled buffers and tubes.

1. Resuspend the cell pellet in 400 µL of ice-cold HLB by pipetting up and down five times. Incubate on ice for 15 min (*see* **Note 8**).
2. Mix by vortexing briefly and centrifuge for 3 min at 1,000× *g* at 4 °C.
3. Carefully transfer the supernatant to a new 2.0 mL microtube. This is the cytoplasm fraction (CYT-PR—for proteins analysis; CYT-R1/R2/R3—for RNA isolation). Keep the pellet (nuclei) on ice.
4. Take out 5 µL of CYT-PR for protein quantifications (*see* Subheading 3.4). Store this aliquot ($CYT\text{-}PR^{BCA}$) and the rest of CYT-PR at −20 °C.
5. To the tubes labelled CYT-R1/R2/R3, add 3 volumes of ice-cold RPS (up to 1.2 mL) (*see* **Note 9**). Incubate for at least 1 h at −20 °C (*see* **Note 10**).
6. To the pellet (nuclei), add 1 mL of ice-cold HLB without supplements and resuspend by pipetting once up and down.
7. Centrifuge for 2 min at 300× *g* at 4 °C.
8. Repeat **steps 6** and **7** twice for a total of three washes.
9. To the tubes labelled CHR-R1/R2/R3, add 400 µL of supplemented MWS.
10. To the tube labelled CHR-PR, add 200 µL of supplemented MWS.
11. Mix all tubes by gentle vortexing for 30 s at mid power. Do not pipette (*see* **Note 11**). Incubate on ice for 5 min.

12. Mix again by vortexing for 30 s and incubate for another 10 min on ice.
13. Centrifuge for 3 min at 1,000× *g* at 4 °C.
14. Carefully transfer the supernatant to a new 2.0 mL microtube (*see* **Note 11**). This is the nucleoplasm fraction (NUC-PR—for proteins analysis; NUC-R1/R2/R3—for RNA isolation). Keep the pellet (chromatin) on ice.
15. Take out 5 μL of NUC-PR for protein quantifications (*see* Subheading 3.4). Store this aliquot ($NUC\text{-}PR^{BCA}$) and the rest of NUC-PR at −20 °C.
16. To the tubes labelled NUC-R1/R2/R3, immediately add 3 volumes of ice-cold RPS (up to 1.2 mL) (*see* **Note 9**). Incubate for at least 1 h at −20 °C (*see* **Note 10**).
17. To the pellet (chromatin), add 1 mL of ice-cold MWS without supplements and resuspend by vortexing for 2 s. Do not pipette.
18. Centrifuge for 2 min at 500× *g* at 4 °C.
19. Repeat **steps 17** and **18** twice for a total of three washes.
20. To the tubes labelled CHR-R1/R2/R3 with the pellet, add 10 μL of 0.5 M EDTA and 700 μL of Qiazol. Flash-freeze in liquid nitrogen or dry ice and store at −80 °C.
21. To the sample CHR-PR with the pellet add 100 μL of supplemented NLB. To fragment and solubilize the chromatin, sonicate for 5 min (Diagenode Bioraptor pico 30 s ON/OFF, power—high) at 4 °C (*see* **Note 12**).
22. Centrifuge the sonicated CHR-PR sample for 15 min at >10,000× *g* at 4 °C.
23. Transfer the supernatant to a new 1.5 mL microtube tube. This is the solubilized chromatin fraction of proteins (sCHR-PR).
24. Take out 5 μL of sCHR-PR for protein quantifications (*see* Subheading 3.4). Store this aliquot ($sCHR\text{-}PR^{BCA}$) and the rest of sCHR-PR samples at −20 °C.

3.3 RNA Isolation from Cytoplasm, Nucleoplasm, and Chromatin Fractions

1. Take out CYT-R1/R2/R3 and NUC-R1/R2/R3 from −20 °C and centrifuge for 30 min at 14,000× *g* at 4 °C to precipitate RNA.
2. Completely remove the supernatant by pipetting (*see* **Note 13**).
3. To the pellets, add 700 μL of Qiazol and 10 μL of 0.5 M EDTA. Optional: flash-freeze in liquid nitrogen or dry ice and store at −80 °C. Alternatively, proceed with the next step.
4. Take out CHR-R1/R2/R3, CYT-R1/R2/R3, and NUC-R1/R2/R3 from −80 °C and immediately incubate at 65 °C for

3–5 min with regular vortexing until the pellet is fully dissolved.

5. Chill the samples to room temperature.
6. To each tube, add 140 µL of chloroform under a chemical hood, mix for 10 s by vortexing and incubate for 5 min at room temperature.
7. Centrifuge for 15 min at 14,000× *g* at 4 °C.
8. Collect the upper aqueous phase and proceed with the conventional RNA extraction procedure (*see* **Note 2**).
9. Recover RNA in 50 µL of water by eluting or resuspending, depending on the purification method chosen.
10. Quantify RNA (*see* **Note 14**) and store at −80 °C. RNA can now be used for single-gene analyses such as RT-qPCR (*see* Subheading 3.5) or nothern blot or as an input for RNA sequencing library preparations (*see* **Note 15**).

3.4 Protein Quantification and Western Blot

Western blot analysis enables the quality control of fractionation by detecting proteins specific for each fraction.

1. Take out the 5 µL aliquots (CYT-, NUC-, sCHR-PR^{BCA}) from the three fractions out of −20 °C. Add 15 µL of water.
2. Prepare nine BSA standards, diluted in water according to manufacturer's procedure. Take out 20 µL of each for absorbance measurements.
3. Prepare the BCA working solution for your samples and nine BSA standards by mixing Reagent A and Reagent B in a 50:1 ratio, using 1 mL per sample.
4. Add 980 µL of the BCA working solution to the protein aliquot samples and to nine BSA standards.
5. Incubate for 30 min at 37 °C.
6. Perform absorbance measurements at 562 nm in 1 mL cuvettes (NanoDrop BCA assay or another specrometer). Calculate protein concentrations relative to the standard BSA curve for each aliquot (*see* **Note 16**).
7. Calculate the stock concentration of proteins in three fractions. Prepare samples for the loading on a SDS-PAGE gel at the final concentration of 1 µg/µL in 1× SDS Sample Loading buffer. Heat at 98 °C or boil in a water bath for 5 min, spin down for 10 s at 14,000× *g* prior to loading on the gel.
8. Load the MWL and 10 µL (10 µg) of each sample per well onto the 10- or 12-well 4–12% SDS-PAGE gel: MWL, CYT-PR, NUC-PR, CHR-PR (*see* **Note 17**). Run the gel at 30 mA till the lowest MWL marker of 10 kDa reaches the bottom.

9. Transfer proteins from the gel to a nitrocellulose or PVDF membrane using a semi-dry or wet transfer system (*see* **Note 3**).
10. Check the quality of transfer by incubating the membrane with the Ponceau S staining solution for 5 min. Wash abundantly with distilled water. Proteins appear as red bands over the white background (Fig. 2a).
11. Cut the membrane into several pieces based on the migration of the MWL for individual antibody incubations, depending on the proteins chosen for detection (*see* **Note 18**), (Fig. 2a). Block the membrane with 5% skimmed milk for 30 min to 1 h at room temperature.
12. Perform the western blot with antibodies against proteins specific to each subcellular fraction to control the quality of separation between chromatin, nucleoplasm, and cytoplasm fractions (*see* **Notes 4** and **18**), (Fig. 2a).

3.5 Reverse Transcription and Quantitative PCR (RT-qPCR)

Prior to transcriptome-wide analysis by RNA sequencing or any other technique of your choice, RT-qPCR enables the control of the quality of fractionation by assessing the levels of intron and exon–exon junction sequences of GAPDH as a proxy for nascent and mature RNA between the chromatin and cytoplasm fractions.

1. Use 5 μL out of 50 μL of each RNA sample per RT. You must not adjust the concentration of each sample to the same level as RNA yields are variable between fractions.
2. Reverse transcribe RNA using either random hexamer primers (for all applications), or oligo-dT to enrich for mature polyadenylated transcripts with the RT kit of of your choice following the manufacturer's procedure.
3. For the total RT volume of 20 μL, dilute 15 μL of each RT reaction 10 times in water (135 μL of water). Pool the rest 5 μL of all RT reactions together and dilute the pool five times (this is the standard 1, ST1). To prepare a standard curve for the absolute cDNA quantification, perform nine additional two-fold serial dilutions starting from ST1 (in total, ten standards, from ST1 to ST10).
4. Prepare the qPCR Master Mix following the manufacturer's procedure with the qPCR primer sets specific to GAPDH exon–exon junction, intron and exon sequences, and to your cDNAs of interest (*see* **Note 19**).
5. Set up the qPCR plate, each sample in two to three replicates as following: CYT-R1/R2/R3, NUC-R1/R2/R3, sCHR-R1/R2/R3 (RT reactions), H_2O (negative control), ST1 to ST10 (standard curve).

6. Run a qPCR program according to the manufacturer's procedure attributing the arbitrary values to each standard, e.g., ST10 = 1; ST9 = 2; and ST8 = 4.
7. Recover the raw qPCR data of absolute quantification of cDNA in each RT reaction. Calculate the mean and standard deviation (SD) values of each qPCR sample and then the mean and SD for three RT replicates of each fraction. The result can be expressed as an histogram of distribution (raw or relative to GAPDH exon) of each transcript across the three fractions (*see* **Note 19**). Nascent RNAs are highly enriched in the chromatin while mature mRNAs are more abundant in the cytoplasm (Fig. 2b). Splice variants may exhibit differential distribution between chromatin and cytoplasm depending on intrinsic properties of each transcript, as is the case with a lncRNA expressed as three isofoms, V1, V2, and V3 in LNCaP cells (Fig. 2c).

4 Notes

1. Buffers HLB, MWS, and NLB without supplements can be stored at 4 °C for at least 6 months.
2. miRNeasy kit (Qiagen) allows to recover RNAs of wide size range, but any other RNA extraction kit or phenol-based protocol is suitable. Follow the manufacturer's procedure. The DNase treatment step is not essential but preferable, especially for RNA sequencing applications.
3. For proteins with a wide range of molecular weights (from 15 to 250 kDa), use the wet transfer system.
4. Antibodies: anti-RNA Polymerase II (clone N-20, Santa Cruz Biotechnology), diluted in 5% milk at 1:500; anti-Nuclear Pore Complex (clone MAb414, Covance; of note, this antibody is no longer available from Covance but can be purchased elsewhere), diluted in 5% milk at 1:5,000; anti-GAPDH (Merck), diluted in 5% milk at 1:500; anti-Histone H3K4me3 (Abcam), diluted in 5% milk at 1:1,000; HRP conjugated anti-Rabbit IgG (Sigma-Aldrich), diluted in 5% milk at 1:5,000; HRP conjugated anti-Mouse IgG (Sigma-Aldrich), diluted in 5% milk at 1:5,000. These antibodies can be replaced by others against any other fraction-specific proteins.
5. For some applications, RT can be done with oligo-dT or transcript-specific reverse primer.
6. Primers are chosen according to the experiment purpose: for quantification of nascent transcripts, primers should map to a single intronic region and avoid spanning exons; for

quantification of mature transcripts, primers should map to two adjacent exons and amplify a cDNA fragment spanning a junction.

7. The experiment can be scaled up for more material-demanding applications. In this case amounts of all reagents should be also upscaled.
8. For some cell types of large size as LNCaP, the volume of HLB buffer should be increased. For example, cell pellets from HeLa or HCT116 cells are equivalent to a volume of approximately 40 μL and thus are resuspended in 400 μL of HLB. The LNCaP cells' pellet occupies an approximate volume of 80 μL and should be resuspended in 800 μL of HLB. If at the end of the protocol the yields of RNA and proteins in all fractions are low, or nucleoplasm or chromatin fractions show presence of cytoplasmic proteins (as GAPDH) by western blot, the hypotonic cell lysis is not efficient enough and should be optimized. The volume of HLB and the time of incubation must then be increased.
9. The RPS solution enables RNA precipitation. Use 5 volumes of RPS and glycogen (or equivalent) to recover small RNA species. Ensure that the addition of glycogen is compatible with the downstream RNA analysis of your choice.
10. RNA in the RPS solution should be kept at −20 °C for a minimum of 1 h, but it can also be stored for a longer period if interruption of the experiment is necessary. For long-term storage prior to extraction, −80 °C is preferable.
11. Pipetting or aggressive vortexing of nuclei suspensions in MWS can lead to chromatin fragmentation and contamination of the nucleoplasm fraction with chromatin-associated proteins. After centrifugation, if the pellet is cloudy and detaches easily, repeat the centrifugation and carefully remove as much supernatant as possible, taking care to avoid disturbing the pellet.
12. Chromatin fragmentation conditions should be adapted for each sonicator. Excessive sonication may lead to fragmentation of RNA and proteins and alter their quality and detection.
13. At this step, there is no need for a pellet wash with 70% ethanol since any remaining traces of the RPS solution in the tubes will not affect the downstream steps.
14. RNA quantity can be determined either by Nanodrop or Qubit RNA quantification methods. The RNA yields depend on the cell type and the starting quantity of cells. As an example of RNA yields, one can obtain 1.5 μg/μL of CYT-RNA, 700 ng/μL of NUC-RNA, and 800 ng/μL of CHR-RNA upon elution in 50 μL.

15. Once extracted from the different fractions, RNA may be used for transcriptome-wide analyses. While mRNA-specific methods may be suitable for the cytoplasm fraction, it is preferable to use total RNA sequencing methods as they enable the detection of both polyadenylated and non-polyadenylated transcripts, including nascent and other premature RNAs in the nucleoplasm and chromatin fractions.
16. The protein yields depend on the cell type and the starting quantity of cells. As an example of protein yields, one can obtain 2 μg/μL of CYT-PR in a total volume of 350 μL, 1.5 μg/μL of NUC-PR in a total volume of 180 μL, and 1 μg/μL of sCHR-PR in a total volume of 80 μL.
17. If the protein yield of the sCHR-PR samples is below 1 μg/μL, it can be loaded in a smaller quantity (up to 1 μg), depending on the detection limit of the chosen proteins by western blot.
18. The membrane can be cut into several pieces to enable the detection of several proteins of different molecular weight in the same gel. For example, RNA Polymerase II (250 kDa) or H3K4me3 or H3 (15–17 kDa) in the chromatin fraction, NUP62 (62 kDa) or Lamin A (74 kDa) in the nucleopasm fraction, and GAPDH (35 kDa) in the cytoplasm fraction.
19. qPCR with the GAPDH intron primer set enables the detection of nascent GAPDH pre-mRNA and shows high enrichment in the chromatin fraction. The GAPDH junction primer set detects the spliced, mature mRNA which is preferentially enriched in the cytoplasm, GAPDH exon primer set quantifies all types of GAPDH transcripts. The GAPDH exon data can be used for normalization of the raw data in each sample, if comparison between different experimental conditions is required. Although GAPDH is used here, the same process can be applied to other control genes specific to other experimental settings. Results can be expressed as a ratio of intron to exon and junction to exon signal in the histogram of relative distribution of nascent and mature transcripts, respectively (Fig. 2b). Telomerase RNA component or TERC is an example of a housekeeping noncoding RNA primarily localized to chromatin, most specifically in Cajal bodies where it functions as part of the Telomerase complex [7].

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Chapter 18

Concurrent Profiling of Localized Transcriptome and RNA Dynamics in Neurons by Spatial SLAMseq

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Abstract

The asymmetric distribution of RNA within a cell plays a pivotal biological role, ensuring the distinctive shapes and functionality of subcellular compartments. In neurons, these mechanisms are fundamental to cellular growth, synaptic plasticity, and information processing. To understand these mechanisms, diverse methods have been developed to analyze localized transcripts. Here, we outline our optimized method for measurement of mRNA half-lives in subcellular neuronal compartments—neurites, and cytoplasmic and nuclear fractions of cell bodies. We call this method spatial SLAMseq, as it combines SLAMseq with subcellular compartment separation techniques. Spatial SLAMseq facilitates the concurrent measurement of mRNA dynamics and steady-state RNA levels within neuronal subcellular compartments.

Key words mRNA localization, mRNA stability, Local translation, SLAMseq, High-throughput sequencing, Neuron

1 Introduction

Neurons exhibit distinct polarity crucial for their function. They are composed of cell body (soma) and neurite extensions (axons and dendrites). This polarity, essential for neural signaling and information processing, relies in large on the asymmetric distribution of RNAs and proteins [1, 2]. We previously reported that the localized transcriptome is the key determinant of localized proteome [3]. Additionally, we have shown that high mRNA stability serves as a predictive factor for mRNA localization to neurites [4]. Here, we provide a detailed account of our optimized method for measuring mRNA half-lives in distinct subcellular neuronal compartments: neurites, as well as the cytoplasmic and nuclear fractions of cell bodies. We have named this method "spatial SLAMseq" [4], as it combines SLAMseq (a thio-uracil-based metabolic RNA labeling technique coupled with next-generation sequencing) [5] with subcellular compartment separation techniques. SLAMseq has

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emerged as a revolutionary technique to study RNA dynamics within living cells. By incorporating synthetic nucleotides into the cellular RNA pool, SLAMseq enables researchers to track RNA molecules throughout their life cycle. In particular, RNA is labeled with 4-thiouridine (S4U), which is subsequent replacement with unmodified uridine (U), and the reduction in S4U-labeled mRNA is measured over time. The S4U is subsequently converted into cytosine, and the rates of T > C conversion are quantified through RNA-seq to assess mRNA half-lives.

In this chapter, we describe the important considerations for carrying out spatial SLAMseq. We focus on mESC-derived neurons, but this methodology can be applied to other types of neurons, including primary cortical neurons and neuronal cell lines. In our compartment separation method, neurons are cultured on microporous membranes in such a way that cell bodies remain on top of the membrane while neurites grow under the membrane through the pores [3, 6–8]. Cell bodies and neurites are collected from the respective side of the membrane. Cell bodies can be further separated into cytoplasm and nuclei using stepwise lysis and centrifugation. Integrating SLAMseq with our compartment separation scheme allows simultaneous analysis of transcripts' subcellular localization and half-life. This dual insight enhances our understanding of intricate correlations in transcript dynamics.

2 Materials

2.1 ASCL-1 mESC Culture

1. Mouse embryonic stem cells (mESC) with doxycycline (dox)-inducible cassette for Achaete-scute homolog 1 (ASCL1) transcription factor [9] (*see* **Note 1**).
2. 80–20 media: 80% 2i medium, 20% mESC medium.
3. 2i medium: 50% Advanced DMEM/F12 (Thermo) and 50% Neurobasal (Thermo) supplemented with 1 × N2 (Thermo), 1 × B27 (Thermo), 1 mM L-Glutamine, 0.1 mM β-mercaptoethanol (βME), 103 U mL^{-1} LIF, 3 μM CHIR99021 (Biozol), 1 μM PD03259901 (Milenyi Biotec).
4. mESC medium: Knockout DMEM (Thermo) supplemented with 14% fetal bovine serum (FBS; Thermo), 0.1 mM βME, 1 mM L-Glutamine, 1 × MEM non-essential amino acid (Thermo), 1 × nucleosides (Millipore), 103 U mL^{-1} leukemia inhibitory factor (LIF, Millipore).
5. Feeder medium: Knockout DMEM supplemented with 10% FBS, 1 mM L-Glutamine.
6. Cryopreservation medium: DMEM, high glucose, GlutaMAX™ Supplement (Thermo) supplemented with 10% FBS.

7. Gelatin for flask coating: dissolve 0.5 g of porcine gelatin in 500 mL tissue culture-grade water (0.1% w/v). Sterilize by autoclaving (121 °C, 15 psi, 30 min) or by filtration (0.22 μm filter). Store at 4 °C; use within 2 months.
8. T75 flasks.
9. DPBS (w/o: Ca and Mg).
10. Gibco™ TrypLE™ Express Enzyme (1X), phenol red.
11. DMSO, tissue culture grade.

2.2 Neuronal Differentiation of ASCL1-mESC

1. AK medium: 50% Advanced DMEM/F12 supplemented with 50% neurobasal, 10% knockout serum replacement (Thermo), 1 mM L-Glutamine, 0.1 mM βME, ± 3 μg mL^{-1} doxycycline. Prepare the medium without doxycycline and add it freshly before using the medium in culture.
2. Monolayer differentiation medium: Advanced DMEM/F12 supplemented with 1 × B27, 1 × N2, and 3 μg mL^{-1} doxycycline. Doxycycline should be added freshly before using the medium in culture.
3. 10 cm cell culture dishes without coating.
4. 6-well cell culture dishes.
5. Matrigel (Corning). Dilute the stock (9–12 mg/mL) to the final concentration of 0.3 mg/mL with ice-cold Knockout™ DMEM and prepare 1–3 mL aliquots. Since matrigel starts to solidify into a gel above 10 °C, keep it on ice, use pre-cooled tips and tubes, and freeze aliquots immediately.
6. Millicell hanging cell culture insert, PET 3 μm, for 6-well plate (Millipore).

2.3 S4U Labeling

1. SLAMseq Kinetics kit - Catabolic Kinetics Module (Lexogen).
2. Monolayer differentiation medium (with freshly added doxycycline).
3. Tin foil or equivalent to protect samples from exposure to light.

2.4 Compartment Separation

2.4.1 Option 1: Two-Compartment Separation (Soma and Neurite)

1. Forceps.
2. Syringe needles.
3. Cotton swabs (large: 4–5.5 mm^2; small: 2 mm^2).
4. Bench top centrifuge at 4 °C.
5. Ice-cold PBS.
6. Tin foil or equivalent to protect samples from exposure to light.
7. 6-well cell culture dishes.

2.4.2 Option 2: Three-Compartment Separation (Cytoplasm, Nuclei, and Neurite)

1. Everything listed for two-compartment separation.
2. NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo).

2.5 RNA Extraction and Iodoacetamide Treatment

1. SLAMseq Kinetics Kit - Catabolic Kinetics Module (Lexogen).
2. Fume hood.
3. peqGOLD TriFast™ or TRIzol.
4. Chloroform.
5. Isopropanol.
6. RNase-free water. DEPC-treated water prepared as described below can be used. Mix 0.1% (v/v) DEPC to double deionized water (for example: milli-Q water) using an orbital shaker or a magnetic stirrer for 1 h to overnight (o/n). Remove DEPC by autoclaving: 15–45 min at 15 psi on a liquid cycle. Traces of DEPC can modify purine residues in RNA by carboxymethylation. As a precaution, always autoclave or heat solutions or containers at 100 °C for 15 min to remove DEPC.
7. 1.5 mL nuclease-free DNA low-binding microfuge tubes.
8. 75% ethanol, molecular biology grade, diluted with Rnase-free water.
9. Tin foil or equivalent to protect samples from exposure to light.

2.6 Separation Validation and RNA Quality Control

1. Qubit 2.0 or later model.
2. Qubit RNA HS Assay Kit (Thermo).
3. Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo) or equivalent.
4. DNase I, RNase-free.
5. SensiFast SYBR no-rox (Bioline) or equivalent.
6. 0.2 mL PCR tubes, nuclease free.
7. RT-qPCR primers for neuritic and somatic enrichment quality control (*see* Table 1).
8. Thermal cycler.
9. RT-qPCR system.
10. Bioanalyzer (Agilent) Systems and Bioanalyzer Nano or Pico analysis kit (Agilent) (*see* **Note 2**).

2.7 NGS Library Preparation

1. QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (REV) with Custom Sequencing Primer (Lexogen).
2. 1.5 mL nuclease-free DNA low-binding microfuge tubes.
3. 0.2 mL PCR tubes, nuclease free.
4. Lexogen PCR Add-on Kit for Illumina.

Table 1
List of primers for qPCR validation of subcellular compartment separation

Target name	Direction	Sequence
Neurite-enriched		
Tagln	Forward	TAGACCCCAGCGGCAACTAT
Tagln	Reverse	GTTCCAGGTTCCAAGTCCACC
Soma(nuclei)-enriched		
Snord15b	Forward	ATGGCCACGTCTTGCTCTTG
Snord15b	Reverse	ACACTTTTGCCAAGGGAACC
Equally distributed		
18S rRNA	Forward	AAACGGCTACCACATCCAAG
18S rRNA	Reverse	CCTCCAATGGATCCTCGTTA
Gapdh	Forward	TGACCTCAACTACATGGTCTACA
Gapdh	Reverse	CTTCCCATTCTCGGCCTTG
Thyn1	Forward	CCCTAAATGGTCGATGGTGGA
Thyn1	Reverse	TTTGTGGGCTTGGTGATAGGT

5. SYBR Green I. Diluted to 2.5× in DMSO. Protect from the light.
6. Bioanalyzer (Agilent) System and Bioanalyzer DNA 1000 analysis kit (Agilent) (*see* **Note 2**).
7. Qubit dsDNA HS Assay Kit.
8. Magnetic stand for 0.2 mL PCR tubes.
9. Thermal cycler.

2.8 Sequencing

1. NextSeq 500/550 High Output Kit v2.5 (300 Cycle, Illumina) (*see* **Note 3**).
2. HT1 Hybridization Buffer (Illumina, supplied with NextSeq 500/550 High Output Kit (*see* **Note 4**).
3. 1 N NaOH, molecular biology grade.
4. 200 mM Tris-HCl, pH 7.0.
5. Other flow cells can be used.

3 Methods

3.1 ASCL-1 mESC Culture

1. Culture ASCL1-mESC cells in 80–20 medium in gelatin-coated T75 flasks at 37 °C and 5% CO_2. Ensure that the cells are not exceeding 75% confluency.

2. To coat with gelatin, apply 0.1% (w/v) gelatin solution to the cell culture flask to cover the bottom, and incubate at room temperature (RT) for 20 min. Remove gelatin and rinse once with PBS. The gelatin-coated flask must not dry.
3. Passage cells 1:10–1:25 every 3–4 days. Change the media every second day between passages.
4. To passage, wash the cells once with PBS, and incubate with TrypLE for 1 min at 37 °C (1 mL/T75 flask).
5. Dilute TrypLE with 5× excess Feeder media, resuspend cells by pipetting up and down in a 10 mL pipette, and transfer the cell suspension into a 15 mL conical tube.
6. Centrifuge the tube at 150× *g* for 4 min and discard supernatant.
7. Resuspend the cell pellet in 80–20 medium and plate 1/10–1/25 into a fresh gelatin-coated T75 flask.
8. For cryopreservation of the ASCL1-mESC cells, prepare cells at 75% confluency, preferably at an early passage (ideally before P5). A flask contains ~1.0×10^7 cells.
9. Follow the passage procedure (Subheading 3.1, **step 2**), but resuspend the cell pellet in 3 mL of cryopreservation medium and count the cells.
10. Add cryopreservation medium to achieve concentration of $2–3 \times 10^6$ cells/mL.
11. Add DMSO dropwise, ensuring that the final DMSO concentration reaches 5%, and slowly mix by pipetting up and down.
12. Make 1 mL aliquots in 2 mL cryovials, position the vials in an isopropanol freezing container and store them at −80 °C overnight. Transfer vials to liquid nitrogen vapor for permanent storage.

3.2 Neuronal Differentiation of ASCL1-mESC

1. Estimate the number of cells for differentiation according to the final required material amount. 0.5×10^6 mESCs will be sufficient for 1 × 6-well filter, which will be used for a replicate from a time-point. For example, if samples are collected at 5 time-points in triplicates, 15 filters (5 time-points × 3 replicates) are necessary. It is advisable to prepare 2–3 filters as backups (*see* **Notes 5** and **6**).
2. We recommend sample collection at 5 time-points: 0, 2, 4, 8, and 16 h after labeling stop. Estimated yield from a filter is 0.3–1 μg of neuritic and 3–10 μg of somatic RNA (1.5–5 μg of cytoplasmic and 0.3–1 μg of nucleic RNA).
3. For differentiation, use cells with 75% confluency. On day 1, follow passaging procedure (Subheading 3.1, **steps 4–6**), and resuspend the cell pellet in 1 mL of AK medium. Ensure

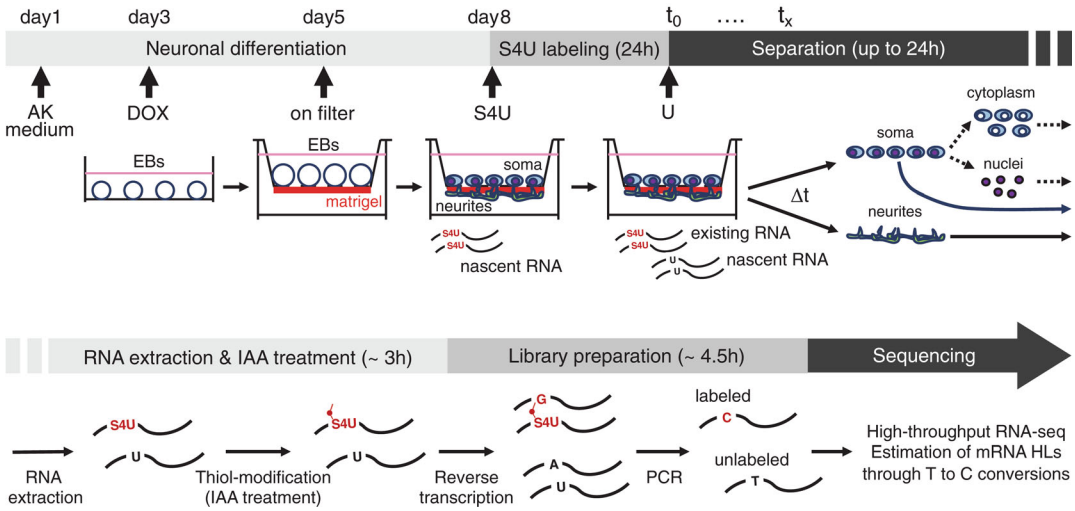

Fig. 1 Spatial SLAMseq schematic workflow. mESC-derived neurons are grown on microporous membranes. Cells are pre-labeled with S4U, which is afterwards exchanged for unlabeled uridine (U) in media at t_0. Compartment separation (sampling) is carried out over a time course (tx), allowing to monitor reflecting the decay of S4U-labeled mRNA. RNA from each compartment is isolated, modified with Iodoacetamide (IAA), and used for RNA-seq libraries preparation. The measurement of mRNA half-lives (HLs) is conducted based on T to C conversions resulting from the incorporated S4U. Working time for labeling, IAA treatment, and library preparation using Lexogen SLAMseq and QuantSeq kits are indicated

thorough dissociation of the pellet into single cells by trituration using a P1000 pipette.

4. Add 5 mL of AK medium and count the cells.
5. Plate 1×10^6 cells in 10 mL of AK medium per one 10 cm dish. Important: the dish used at this point must not be coated with gelatin or other coating materials, to facilitate the growth of cells in suspension and the formation of embryoid bodies (EBs) (*see* Fig. 1).
6. On day 2 (24 h after plating), the majority of the cells form EBs, which should have uniform size and round shape (*see* Fig. 1).
7. On day 3 (48 h after plating), initiate ASCL1 expression by splitting the EBs in 1:2 in AK media supplemented with doxycycline. For that, collect the EBs in a 50 mL conical tube and centrifuge at 100× *g* for 3 min.
8. Resuspend the EBs in 2 mL of AK media containing 3 μg/mL of doxycycline (AK + dox).
9. Add 1 mL of the EB suspension to a 10 cm plate containing 9 mL of AK + dox media and let them grow for an additional 2 days.
10. On day 4 or in the morning of day 5, thaw matrigel at 4 °C. To do this, place required number of matrigel aliquots (~0.5 mL

for a filter, ~3 mL for one 6-well plate) in an ice bucket filled with ice and leave it in a refrigerator or in the cold room for ~3 h to overnight. Keep in mind that matrigel will polymerize rapidly if left at room temperature.

11. On day 5, apply a coating to the filters with matrigel. For that, place filter inserts in a 6-well plate, close the lid and invert the plate. Carefully remove the plate bottom so that inserts remain standing on the plate lid.
12. Pipette ~500 μL of matrigel on each filter and place the plate bottom back onto top of the lid with filters. Incubate for 3 h at 37 °C (in a cell culture incubator).
13. On the same day (48 h after ASCL1 induction), seed the EBs on matrigel-coated filters, using one 10 cm dish of EBs for each filter.
14. First, remove the excess matrigel from filters. Under the fume food, carefully lift the plate bottom so that matrigel remains on the filter.
15. Aspirate the matrigel and place the plate bottom back onto the plate lid. Flip the plate (the lid is now on the top), rinse filters once with PBS and transfer the filters in a new 6-well plate with monolayer medium supplemented with 3 μg/mL doxycycline (mono + dox, 2 mL per well).
16. Collect the EBs in a 50 mL conical tube and pellet them down by centrifugation at 100× *g* for 3 min.
17. Remove the supernatant, resuspend the EBs obtained from one 10 cm dish in 2 mL of mono + dox, and plate on top of the filter (*see* Fig. 1).
18. On day 8, examine the cells under the cell culture microscope. They should be initiated with differentiation, with axons and dendrites extending towards the lower part of the membrane (*see* Fig. 1).
19. Proceed to S4U labeling (*see* **Note 7**).

3.3 S4U Labeling

1. Follow the instructions from SLAMseq Kinetics Kit - Catabolic Kinetics Module (SLAMseq kit). In this phase, cells undergo incubation with media containing 4-Thiouridine (S4U), and S4U will replace uridine in newly synthesized RNA transcripts.
2. On day 8 of differentiation, prepare monolayer medium supplemented with freshly added 3 μg/mL doxycycline and 200 μM 4-Thiouridine (4SU, from SLAMseq kit) (*see* **Note 8**).
3. Remove medium from the 6-well plates and replace it with S4U-containing medium (2 mL to the top and 2 mL to the bottom of the filter). Important: S4U is light sensitive. Plates

should be wrapped in tin foil once the S4U-containing medium is added to prevent exposure to light.

4. Incubate the plates for 24 h at 37 °C. Exchange media with new medium with freshly added doxycycline and S4U every 3–6 h, to enhance S4U incorporation.

3.4 Labeling Stop

1. Prepare monolayer media containing freshly added 3 μg/mL doxycycline and 20 mM uridine (U, from SLAMseq kit). U should be in 100× excess relative to the initial S4U concentration in the media.
2. Remove the S4U-containing media from all the filters except for those for t_0. Wash the cells twice with PBS, and add the media with excess U to the plates (2 mL to the top and 2 mL to the bottom of the filter).

3.5 Compartment Separation

The workflow for the compartment separation is summarized in Fig. 2.

3.5.1 Option 1: Two-Compartment Separation

1. Perform separation of neurites and soma on t_0 filters (*see* Fig. 2). *See* **Note 9** for tips for working with RNA beforehand. The separation procedure must be done quickly (under 5 min) and at 4 °C to ensure the integrity of the isolated RNA. It is convenient to prepare a large ice box which can be placed with a 6-well plate, a bottle of PBS and a metallic rack for 1.5 mL tubes.
2. Place 1.5 mL tubes (nuclease free, DNA low-binding), as many as the number of t_0 soma and neurite samples, on an ice-cold metallic rack. Add 400 μL of TriFast to the tubes for neurite samples.
3. Place a new 6-well plate on ice and pour cold PBS in the wells. Transfer a t_0 filter in a well of 6-well plates, using forceps, and rinse it in cold PBS. For soma isolation, detach it from the filter top by pipetting ~1 mL of PBS up and down using P1000 pipette (10–20 times, until the top of the filter looks clean).
4. Transfer the PBS containing soma from the top of the filter to a cold 1.5 mL tube and pellet down by centrifugation at 1000× *g* for 3 min at 4 °C.
5. Remove supernatant, immediately add 400 μL of TriFast to the pellet and lyse the soma by pipetting.
6. Isolate neurites during the centrifugation to pellet the soma. Add ~1 mL of cold PBS to the top of the filter. With cotton swabs, dipped in cold PBS, wipe the top of the filter to remove the remnants of soma.
7. Discard PBS containing soma debris and repeat the wash 2–3 times in fresh PBS. It is convenient to use large cotton swab to

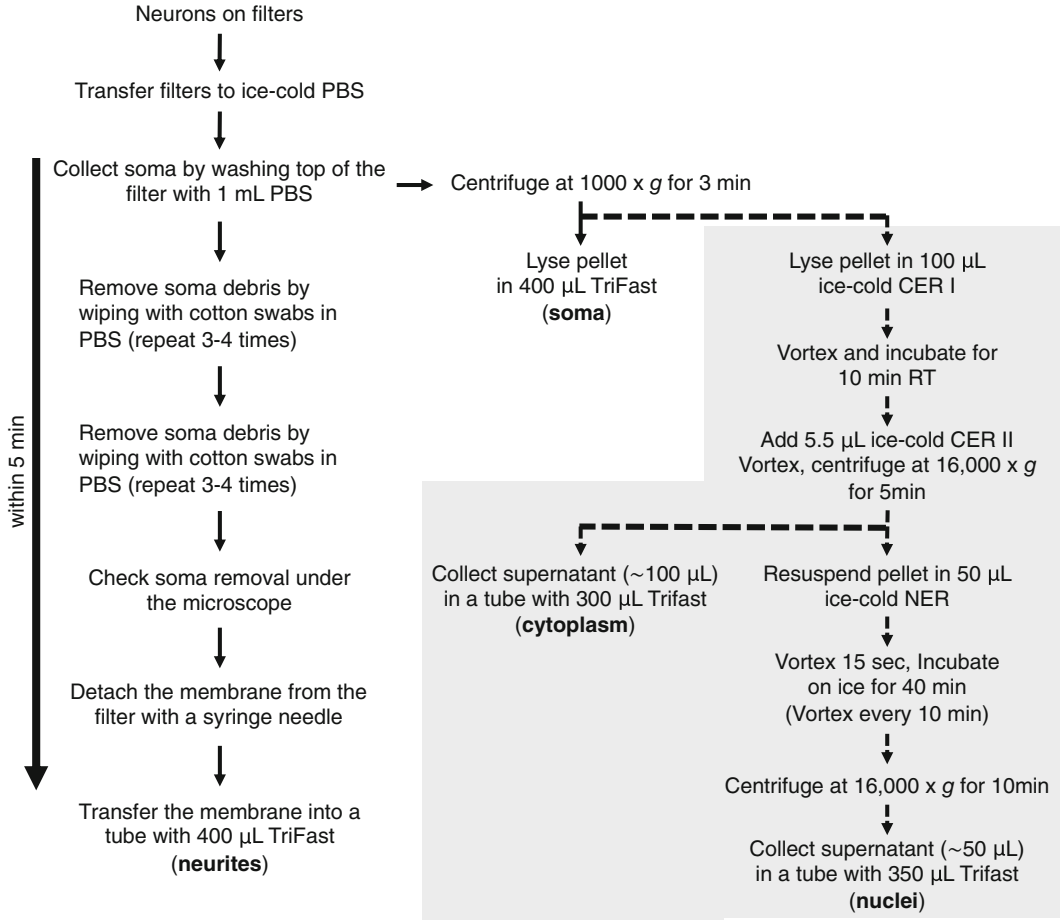

Fig. 2 Workflow for subcellular compartment separation of mESC-derived neurons. The pathways shaded in gray are for three-compartment separation. c.f. centrifuge

wipe most of the filer and small cotton swab near the perimeter where filter is glued to plastic ring.

8. Verify efficient soma removal under a cell culture microscope. Pierce the membrane at a perimeter and tear the membrane along the plastic ring using a syringe needle and place it with forceps into a tube congaing 400 µL TriFast. Vortex the tube to lyse the neurite.
9. At each time point (Δt), repeat the separation procedure. Δt can be extended up to 24 h. Safe stopping point: the samples can be stored at −80 °C.

3.5.2 Option 2: Three-Compartment Separation

1. Alternatively, mESC-derived neurons can be separated into nucleic, cytoplasmic, and neurite compartments. Follow the instructions for two-compartment separation (Subheading 3.5.1) until centrifugation of soma. Follow the instructions of

NE-PER Nuclear and Cytoplasmic Extraction Kit (NE-PER kit) for protein samples, but perform the final sample lysis using TriFast.

2. Keep the buffers from NE-PER kits on ice. Prepare new 1.5 mL tubes equal to the number of samples in a metal rack on ice. Add 300 µL of TriFast to the tubes for cytoplasmic samples and 350 µL of TriFast to the tubes for nuclear samples.
3. After the centrifugation of soma, remove the supernatant and resuspend the pellet in 100 µL of ice-cold CER I (from NE-PER kit). The expected volume of the pellet from a filter is about 10 µL (*see* **Note 10**).
4. Vortex the tube vigorously for 15 s and incubate for 10 min at RT.
5. Add 5.5 µL of ice-cold CER II (from NE-PER kit) to the tube, vortex vigorously for 5 s and centrifuge for 5 min at maximum speed (~16,000× *g*).
6. Immediately transfer the supernatant (~100 µL, cytoplasmic extract) to a new pre-cooled 1.5 mL tube containing 300 µL of TriFast. Mix well by vortexing. Keep the tube on ice until RNA extraction or storage.
7. Resuspend the pellet (nuclei) in 50 µL of ice-cold NER (from NE-PER kit).
8. Vortex vigorously for 15 s and incubate on ice for 40 min, with vortexing every 10 min. To save time, separation on a new filter can be performed during 10 min intervals of incubation.
9. Centrifuge the tube at maximum speed (~16,000× *g*) for 10 min. Immediately transfer the supernatant (nuclear extract) to a new 1.5 mL tube containing 350 µL of TriFast. Place on ice. Safe stopping point: samples can be stored at −80 °C.

3.6 RNA Extraction

Perform all the steps involving phenol and chloroform under the fume hood. Follow the instruction manual of SLAMseq kit and TriFast or another phenol-based reagent you use. For the scheme of the workflow, *see* Fig. 3a. Important: RNA extraction must be carried out in the darkness or protected from exposure to light, using a cover or wrapping the tubes with tin foil. Additionally, ensure that samples treated with S4U are kept under reducing conditions using Reducing Agent (RA, from SLAMseq kit). RA must be added at 1/1000th of the aqueous volume in isolation and wash buffers, and at 1/100th of the volume in elution buffer.

1. After lysing the material with TriFast reagent (Subheading 3.5), incubate the samples for 5 min at RT to ensure complete dissociation of the RNP complexes. If the samples were frozen, thaw the lysate and then incubate for 5 min at RT.

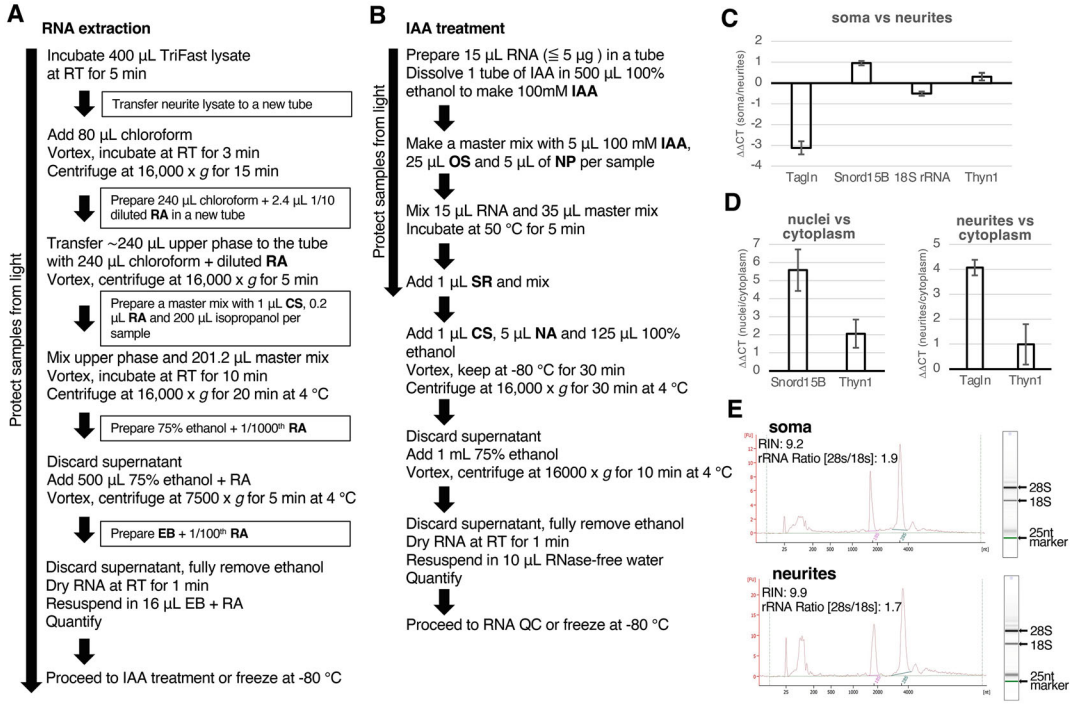

Fig. 3 RNA extraction and Iodoacteamide (IAA) treatment. (**a**) Workflow of RNA extraction protocol. The reagents from SLAMseq kit are in bold letters and abbreviated. RA: Reducing Agent, CS: Carrier Substrate, EB: Elution Buffer. (**b**) Workflow of IAA treatment protocol. The reagents from SLAMseq kit are in bold letters and abbreviated. OS: Organic Solvent, NP: Sodium Phosphate, SR: Stopping Reagent, NA: Sodium Acetate. (**c**) Representative qRT-PCR results to validate two-compartment separation and (**d**) three-compartment separation. The results show enrichment of Tagln in neurites, enrichment of Snord15B in soma and nuclei, equal distribution of 18S rRNA and Thyn1 between soma and neurites, and equal distribution of Thyn1 between cytoplasm, nuclei, and neurites. Bar: mean; error bar: SD, $n = 3$ soma/neurite, cytoplasm/neurite and cytoplasm/nuclei pairs, normalized to Gapdh. (**e**) Examples of expected Bioanalyzer profile of somatic and neuritic RNA. RIN: RNA Integrity Number

2. Transfer the TriFast lysate of neurite samples to fresh 1.5 mL tubes to remove the filters. For transfer, use a P200 pipette to extract as much as possible, squeezing the lysate out of filters.
3. Add 80 µL of chloroform (1/5th volume of the initial TriFast lysate, *see* **Note 11**) to each 400 µL of the lysate from soma and neurite, vortex vigorously, and incubate for 3 min at RT.
4. Centrifuge the tube at 15 min at 16,000× *g* to separate the material into three phases: lower red phase (phenol-chloroform), white interphase,.and the upper aqueous phase, which contains RNA.
5. During centrifugation, prepare a 1:10 dilution of RA and pipette 2.4 µL into fresh 1.5 mL tubes (as many as the number of the samples).

6. Transfer the upper phase into the 1.5 mL tube containing diluted RA. The volume of this phase is about 240 μL (60% of the volume of the initial TriFast lysate).
7. To eliminate any traces of phenol, add 240 μL of chloroform (equivalent to the volume of the transferred upper phase) to the tube. Vortex and centrifuge the tube at 5 min at 16,000× *g*. Collect the upper phase (about 200–230 μL) into a fresh 1.5 mL tube (*see* **Note 12**).
8. To precipitate RNA, prepare a mastermix containing 1 μL of Carrier Substance (CS from the SLAMseq kit), 0.2 μL of Reducing Agent (RA from the SLAMseq kit, ~1/1000th of the upper phase volume), and 200 μL of isopropanol (~1:2 of the initial TriFast volume) per sample.
9. Add 201.2 μL of the master mix to the tube and vortex. Incubate the samples for 10 min at RT and centrifuge for 20 min at 16,000× *g* at 4 °C. The RNA pellet is visible at the bottom of the tube as a white precipitate.
10. During centrifugation, prepare 75% ethanol containing 1/1000th volume of RA (mix 25 mL of 75% ethanol and 25 μL of RA, for instance) (*see* **Note 13**). Keep the mixture on ice.
11. When centrifugation is done, carefully discard the supernatant using a P1000 pipette. Wash the RNA pellet with 500 μL of 75% ethanol containing RA. Vortex well and centrifuge the tube for 5 min at 7500× *g* at 4 °C. Discard the supernatant.
12. Quickly spin the tube again to collect the remaining ethanol at the bottom of the tube.
13. To dry the pellet, remove the ethanol thoroughly with a P10 pipette and leave the tube open for about 1 min until the edge of the pellet becomes transparent. Do not let the pellet dry completely as it will be difficult to dissolve the RNA.
14. Mix Elution Buffer (EB, from the SLAMseq kit) and 1/100th volume of RA (mix 800 μL of EB and 8 μL of RA, for instance). Resuspend the pellet in 16 μL of EB containing RA (*see* **Note 14**).
15. To quantify the amount of RNA isolated, measure the concentration using a Qubit analyzer and a Qubit RNA HS Assay Kit following the manufacturer's instructions. Expected total RNA yield from one 6-well filter is 0.3–1 μg for neurites and 3–10 μg for soma.
16. The sample can also be analyzed by a spectrophotometer (NanoDrop, for instance) to check the purity of the sample. Ensure that there is no phenol or protein contamination as it can affect NGS library preparation (*see* **Note 15**). Safe stopping point: RNA can be stored at −80 °C (protect from exposure to light).

3.7 Iodoacetamide Treatment

In this section, the 4-thiol groups on S4U-labeled transcripts are alkylated by Iodoacetamide. For the scheme of the workflow, *see* Fig. 3b. In the subsequent NGS preparation, this results in the incorporation of Guanine (G) instead of Adenine (A) at the alkylated S4U nucleotide positions. Follow the instruction manual of SLAMseq kit. Important: the initial steps must be carried out in the dark or protected from exposure to light, using a cover or wrapping the tubes with tin foil.

1. If RNA samples were previously frozen, defrost it avoiding exposure to light. Keep the RNA samples cold to ensure the integrity.
2. Transfer 15 μL of each RNA sample to new 1.5 mL tube. If the concentration is higher than 0.33 μg/μL, dilute the sample with RNAse-free water (H_2O, from SLAMseq kit) to ensure that the total RNA amount within 15 μL does not exceed 5 μg.
3. Dissolve 1 tube of Iodoacteamide (IAA, from the kit) in 500 μL of 100% ethanol (100 mM final concentration). Important: use only freshly prepared IAA, do not reuse it after dissolving. Treat all samples in parallel.
4. Prepare a mastermix containing 5 μL of the freshly prepared 100 mM IAA, 25 μL of Organic Solvent (OS, from SLAMseq kit), and 5 μL of Sodium Phosphate (NP, from SLAMseq kit) per sample. Important**:** NP can form salt aggregates when mixed with OS. Prepare a slightly larger mastermix and use only the supernatant for the reaction (*see* **Note 16**).
5. Add 35 μL of the IAA/OS/NP mastermix to the tube containing 15 μL of RNA and incubate at 50 °C for 15 min.
6. Stop the reaction by adding 1 μL of Stopping Reagent (SR, from SLAMseq kit) and mix well. After this step, samples can be exposed to light.
7. Add 1 μL of Carrier Substance (CS, from SLAMseq kit), 5 μL of Sodium Acetate (NA, from SLAMseq kit), and 125 μL of 100% ethanol. Vortex and keep at −80 °C for 30 min for precipitation. Centrifuge at 16,000× *g* for 30 min at 4 °C.
8. Remove the supernatant, wash the pellet with 1 mL of 75% ethanol, and vortex. Centrifuge at 16,000× *g* for 10 min at 4 °C.
9. Discard the ethanol and quickly spin the tube again to collect the remaining ethanol on the bottom of the tube.
10. Use a P10 pipette to remove ethanol completely and leave the tube open for about 5 min to dry the RNA pellet. Do not let the pellet overdry.
11. Resuspend in 10 μL of RNase-free water (*see* **Note 17**).

12. To quantify the amount of RNA recovered, measure the concentration using a Qubit analyzer and a Qubit RNA HS Assay Kit following the manufacturer's instructions. Do not use more than 1 μL for the measurement to save the RNA for the quality control and library preparation. Expected total RNA amount from 200–5000 ng of input RNA is 200–700 ng for both soma and neurite samples. Safe stopping point: RNA can be stored at −80 °C.

3.8 RNA Quality Control

To validate the efficiency of neurites and soma separation, perform RT-qPCR on RNA samples (*see* **Note 18**). Selected neuritic and somatic markers (neuritic and nucleic if three-compartment separation is performed) and loading controls are provided in Table 1. We use the Maxima first-strand cDNA synthesis kit (Maxima kit) for cDNA generation and the sensiFAST SYBR No ROX qPCR kit (sensiFAST kit) for qPCR reaction. Instructions for RT-qPCR using these kits are described below. However, other analogous reagents can also be used.

1. For RT-qPCR, include technical duplicate and biological triplicates in the analysis.
2. Prepare 40–50 ng of total RNA per sample in a PCR tube and add water to make a total volume of 4 μL.
3. Add 0.5 μL of 10× reaction buffer (supplied with DNase I) and 0.5 μL of DNase I to RNA, mix well with tapping. Incubate for 30 min at 37 °C in a thermal cycler.
4. Stop the reaction by adding 0.5 μL of 50 mM EDTA (supplied with DNase I) and incubating for 10 min at 65 °C.
5. To the 5.5 μL of DNase-treated RNA mixture from the above reaction, add 4 μL of 5× Reaction Mix (from Maxima kit), 2 μL of Maxima Enzyme Mix (from Maxima kit), 8.5 μL of RNase-free water.
6. Incubate on a thermal cycler for 10 min at 25 °C, then for 15 min at 50 °C.
7. Stop the reaction by incubating at 85 °C for 5 min.
8. Dilute the reaction mixture by adding 40–80 μL of RNase-free water, depending on the required number of reactions in qPCR. The diluted cDNA samples can be stored at −20 °C for up to a week or at −80 °C for longer storage.
9. For qPCR reaction, prepare a 20 μL reaction mixture consisting of: 10 μL of 2× SYBR, 0.8 μL of 10 μM forward primer, 0.8 μL of 10 μM reverse primer, 5 μL of diluted cDNA and 3.4 μL of water.

10. Run the qPCR reaction with the following settings in a qPCR instrument: 95 °C for 2 min; 40 cycles of 95 °C for 5 s, 63 °C for 10 s, and 72 °C for 20 s; hold at 4 °C.
11. *See* Table 1 for the suggested primers. Tagln is enriched in neurites, Snord15b is enriched in soma, and Gapdh, Thyn1, and 18S rRNA are equally distributed between neurites and soma (Fig. 3c). If three-compartment separation was conducted, check the enrichment of Snord15b in nuclei and the enrichment of Tagln in neurites compared to in cytoplasm (Fig. 3d).
12. To determine $\Delta\Delta C_T$ for comparing soma and neurite, begin by computing the mean threshold cycle (CT) for each target by averaging over technical replicates. Subsequently, calculate ΔC_T for a specific target in each compartment ($\Delta C_T^{\text{neurites}}$; ΔC_T^{soma}) by normalizing to a reference transcript:

$$\Delta C_T^{\text{neurites}} = C_{\text{target}}^{\text{neurites}} - C_{\text{reference}}^{\text{neurites.}}$$
$$\Delta C_T^{\text{soma}} = C_{\text{target}}^{\text{soma}} - C_{\text{reference}}^{\text{soma}}$$

We suggest using rRNA, Gapdh, or Thyn1 for normalization (reference transcript). Then compute $\Delta\Delta C_T^{\text{soma/neurites}}$:

$$\Delta\Delta C_T^{\text{soma/neurites}} = \Delta C_T^{\text{neurites}} - \Delta C_T^{\text{soma}}$$

Calculate $\Delta\Delta C_T^{\text{nuclei/cytoplasm}}$ and $\Delta\Delta C_T^{\text{cytoplasm/neurites}}$ using the same method to validate three-compartment separation.

13. Analyze the RNA integrity using Agilent Bioanalyzer or an equivalent automated electrophoresis system. If using Bioanalyzer, choose the Bioanalyzer chip based on the Qubit measurement from Subheading 3.7, **step 12**. For a Nano Chip, RNA concentration should be within 25–500 ng/μL; for a Pico Chip, RNA concentration should be 50–250 pg/μL. Follow Bioanalyzer manufacturer's instructions to assess the integrity of the RNA. Expected Bioanalyzer profile for RNA from each compartment is provided in Fig. 3e.
14. Use only intact RNA with distinct 18 and 28S peaks (28S:18S rRNA ratios ~2) for library preparation. RNA integrity number (RIN) should be above 7. Neurite samples can have a high peak around 20–150 nt. This is mainly from small RNA species (unpublished data) and does not affect the library preparation.

3.9 NGS Library Preparation

1. Prepare NGS library using the samples obtained in Subheading 3.7, following manufacturer's instructions. We recommend using Lexogen QuantSeq 3'mRNA-Seq Library Prep Kit for Illumina (REV) with Custom Sequencing Primer (QuantSeq kit) for fast and quantitative library preparation, but it is also possible to use other library preparation kit (from Illumina, for example).

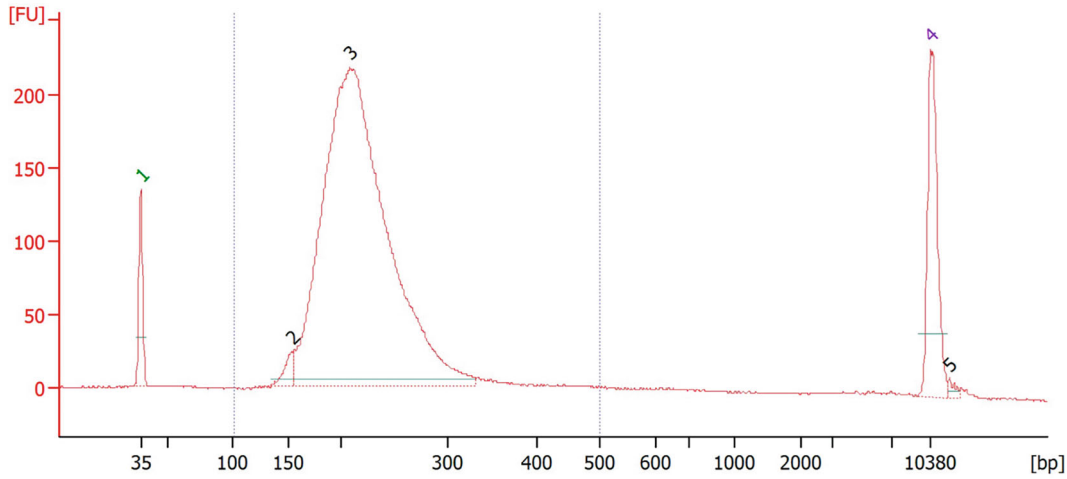

Fig. 4 Representative BioAnalyzer profiles of a spatial SLAMseq library prepared using QuantSeq kit

2. After the library preparation, to quantify the library, measure the concentration using a Qubit analyzer and a Qubit dsDNA HS Assay kit. If using QuantSeq kit, expected library concentration is 20–50 ng/μL (the expected total amount of libraries is 300–850 ng) for both soma and neurite samples.
3. Analyze the libraries using a Bioanalyzer or an equivalent analyzer to assess their profiles in gel electrophoresis. The libraries should have a peak between 200–300 bp (*see* Fig. 4). If you see a shoulder or a secondary peak in the longer range, the libraries are overamplified and the expression levels cannot be accurately calculated during data analysis. In such case, regeneration of the NGS libraries is recommended.

3.10 Sequencing

1. Run the libraries in NextSeq 500/550 or an equivalent sequencer (CSP Version 5 is optimized to be used on NextSeq 500/550).
2. Specific instructions for running samples prepared by using QuantSeq kit in NextSeq 500 are explained below.
3. Prepare the reagent cartridge and flow cell according to the manufacturer's instructions (*see* **Note 19**).
4. Denature and dilute the library following the instructions from NextSeq 500 and 550 Sequencing Systems Denature and Dilute Libraries Guide (Illumina). For a high output sequencing (recommended), dilute the library to the final concentration of 1.8 pM in 1.3 mL. For mid-output sequencing, dilute the library to 1.5 pM in 1.3 mL.
5. Library prepared with QuantSeq kit is not compatible with PhiX control because they require different Read1 primers. It

is also not recommended to multiplex QuantSeq library with libraries prepared using other kits.

6. Dilute the Custom Sequencing Primer (CSP from QuantSeq kit). Spin down the tube of CSP and add 6 μL of CSP (100 μM) to 1994 μL of HT1 buffer (final volume 2000 μL). Mix well and spin down.
7. At the sequencer, load the 1.3 mL of 1.8 pM libraries onto #10, and load the 2 mL of 0.3 μM CSP onto #7 (for a Custom Read1 Primer).
8. If using Local Run Manager run mode, specify custom primers on the "Create Run" page of the Local Run Manager software. If using manual run mode, select the checkbox for Custom Read1 Primer on the NCS Run Setup screen. During the run, the software directs the sippers to extract primers from the designated reservoir.
9. Regarding the sequencing format, Lexogen suggests single-read 100–150 bp (SR100–150) for the library prepared using SLAMseq kit and QuantSeq kit. The cycle number can be set lower as QuantSeq library is optimized for shorter reads (SR75–100).

3.11 Computational Analysis

For data analysis methods and expected results, please consult our publication where we performed spatial SLAMseq on ASCL1-mESC-derived and primary cortical neurons [4]. The pipeline utilized in the publication is available at https://doi.org/10.5281/zenodo.8042212.

4 Notes

1. While this protocol is designed specifically for special SLAMseq using ASCL1-mESC cells, it has the potential to be applied to primary neurons as well. Our experiment validated S4U incorporation in murine primary cortical neurons [4]. We recommend using hanging cell culture inserts with 1 μm pores for primary neurons.
2. Other automated electrophoresis systems such as TapeStation Systems (Agilent) or Fragment Analyzer Systems (Agilent) can be used.
3. Earlier versions of QuantSeq kits were supplied with a different custom sequencing primer (CSP) version (Version 2, predating 2019), which may not be compatible with all Illumina instruments. Verify the CSP version number indicated on the provided tube before initiating the sequencing process.

4. HT1 Hybridization Buffer is a typical component of sequencing kits from Illumina. It can be purchased separately if necessary.
5. An additional filter can be prepared for no-S4U control (*see* **Note 7**). Preparation of backup filters are recommended as accidental loss of materials can happen during compartment separation. The most common example of loss occurs during the breakage of the filter membrane while removing soma from the filter using cotton swabs.
6. Optionally, additional filters can be prepared to validate compartment separation through western blotting. Expected yield from a filter is ~30 μg of neuritic and ~375 μg of somatic protein (~250 μg cytoplasmic and ~50 μg nucleic protein). For two-compartment separation (Subheading 3.5.1), lyse each soma and neurite samples from a filter in 400 μL of Urea buffer (8 M Urea, 0.1 M Tris-HCl pH 7.5) instead of TriFast. Sonicate the lysate with Bioruptor using the following settings: 15 s ON, 45 s OFF, high, 4 cycles. Centrifuge at 14,000× *g* for 3 min at 4 °C to remove the cell debris. Transfer the supernatant into a fresh tube. The sample is ready to be used in western blotting. For three-compartment separation (Subheading 3.5.2), dissect soma pellet into cytoplasmic and nucleic fractions using NE-PER Nuclear and Cytoplasmic Extraction Kit. Follow the manufacturer's instructions. We suggest checking enrichment of Histone H3 in soma/nuclei, Beta-3 Tubulin in cytoplasm and Neurofilament in neurites.
7. Optionally, the protocol can be shortened by 2 days, by generating EBs in AK supplemented with doxycycline on day 1 and plating them on filters on day 3 (one 10 cm dish of the EBs per one filter) [3, 7]. In this case, coat the filters with matrigel either in the evening of day 2 or in the morning of day 3.
8. The concentration of S4U is adjusted for the protocol. A control filter without 4SU treatment (use water instead) can be prepared to assess cell viability during S4U labeling.
9. When working with RNA samples, to minimize RNA degradation, use RNase- free reagents and plastic, filter tips. Wear clean gloves and keep RNA on ice, unless specified otherwise. Work fast during the compartment separation to obtain intact RNA. After isolation, aliquot RNA to avoid multiple freezing-defrosting cycles, and store the aliquots at −80 °C.
10. Adjust the buffer volumes if the volume of the pellet differs significantly. For 10 μL of pellet, 100 μL of CERI, 5.5 μL of CERII, and 50 μL of NER should be used.
11. Chloroform:isoamyl alcohol mix (24:1) can also be used instead of chloroform.

12. The second chloroform extraction step (Subheading 3.6, **step 7**) is optional, but it markedly decreases the chance of phenol contamination in isolated RNA. Although it is possible to eliminate phenol contamination after the extraction (*see* **Note 15**), we recommend the second chloroform extraction for convenience.
13. Prepare 75% ethanol freshly each time, as ethanol is highly volatile.
14. Although the SLAMseq kit manual suggests incubation at 55 °C for 10 min to dissolve RNA, we don't recommend it due to the increased risk of RNA degradation.
15. Phenol contamination is observed as a peak with absorbance maximum at 270 nm, while protein contamination is observed as a peak at 280 nm on a spectrophotometer. To remove a considerable contamination, another chloroform extraction can be performed. To do this, dilute the RNA to 100–200 μL and add an equal volume of chloroform. Vortex and centrifuge the tube for 5 min at 16,000× *g* and transfer the upper phase to a new tube. Perform the RNA precipitation as described in **steps 8–9** of Subheading 3.6, but include 3 M sodium acetate at 1/10th volume relative to the upper phase. Continue from the RNA wash step (Subheading 3.6, **steps 10–11**).
16. The SLAMseq kit manual indicates that the presence of aggregates does not impact the reaction, although it is advisable to use only the supernatant.
17. The SLAMseq kit manual specifies the elution volume of 5–10 μL. Decrease the elution volume if necessary.
18. Optional western blotting, as described in **Note 6**, can be conducted at this point.
19. Wear protection equipment such as gloves and glasses while handling the reagent cartridge, as reagents are potentially hazardous.

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Part VIII

Methods for the Characterization of RNA Decay Targets and Intermediates

Chapter 19

Analysis of Cytoplasmic RNA Decay Targets Using the Auxin Degron System

Dominika Foretek, Marc Gabriel, and Antonin Morillon

Abstract

RNA degradation in mammalian cells is performed by multiple enzymes and cofactors making it difficult to identify the specific impact of each of them separately. The auxin-inducible degron system enables direct depletion of a protein of interest limiting the time of depletion and thus reducing secondary effects due to cell adaptation. In this chapter, using XRN1 as an example of cytoplasmic RNA decay enzyme, we describe a combination of methods to introduce the auxin-inducible degron by CRISPR-Cas9, together with downstream analyses of RNA levels after protein depletion.

Key words RNA decay, Auxin degron, CRISPR-Cas9, RNA-seq

1 Introduction

The siRNA and ASO technologies are commonly used approaches in mammalian cells for knocking down gene expression at the RNA level and as a result reducing the protein levels in the cell. However, such type of depletion requires often prolonged time of treatment, which can result in cell adaptation. Also unspecific side effects and/or toxicity have to be considered during experimental design [1]. An alternative approach of auxin-inducible degron (AID) system enables a direct and rapid depletion of the targeted protein. First, CRISPR/Cas9-based approach for introduction of this system in human cells was proposed by the group of Masato Kanemaki [2, 3], where an AID tag is added to the protein of interest. The AID tag is recognized by the auxiliary introduced plant auxin receptor F-box protein Tir1 that forms a part of SCF^{TIR1} ubiquitin ligase complex, which upon addition of indole-3-acetic acid (IAA, referred to here as auxin), leads to the degradation of the tagged protein. This approach can substantially shorten the time of depletion, revealing the direct effects of the loss of enzymes/cofactors on RNA levels in the cell.

Maxime Wery (ed.), *mRNA Decay: Methods and Protocols*, Methods in Molecular Biology, vol. 2863,
https://doi.org/10.1007/978-1-0716-4176-7_19,

In the cytoplasm of mammalian cells, RNA degradation is an orchestrated process in which the transcripts' 3′ end is first deadenylated by the PAN2-PAN3 and CCR4-NOT complexes, followed by 5′ cap removal prior the 5′ end digestion by the XRN1 5′-3′ exoribonuclease. The 3′-5′ decay pathway is performed by the exosome complex containing the DIS3 or DIS3L catalytic subunits, degrading incorrectly polyadenylated, unspliced, or endonucleolytically cleaved RNAs. One of the key exosome cofactors is the SKI-complex with a helicase activity, functioning mainly in co-translational surveillance-decay pathway. Moreover, in the cytoplasm, the DIS3L2 enzyme that does not associate with the exosome degrades RNAs via an oligo-uridylation-dependent pathway (reviewed in [4]). The numerous enzymes and cofactors of RNA decay pathways require an adapted approach to identify specific target RNAs and mechanisms. Here, we describe a combination of the use of auxin-degron together with a stranded total RNA-seq protocol and customized analysis of RNA-seq data to identify the specific RNA-decay targets. This chapter exemplifies the construction of a cell line expressing an AID-tagged version of the 5′-3′ exoribonuclease XRN1.

2 Materials

2.1 Cell Lines and Vectors

1. HCT116 cell lines harboring Tet-OsTIR1 (RIKEN Cell Bank).
2. HEK293T cell line.
3. pX459 vector (Addgene).
4. Optional AAVS1-Tet-OsTIR1(WT)-V5 (Addgene).
5. AID tag vectors for cloning into HDR cassette.

2.2 Reagents

1. Phusion HF polymerase.
2. 10 mM dNTP mix.
3. 10× NEBuffer 3.1: 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 μg/mL BSA, pH 7.9.
4. AflIII restriction enzyme.
5. XbaI restriction enzyme.
6. T4 DNA ligase.
7. Chemically competent *E. coli* cells (e.g. One Shot™ Stbl3™).
8. LB agar plates with 100 μg/mL ampicillin.
9. Liquid LB medium with 100 μg/mL ampicillin.
10. MspI or BbsI restriction enzyme.
11. Cell culture media supplemented with 10% fetal bovine serum (FBS).

12. 1× Dulbecco's phosphate buffered saline (DPBS).
13. Trypsin (e.g. TrypLE™ Express (1X), phenol red).
14. Cell lysis buffer: 10 mM Tris pH 8.0, 100 mM NaCl, 25 mM EDTA, 0.5% SDS.
15. Proteinase K, 20 mg/mL.
16. Pure phenol, pH 4.3.
17. Phenol:chloroform:iso-amyl alcohol (25:24:1) solution (pH 4.3–4.7).
18. Chloroform.
19. 3 M sodium acetate, pH 5.2.
20. 100% and 70% ethanol.
21. Transfection kit.
22. 10× NEBuffer 2: 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9.
23. T7 Endonuclease I (NEB).
24. Kit for the purification of DNA from agarose gel (e.g. QIAquick gel extraction kit).
25. Kit for PCR product purification (e.g. QIAquick PCR purification kit).
26. Auxin—IAA (indole-3-acetic acid), 500 mM stock.
27. Doxycycline, 10 mg/mL.
28. RNA extraction kit (e.g. miRNeasy Micro Kit).
29. RIPA lysis buffer: 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS.
30. Protease inhibitor (e.g. Halt™ Protease Inhibitor Cocktail, 100X).
31. TruSeq Stranded Total RNA kit (Illumina).
32. DNase I.

2.3 Consumables

1. 6-well cell culture plate.
2. 100 mm cell culture dish.
3. 24-well cell culture plate.
4. Cell scrappers.
5. Safe-lock 1.5 mL and 2 mL nuclease-free tubes.
6. Nuclease-free PCR tubes.
7. Aspiration pipettes.
8. Sterile pipettes.

2.4 Equipment

1. Heating block for 1.5 mL tubes.
2. PCR thermocycler with programmable temperature ramp rate.
3. UV spectrophotometer or fluorometer.
4. Agilent 2100 Bioanalyzer or 4200 TapeStation system.
5. Agarose gel electrophoresis system.
6. Acrylamide gel electrophoresis system.
7. Agarose gel analysis system.
8. Vacuum concentrator.
9. Protein transfer system.
10. Acquisition system for western blot analysis.
11. Chemical hood.
12. Cell culture hood.
13. Microscope with fluorescence adapted to cell culture dishes.
14. Vortex.
15. Microcentrifuge.
16. Tabletop centrifuge.
17. Pipettes.
18. Pipetboy.
19. Sonicator.

2.5 Computer and Software

1. Benchling (https://www.benchling.com/crispr).
2. TrimGalore (https://github.com/FelixKrueger/TrimGalore).
3. STAR 2.6.1a [5].
4. Scallop [6] (https://github.com/Kingsford-Group/scallop).
5. Cufflinks [7] (https://github.com/cole-trapnell-lab/cufflinks).
6. BEDTools [8].
7. featureCounts [9].
8. Subread package (https://bioconductor.org/packages/release/bioc/html/Rsubread.html).
9. DESeq2 [10].
10. Integrative Genome Browser (https://www.igv.org/).
11. A computer with at least 30 GB of RAM and a Unix like system or access to a cluster.

3 Methods

3.1 Creation of an Auxin-Degron Cell Line

3.1.1 Guide RNA Design

To be able to introduce the tag into the genomic DNA, a cut has to be introduced in the specific location. Thus, the first step in the process is the design and selection of sgRNAs that will guide the Cas9 enzyme into the selected site (*see* **Note 1** and Fig. 1a).

1. Select your DNA sequence of interest and input the region into the Benchling software (or the software of your choice).

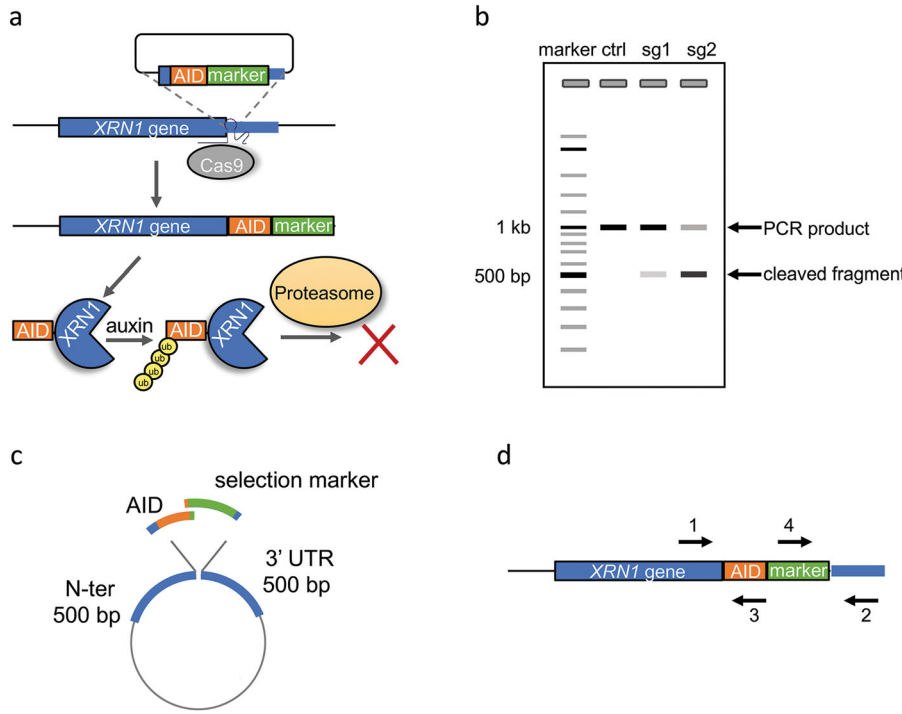

Fig. 1 Design and validation of Cas9-based insertion of the auxin-inducible degron. (**a**) Schematic representation of the experimental workflow for the preparation of AID tag insertion into the genomic locus using Cas9 and a gRNA. The tagged protein is expressed in a cell line expressing the plant Tir1 receptor that forms a complex with the endogenous ubiquitin ligase cofactors [3]. Upon addition of auxin to the media, Tir1 recognizes the AID tag and leads to the ubiquitylation of the AID tagged protein and its degradation by the proteasome. (**b**) Schematic representation of agarose gel readout of the T7- endo assay: in the control (ctrl) lane, no cleavage and only the full-length PCR product is observed; in the lanes with sgRNA 1 and 2 showing low and high cleavage efficiency, respectively, the proportion between the full-length amplicon and cleaved fragment changes. (**c**) Schematic representation of the design of the fragments for Gibson assembly. The circle represents a vector with synthetic DNA inserts corresponding to the 500 nt regions flanking the insertion site. The two lines above represent the two PCR fragments containing the selected tag (in orange) and marker (in green). Each PCR fragment also contains an overlapping sequence (respectively in blue, orange, and green) for the enzyme to be able to assemble the construct in the correct orientation. (**d**) A scheme presenting the primer pairs that should be used for testing the insertion of the HDR donor DNA into the genomic region. Primers 1–2 amplify the full region. Primers 1–3 or 2–4 amplify a part of the insert and the XRN1 flanking regions

Table 1
Oligonucleotides for PCR cloning of sgRNA

Name	Sequence
Fw1	5′ TTACGGTTCCTGGCCTTTTG 3′
Rev1[a]	5′ AAAAC**TCTGAGTAAATTTGGCTCTT**CGGTGTTTCGTCCTTTCCACAA 3′
Fw2[a]	5′ACACCG**AAGAGCCAAATTTACTCAGA**GTTTTAGAGCTAGAAATAGCAAGT TAAAAT 3′
Rev2	5′ GGGCCATTTACCGTAAGTTATGT 3′

[a]Bold underlined: sequence of a specific gRNA used for XRN1 tagging
Fw1 and Rev2 amplify the pX459 regions containing the AflIII site and the XbaI site, respectively

2. Among the proposed guides, select the sgRNA with the highest target score and the lowest off-target score (preferably with no off-target in protein coding genes), in a distance of up to 20 nt from the site where the mutation should be introduced (*see* **Note 2**).
3. To clone the gRNA into the vector, design oligonucleotides for sequential PCR amplification around the AflIII and XbaI restriction sites. Primers are listed in Table 1.
4. Prepare a 50 μL reaction mix: 10 μL of 5× HF buffer, 1 μL of 10 mM dNTP mix, 1 μL of Phusion HF polymerase (2 U/μL), 50 ng of pX459, 0.25 μL of forward primer (100 μM), 0.25 μL of reverse primer (100 μM), and H_2O up to 50 μL.
5. Perform a PCR using the primer pairs specific for each fragment. For the amplification of the PCR fragment 1 (314 bp), use primers Fw1 and Rev1, with 5 s of elongation. For the amplification of the PCR fragment 2 primers (224 bp), use primers Fw2 and Rev2, with 4 s of elongation.
6. Run the following program (adjusting the time of elongation depending on the product size): 30 s at 98 °C; 40 cycles of 10 s at 98 °C, 30 s at 55 °C, 4 or 5 s at 72 °C; final elongation of 5 min at 72 °C; hold at 10 °C.
7. Load the PCR products on a 0.8% agarose gel and purify the amplified fragments (*see* **Notes 3** and **4**).
8. Prepare a second PCR mix with both purified fragments: 10 μL of 5× HF buffer, 1 μL of 10 mM dNTP mix, 1 μL of Phusion HF polymerase (2 U/μL), 50 ng of fragment 1, 50 ng of fragment 2, 0.25 μL of Fw 1 primer (100 μM), 0.25 μL of Rev. 2 primer (100 μM), and H_2O up to 50 μL.
9. Run the following program: 30 s at 98 °C; 40 cycles of 10 s at 98 °C, 30 s at 55 °C, 8 s at 72 °C; final elongation of 5 min at 72 °C; hold at 10 °C.

10. Run the PCR product on a 0.8% agarose gel. The size of the amplicon should be 508 bp.
11. Purify the amplicon from the agarose gel and quantify the DNA using a UV spectrophotometer (*see* **Note 3**).
12. Digest 1 μg of pX459 vector and 500 ng of purified PCR product with 2 μL of 10× NEBuffer 3.1, 1 μL of AflIII enzyme, 1 μL of XbaI enzyme, and H_2O up to 20 μL.
13. Incubate for at least 2 h (up to overnight) at 37 °C.
14. Load the digested products on a 0.8% agarose gel.
15. Purify the digested vector and insert from the agarose gel (*see* **Note 3**).
16. Quantify the purified vector and insert using a UV spectrophotometer.
17. Prepare a ligation reaction by mixing 100 ng of digested pX459 vector, 20 ng of digested insert, 2 μL of 10× T4 ligase buffer, 1 μL of T4 DNA ligase and H_2O up to 20 μL.
18. Incubate the reaction overnight at 16 °C.
19. Transform chemically competent bacteria with the ligation product and seed on LB plates with ampicillin (100 μg/mL).
20. Incubate the plates overnight at 37 °C.
21. Inoculate liquid LB medium (with 100 μg/mL ampicillin) using single colonies and grow overnight in air shaker at 37 °C.
22. Purify the plasmid DNA using the kit of your choice.
23. Screen the clones by digestion of the plasmid DNA with MspI or BbsI. The positive clones should lose the MspI and BbsI restriction sites. Include an empty pX459 vector as a positive control.
24. Check the sequence of the insert by Sanger sequencing.

3.1.2 Testing Guides Efficiency

The homology-directed repair can have a different efficiency depending on the cell line. Therefore, before performing the time-consuming experiment of growing and selecting single clones, it is important to first select the sgRNAs that give the highest efficiency for cutting the DNA. The assay described in this section is based on the DNA reannealing after the PCR reaction from the region around the site of Cas9 cleavage. When denatured DNA reanneals with a fragment that is mutated, it creates structural deformities that can be detected and cut by the T7 endonuclease, creating shorter fragments, that can be detected on agarose gel.

1. Seed 600×10^3 exponentially growing HEK293T cells per well of a 6-well plate (*see* **Note 5**).

2. The next day, transfect transiently the cells in a well with 1 μg of pX459 plasmid with your gRNA insert. Include one well with no gRNA for negative control (*see* **Note 6**).
3. Extract the genomic DNA from whole cell population 2 days after transfection.
4. Remove the cell culture media from the plate and wash once with 1× DPBS.
5. Remove the residual liquid and add 1 mL of DPBS and scrape the cells with a scrapper.
6. Transfer the cell suspension into a 2 mL tube.
7. Repeat **steps 5** and **6** to collect any residual cells.
8. Centrifuge for 3 min at 2000 rpm in a tabletop centrifuge to pellet the cells at room temperature.
9. Remove the supernatant and then add 300 μL of cell lysis buffer and 1.5 μL of proteinase K (20 mg/mL). Incubate overnight at 55 °C (it is possible to freeze the cells and store them at −20 °C after this step).
10. Add an equal volume (300 μL) of pure phenol.
11. Mix by inverting the tube for 15 s.
12. Spin for 10 min at maximal speed and at room temperature in a tabletop centrifuge.
13. Transfer the aqueous phase into a new 1.5 mL tube.
14. Add an equal volume (300 μL) of phenol:chloroform:isoamyl alcohol.
15. Mix by inverting the tube for 15 s.
16. Spin for 10 min at maximal speed and at room temperature in a tabletop centrifuge.
17. Transfer the aqueous phase into a new 1.5 mL tube.
18. Add an equal volume (300 μL) of chloroform.
19. Mix by inverting the tube for 15 s.
20. Spin for 10 min at maximal speed and at room temperature in a tabletop centrifuge.
21. Transfer the aqueous phase into a new 1.5 mL tube.
22. Add 1/10 volume (30 μL) of 3 M NaAc pH 5.2.
23. Precipitate the DNA with 2–2.5 volumes (700 μL) of ice-cold 100% ethanol.
24. Pellet the DNA by centrifugation for 10 min at maximum speed at room temperature in a tabletop centrifuge (*see* **Note 7**).
25. Discard the supernatant and wash the pellet with 700 μL of ice-cold 70% ethanol.

26. Centrifuge as above and remove the residual ethanol.
27. Dry the DNA for 10 min in a vacuum concentrator (no heating required).
28. Resuspend the DNA in nuclease-free water (the volume depends on the size of the DNA pellet).
29. Allow the DNA to dissolve overnight (up to few days) at 4 °C.
30. Measure the concentration of the extracted DNA using a UV spectrophotometer (or a fluorometer) and then store at −20 °C.
31. Design PCR primers flanking the region where the cut has been introduced (*see* **Note 8**).
32. Perform a PCR reaction using the conditions optimized for your PCR primers and amplicon size (*see* **Note 9**).
33. Fill a 1.5 mL tube with water, and then preheat this tube at 95 °C in a heating block. Open the 1.5 mL tube and place your PCR tube in it so that it is in contact with the water. Incubate the PCR reaction (no purification needed) at 95 °C for 10 min.
34. Remove the block and let it slowly cool down to room temperature. This is the most critical step. The reannealing needs to be very slow, more than 1 h (*see* **Note 10**).
35. Prepare the following mix: 8.5 μL of reannealed PCR product, 1 μL of 10× NEBuffer 2, 0.5 μL of T7 Endonuclease I.
36. Incubate for 30 min at 37 °C.
37. Add 2 μL of 6× loading dye (preferably orange to not obscure the bands) and load in a 2% agarose gel (leave on ice until you load on gel) (*see* **Note 11**).
38. Select the guides that show the highest efficiency of cutting based on the relative proportion of the upper and lower bands (*see* schematic representation in Fig. 1b).

3.1.3 Creation of the Donor DNA for Homology-Directed Repair

To introduce the auxin-inducible degron tag into the protein of interest, you need to design a donor template DNA that will be introduced by the homology-directed repair (HDR) mechanism into the Cas9 cleavage site by the cell DNA repair machinery.

1. Select the most appropriate tag for your application. The Kanemaki group provides a vast set of vectors for C- or N- terminal tagging [2, 11] (*see* **Note 12**).
2. Order a synthetic gene flanking 500 nt of each side where the insertion will be introduced. A silent mutation in the PAM sequence of the guide should be introduced in the synthetic gene to make sure that Cas9 does not cut the HDR donor DNA template.

3. Perform Gibson cloning [12] or PCR amplification and classical cloning to introduce the degron and the selection marker into your HDR donor vector (*see* **Note 13** and Fig. 1c).
4. Check the sequence of your vector by Sanger sequencing.
5. Linearize the vector by enzymatic digestion and purify it after migration on agarose gel to prepare the HDR donor template that will be used for cell line transfection (*see* **Note 14**).

3.1.4 Creation of the Cell Line with Tagged Protein

1. If working with the HCT116 cell line, we recommend seeding 250×10^3 cells per well of a 6-well plate of exponentially growing cells (in McCoy 5A media with 10% FBS) the day before transfection.
2. Transfect the cells with 1 μg of the all-in-one plasmid (containing Cas9 and gRNA) and 1 μg of HDR donor template/templates (*see* **Note 15**), with conditions optimized to your cell line.
3. Change the media after 24 h.
4. After 48 h, pass the cells into 100 mm dishes (*see* **Note 16**) in media containing the drug specific to the selection marker used in the HDR template (*see* **Note 17**). Resistant colonies should appear within 7–10 days.
5. Make a mark around the colonies under a microscope.
6. Transfer colonies into 24-well plates using sterile tips (*see* **Note 18**).
7. Split the cells depending on their confluence. Prepare replicates of wells for each clone for DNA and protein extraction.
8. Design a set of primers to amplify the whole region of the insertion and primers inside the insert. Using series of specific PCRs, evaluate the correct genomic insertion of AID tag and if it is homozygous or heterozygous (*see* **Note 19** and Fig. 1d).
9. Assess the expression of the tagged protein by western blotting (*see* **Note 20**). *See* Fig. 2 for the detection of a tagged version of the cytoplasmic 5′ exoribonuclease XRN1.
10. Perform a time course experiment to determine the optimal timing for protein depletion using final concentration of 2 μg/mL doxycycline and 500 μM auxin, followed by protein extraction and western blotting.
11. Expand and freeze the positive clones expressing the tagged protein (*see* **Note 21**).

3.2 Cell Culture and RNA Extraction

1. After defreezing the AID-tagged and control cell lines, make sure to give them enough time to recover and keep them in exponential growth phase.

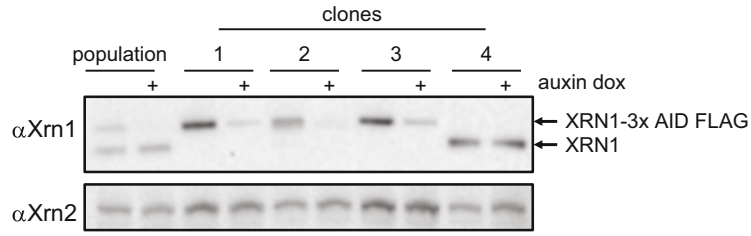

Fig. 2 Example of western blot for testing XRN1-3miniAID-3FLAG clones. The cells were cultured with or without auxin to estimate depletion efficiency. Both untagged and AID-tagged XRN1 are detected in the population of transfected cells. Unique clones 1–3 contain at least one copy of tagged XRN1, the level of which decreases upon addition of auxin and doxycycline to the media. In contrast, clone 4 does not have the proper insert. Untagged XRN2 was used as a loading control

2. Depending on the timing of experiment and the cell culture dish size, test different seeding densities to reach a 60–70% confluency when harvesting cells. The size of the culture depends on the desired yield of RNA.
3. Treat the cells with doxycycline and/or auxin for determined time (*see* **Note 22**).
4. Cool down a tabletop centrifuge to 4 °C.
5. Transfer the treated cells under a chemical hood, quickly remove the media and aspirate the residual liquid with a pipette. Add Qiazol or phenol depending on your preferred method for RNA extraction (for 100 mm dish, we add 700 μL of Qiazol). For adherent cells, use a scrapper to collect the cells. Transfer the cells to a 1.5 mL RNase-free tube, ensuring that they are homogenized, and then incubate for 5 min at room temperature.
6. Add 140 μL of chloroform. Ensure that the tube is well closed and vortex for 15 s.
7. Keep the tubes in a stand for 2–3 min at room temperature.
8. Spin the tubes for 15 min in a tabletop centrifuge at 12,000× *g*, at 4 °C.
9. During the centrifugation, collect the samples for protein extraction. Remove the cell culture media, wash the cells with ice-cold 1× DPBS and add RIPA buffer supplemented with protease inhibitor to lyse the cells. For adherent cells, use a scrapper to collect the cells and transfer to 1.5 mL tubes. Sonicate the samples for 8 cycles (30 s on, 30 s off) at 4 °C, then spin for 10 min at maximum speed at 4 °C. Collect an aliquot for measuring the protein concentration. Store the samples at −20 °C for further use.

10. After the centrifugation of the cell lysates in Qiazol (**step 8**), carefully collect the top aqueous phase into a new 1.5 mL RNase-free tube (do not disturb the interphase). Add 1.5 volume of 100% ethanol, and mix thoroughly by pipetting.
11. Transfer the sample including any precipitate into an RNeasy Mini column (up to 700 μL). Spin for 30 s at ≥8000× *g*, at room temperature. Remove the flow-through.
12. If there is any sample remaining, repeat **step 11**.
13. Add 700 μL of RWT buffer to the column, close the lid and spin for 30 s at ≥8000× *g*, at room temperature. Remove the flow-through.
14. Add 500 μL of RPE buffer to the column, close the lid, and spin for 30 s at ≥8000× *g*, at room temperature. Remove the flow-through.
15. Repeat **step 14** extending the centrifugation time to 2 min.
16. Transfer the column in new collection tube and spin for 1 min at full speed to remove residual ethanol and dry the membrane.
17. Transfer the column in a new 1.5 mL RNase-free tube and elute the RNA by adding 30–50 μL of RNase-free water into center of the column, without touching the membrane. Close the lid and spin for 1 min at ≥8000× *g*, at room temperature.
18. Measure the RNA concentration using a UV spectrophotometer (or a fluorometer).
19. We recommend performing a western blot using the protein extracts prepared at **step 9** to confirm the efficiency of the depletion of the protein of interest before proceeding to the preparation of the libraries (*see* **Note 23**).

3.3 Preparation of RNA-Seq Libraries

1. Treat all RNA samples with RNase-free DNase I (*see* **Note 24**). Purify the DNase-treated RNAs using phenol-chloroform extraction, followed by ethanol precipitation or by using RNA purification/concentration kit.
2. Check the quality and integrity of your RNAs using a 2100 Bioanalyzer or 4200 TapeStation system.
3. Provide 400 ng of RNA for each sample to your NGS facility for the preparation of the libraries using the TruSeq Stranded Total RNA kit (Illumina) (*see* **Note 25**).
4. Use sequencing parameters to get a minimum depth of 50 M of paired-end reads (100 nt each) per sample.

3.4 Transcriptomic Analysis of Changes in RNA Abundance

1. Check the quality of the data and trim the reads using TrimGalore, with the following parameters:

```
--length 10, --trim-n, --quality 20
```

2. Download the latest version of human genome from https://www.gencodegenes.org/human/.
3. Align the reads to the human genome using STAR 2.6.1a [5], with the following parameters:

```
–outSAMstrandField intronMotif –outSAMattributes All –outSAM-
type BAM SortedByCoordinate –alignIntronMax 1000000 –outFil-
terMismatchNmax 999
–seedPerReadNmax 100000 –outFilterMultimapNmax 20
```

4. Assemble the transcriptome using Scallop [6], with following parameters:

```
–min_transcript_length_base 200 –library_type first –min_spli-
ce_bundary_hits 5
–min_transcript_coverage 10 –min_single_exon_coverage 20
```

5. Merge the obtained GTF annotations for all samples using cuffmerge from v2.2.1 of cufflinks [7], with default parameters.
6. Use v2.29 of BEDTools [8] to filter out novel transcripts for which the exons overlap the GENCODE annotation with parameter: `-nonamecheck -s`
7. Concatenate the filtered non-annotated scallop transcripts with GENCODE annotation using the following command: `cat file1.gff3 file2.gff3`
8. Perform read counting using featureCounts [9] (v2.0.0) from the R package Subread, with the following parameters: `-s 2 -O`
9. Perform a differential gene expression analysis using DESeq2 [10], with the following parameters: `betaPrior=FALSE, independentFiltering=F, cooksCutoff=F`
10. Perform a recursive intersection of the results to keep only the genes that are significantly differentially expressed in at least two auxin-degron clones after treatment, against the control cells, with and without treatment, as illustrated in Fig. 3. The rationale of performing sequential differential analyses for the up- or down- regulated genes is to avoid selection of genes induced by the stress of adding auxin/doxycycline or by the insertion of the tag in absence of auxin treatment. The two intersections of the differential analyses result in two additive lists of up- or downregulated genes following the depletion of the RNA decay factor.
11. Filter the results using an adjusted p-value ≤ 0.05, read counts ≥ 20, and abs($\log_2$FoldChange) ≥ 0.585 or higher for more stringent results (*see* **Notes 26** and **27**).

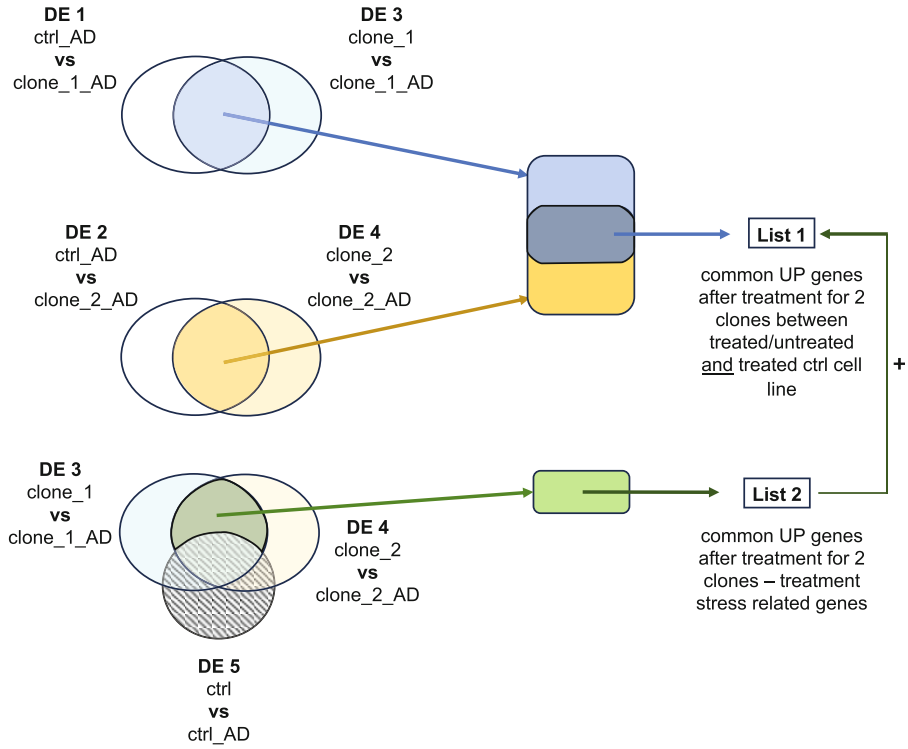

Fig. 3 Scheme of recursive intersection of differential expression analysis. The clonal selection of unique cells or the tagging of the protein and/or the doxycycline treatment can introduce biases in the results, with a nonspecific dysregulation of genes not directly controlled by the RNA decay factor of interest. Here, we propose a strategy in which the data are analyzed in sequential steps, clone by clone and condition by condition, illustrated by an example of intersection of upregulated genes analysis following XRN1-AID depletion in two independent clones. It requires performing differential expression analyses using DEseq2 (DE) for each pair of treated cells (AD indicates addition of auxin and doxycycline) versus (vs.) clone (XRN1-AID clone) without treatment. The upregulated genes are then intersected (Venn diagram) with DESeq2 results of a control cell line (ctrl) after treatment and the clone after treatment (AD). These results are then crossed with the second clone to generate list 1 of upregulated genes. To remove any secondary effect of the drug treatment, the upregulated genes selected from the control cell line after treatment (vs. no treatment) are subtracted from the intersection of differential genes for individual clone (lower line of the scheme), resulting in list 2. These two genes lists are combined and further filtered out by fold-change and adjusted *P*-values

4 Notes

1. There are multiple Cas9 enzymes available including a mutated nicase version that can cut only one DNA strand to reduce off target effect. However, such strategy requires two gRNAs with similar level of efficiency which in case of insertion in a specific region can be limiting. Therefore, we used a transient transfection with all-in-one plasmid (Cas9 and gRNA), to increase the chance of presence of Cas9 and gRNA in the same cell while avoiding constitutive expression of Cas9.

2. When selecting the gRNA from the proposed list, it is recommended that the site of cleavage with gRNA is up to 20 nt of the position where the mutation/insertion should be introduced in the genome. However, a distance up to 50 nt should still give positive result.
3. The DNA was purified with the QIAquick gel extraction kit, but any commercial kit that is adapted to the size of the amplicon can be used for the purification.
4. If the amplification of the second fragment is not efficient, it is recommended to switch to a GC-rich buffer and add DMSO to the PCR reaction.
5. HEK293T cells were used due to their high efficiency of transfection and easy handling.
6. To properly interpret the result of T7-endo essay, it is important to have a highly efficient transfection. We recommend adding 1 μg of a control plasmid expressing GFP to the transfection mix, enabling the control of transfection efficiency by fluorescent microscope.
7. Genomic DNA does not require incubation in cold for precipitation. It is recommended to not leave it in the freezer as it can cause excess precipitation of salts, which can interfere with the future steps.
8. For the facility of detection and analysis of the results of the T7 endo assay, we recommend designing primers to get a product of ~1 kb with the Cas9 cleavage site located in the middle of the amplicon. Thus, the digestion with the endonuclease will produce ~500 bp fragments, easy to separate and distinguish in the agarose gel.
9. We recommend using a high-fidelity polymerase to reduce chances of introduction of additional mutation in PCR product. For our specific fragment, we used the Expand™ Long Template PCR System (Roche): 5 μL of 10× buffer 2, 1.75 μL of 10 mM dNTP mix, 0.75 μL of Expand Long Template Enzyme Mix, 100 ng of DNA, 1.5 μL of Fw primer (10 μM), 1.5 μL of Rev. primer (10 μM), and H_2O up to 50 μL. The PCR program was: 5 min at 94 °C; 34 cycles of 30 s at 94 °C, 30 s at 52 °C, 2 min at 68 °C; final elongation for 5 min at 68 °C; hold at 12 °C.
10. The reannealing step is crucial for the detection of altered DNA structure by the T7 endonuclease. We strongly recommend using a heating block and cooling it slowly on a table.
11. The good acquisition of the gel is essential for proper analysis of the results. We recommend using small wells to concentrate the sample and short migration time.

12. Introduction of the tag can decrease the efficiency of detection by western blot using an antibody specific to the protein of interest. It is possible to detect the tagged protein using an antibody against AID itself. However, in our hands, this antibody gave very low signals. We therefore recommend adding an additional small tag for the detection of the protein of interest, bearing in mind that a large tag is more likely to affect the activity of the protein.
13. We designed two PCR fragments with flanking regions overlapping the insertion site in the vector with the gene specific homology arms. The PCR should be performed with a high-fidelity enzyme. We used the Q5 High-Fidelity DNA Polymerase (NEB) that is recommended for use with the NEBuilder HiFi DNA Assembly Master Mix.
14. The vector should be linearized with an enzyme that does not cut in the HDR donor sequence. The linearization of the vector increases HDR efficiency; however, the linear form is more sensitive to nucleases and degradation.
15. The transfection method should be tested and optimized to get the highest efficiency and the lowest toxicity to increase the chance to obtain clones. For HCT116 cells, we recommend using the Jetprime (Polyplus) reagent, as it gives good level of transfection with low toxicity for these cells.
16. It is essential to sufficiently dilute the cells, while seeding, to have single cells distribution in the plate and enough space between each cell, to ensure that colonies that will grow back later are originating from unique cells. If the experiment is performed with a number of cells different from what is suggested in this protocol, the size of the dishes should be adapted to ensure the most efficient spreading of the cells.
17. The drug concentration and the time necessary to kill the cells without the selection marker should be tested in advance.
18. Some cell lines are sensitive to growth at very low confluency. We recommend mixing a fresh media with 30–50% filtered conditioned media from the same parental cell line grown at 80% confluency.
19. For verifying the insertion of the cassette in the DNA regions, the best strategy is to design a pair of primers that overlap the flanking region of the insert (primers 1 and 2 in Fig. 1d), especially for diploid cell lines. In case of cut but without insertion, a shorter PCR product will be visible on the gel. In case of double insertion (or more) of the cassette, the PCR amplicon will be larger. Finally, two distinct PCR amplicons would indicate that only one copy has been modified, and that the clone is heterozygous. We recommend additional PCR tests with one of the primers flanking the regions of insertion

and the second primer specific to the introduced insert sequence to ensure the proper integration of the donor DNA (primer pairs 1–3 and 2–4 in Fig. 1d). If this second PCR results in a correct amplification but not the first one (using primers 1 and 2), it could indicate that the conditions of the first PCR should be optimized and that the long amplicon could be more difficult to amplify.

20. If using the AID antibody (MBL), we recommend to dilute it at a 1:500 concentration in PBS with 0.05% of Tween and 5% of milk, incubate it overnight with the membrane and detect the signal with a high sensitivity reagent (e.g. SuperSignal™ West Femto Maximum Sensitivity Substrate).
21. It is recommended to use two different selection cassettes and transfect the cells simultaneously with both HDR donor templates. The double drug selection increases the chance of homozygous clones if your cell line is diploid for the chromosome of interest. However, we were able to obtain also homozygous clones using a selection for a single drug.
22. We recommend performing the experiment with more than one clone expressing the tagged protein to avoid any artifact resulting from the clonal selection during the transcriptomic analysis.
23. We usually prepare three replicates for RNA extraction and one plate for protein extraction to have enough material to control the efficiency of protein depletion.
24. This step is essential to remove any possible remaining DNA.
25. We recommend using a total RNA-seq protocol instead of poly-A selection to sequence deadenylated RNA degradation intermediates.
26. For any gene of interest, it is important to check the profile to determine whether this is the mature isoform or a degradation intermediate that is upregulated. We recommend converting the alignment files to BigWig files with RPM normalization (reads per million of mapped reads), using UCSC tools (http://hgdownload.cse.ucsc.edu/admin/exe/linux.x86_64/). Then, for each part and each gene, extract the read coverage and visualize them using the Integrative Genome Browser.
27. In our experience, when studying cytoplasmic RNA decay, direct analysis of the cytoplasmic fraction highlights additional misregulated RNAs that could be missed when working with total RNA. A protocol for purifying RNA from nuclear and cytoplasmic fractions is described by Pinskaya and colleagues elsewhere in this volume (*see* Chapter 17).

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Chapter 20

Detection of Nuclear RNA Decay Intermediates Using a Modified Oxford Nanopore RNA Sequencing Strategy

Kevin He and Guillaume F. Chanfreau

Abstract

The nuclear RNA exosome complex is crucial for noncoding RNA processing and RNA quality control in the nucleus. Identifying substrates and intermediates of RNA decay pathways, such as those mediated by the exosome complex using Oxford Nanopore sequencing can be difficult in part because a simple method to detect them has been lacking and also because some of these RNAs lack abundant poly(A) tails which are required for Oxford Nanopore-based sequencing. Here we describe an Oxford nanopore-based approach which can be used to identify long reads corresponding to precursors and products of nuclear exosome processing. We are able to observe accumulation of unprocessed snoRNAs, cleavage products of the yeast nuclear RNase III endonuclease Rnt1p when the nuclear exosome component Rrp6p is inactivated.

Key words Oxford Nanopore sequencing, Rrp6, RNA exosome, Slu7, Rnt1, RNA degradation, *Saccharomyces cerevisiae*

1 Introduction

The "'lifecycle" of an RNA proceeds through many stages, each of which are subject to elaborate control. RNA degradation provides a key regulatory mechanism which can impact the quantitative and qualitative output of many genes. A large number of RNA decay pathways have been identified (reviewed in [1–4]), but regardless of these pathways, RNA decay intermediates are typically stabilized in cells in which degradative enzymes are experimentally inactivated. Here, our main goal is to outline a protocol for the detection of mRNA decay products or intermediates using the long read sequencing platform offered by Oxford Nanopore, combined with a PCR-cDNA library strategy. We will cover possible strategies to adapt RNAs for the Nanopore sequencing approach and how to check the relative sequencing integrity.

Nanopore sequencing technology relies on a nanoscale protein pore, or "nanopore" [5]. Threading a string of nucleic acids

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through the nanopore produces a dip in ionic current. This change in ionic current is interpreted by a base-calling software to identify the nucleotide bases which made up that nucleic acid polymer. For RNA sequencing what this means is that the maximum read length is theoretically unlimited.

A well-known limitation of short-read sequencing is that RNAs that can vary widely in length need to be reassembled from the short reads and quantified. This becomes technically challenging when processes like alternative splicing and polyadenylation can lead to the expression of isoforms that differ by only a small proportion of their length. Nanopore sequencing removes this obstacle by being able to sequence the entire transcript in one read. One current limitation of long-read sequencing, however, is its modest sequencing depth compared to short-read sequencing. This limitation can prevent the accurate quantification of low abundance transcripts. Another limitation is the relatively low coverage for short RNAs (<80 nt long). This limitation can be overcome using reprocessing of reads, which was used recently to analyze tRNAs [6].

Here we demonstrate in *Saccharomyces cerevisiae* that targets of nuclear exosome processing can be identified and quantified using a simple, robust, and efficient Nanopore sequencing approach. The RNAs to be sequenced are obtained from strains in which nuclear RNA degradation or splicing factors are rapidly exported out of the nucleus using the Anchor-Away technique [7], mediating their functional inactivation. Two components make up the Anchor-away system: an anchor protein (human FKBP12 fused to an endogenous ribosomal protein) and a target protein (in our case an RNA degradation or splicing factor) tagged with the FRB domain of human mTOR. Nuclear export of the target protein is triggered by the addition of rapamycin, which mediates interaction of the anchor protein and of the target protein, and results in the functional inactivation of the target protein.

Extracting and sequencing RNAs by Nanopre from a strain expressing FRB-tagged versions of the splicing factor Slu7p and the nuclear exosome component Rrp6p, we are able to observe the accumulation of unprocessed snoRNAs, cleavage products of the yeast nuclear RNase III endonuclease Rnt1p [8], and identify the site of Spliceosome Mediated Decay (SMD) for the *BDF2* gene [9].

2 Materials

2.1 Preparation of Yeast Cells

1. Yeast Extract Peptone Dextrose (YPD) medium: 1% Yeast Extract, 2% Peptone, 2% Dextrose.
2. 50 mL, 250 mL and 500 mL Erlenmeyer flasks.

3. Spectrophotometer.
4. Rapamycin: stock solution at 10 mg/mL.
5. Sterile water.
6. 50 mL centrifugation tubes.
7. 1.5 mL conical screw cap tubes.
8. Centrifuge.
9. Microcentrifuge.

2.2 RNA Preparation

1. RNA-SDS Buffer: 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM EDTA, 2% SDS.
2. Glass beads (5 mm).
3. RNA Phenol-Chloroform (pH 6.7).
4. Heating block.
5. Microcentrifuge.
6. Vortex.
7. 100% ethanol.
8. 3 M NaOAc, pH 5.2.
9. Nuclease-free water.
10. DNase I with its 10X buffer.
11. Glycoblue coprecipitant.
12. E. coli Poly(A) Polymerase (5000 units/mL).
13. 10 mM ATP.
14. Qubit fluorometer.
15. Qubit RNA HS Assay Kit.
16. Qubit Assay Tubes.

2.3 Library Preparation and Sequencing

1. Nuclease-free PCR tubes.
2. Thermocycler.
3. PCR-cDNA Barcoding Kit (SQK-PCB111.24, Oxford Nanopore): the kit includes the cDNA RT Adapter (CRTA), the Annealing Buffer (AB), the Short Fragment Buffer (SFB), the RT primer (RTP), the Strand Switching Primer II (SSPII), the Barcode Primers 1-24 (BP01-24), the Elution Buffer (EB), the Rapid Adapter T (RAP T), the RAP Dilution Buffer (RDB), the Sequencing Buffer II (SBII), the Loading Beads II (LBII), the Flush Tether (FLT), and Flush Buffer (FB).
4. NEBNext Quick Ligation Reaction Buffer (NEB).
5. T4 DNA Ligase: 2 M U/mL (NEB).
6. RNaseOUT: 40 U/μL.
7. Lambda Exonuclease.
8. USER (Uracil-Specific Excision Reagent) enzyme.

9. Agencourt RNAClean XP beads.
10. Magnet.
11. 10 mM dNTPs.
12. Maxima H Minus Reverse Transcriptase (200 U/μL) with 5× RT Buffer.
13. LongAmp Hot Start Taq 2X Master Mix.
14. Nuclease-free water.
15. Exonuclease I.
16. Agencourt AMPure XP beads.
17. Freshly prepared 70% ethanol in nuclease-free water.
18. 1.5 mL DNA LoBind tubes.
19. Qubit dsDNA HS Assay Kit.
20. Qubit Assay Tubes.
21. Qubit fluorometer.
22. MinION sequencing device (Oxford Nanopore).
23. R9.4.1 flow cell (Oxford Nanopore).

2.4 Softwares

1. MiniKNOW.
2. Minimap2.
3. Integrative Genomics Viewer (IGV).

3 Methods

3.1 Growth of Yeast Strains

S. cerevisiae is typically grown to log phase before changing cellular conditions and/or harvesting because this is when cells are actively dividing.

1. Prepare an overnight culture. Using a sterile loop inoculate a single colony of each strain of interest into approximately 20 mL of appropriate media (in this case YPD) in a sterile 50 mL Erlenmeyer flask. Grow cells for 8 to 16 h while shaking (200 rpm) at 30 °C.
2. Dilute the overnight culture into a new 500 mL Erlenmeyer flask with 100 mL of fresh media to an optical density (OD) at 600 nm of 0.10. Swirl to mix then grow cells to an OD_{600} of 0.40 (~2 doublings) while shaking (200 rpm) at 30 °C.
3. Once the desired OD_{600} has been reached, split the culture into two 50 mL aliquots. One of these aliquots will be your "pre-rapamycin" sample, which is an important experimental control. The other 50 mL aliquot will be the "post-rapamycin" sample, which is where the protein of interest will be anchored away.

4. For the post-rapamycin aliquot, spin it down in a 50 mL tube for 3 min at 2500× *g*. Carefully remove the supernatant without disturbing the pellet. Add 50 mL of fresh media along with enough rapamycin to have a concentration of 1 μg/mL. Invert the tube to mix well and then pour into a clean sterile 250 mL Erlenmeyer flask. Grow this flask while shaking (200 rpm) at 30 °C for 1 h.
5. For the pre-rapamycin, go ahead and spin down culture in a 50 mL falcon tube for 3 min at 2500× *g*. Carefully remove the supernatant without disturbing the pellet. Use 1 mL of sterile water to wash and resuspend the pellet and transfer it to a 1.5 mL conical screw cap tube. Spin down the tube at 2500× *g* for 30 s. Remove the supernatant and flash-freeze in liquid nitrogen. Store at −80 °C until ready for RNA extraction.
6. Once the hour for the post-rapamycin is finished, centrifuge, wash, and freeze the cells as in **step 5**.

3.2 Total RNA Extraction

This section is for the extraction of total RNA from flash-frozen *S. cerevisiae* cells (*see* **Note 1**).

1. To the cell pellets that were previously flash-frozen and stored in 1.5 mL conical screw cap tubes, add 400 μL of glass beads, 500 μL of RNA-SDS buffer, and 500 μL of RNA Phenol-Chloroform (pH 6.7) (*see* **Note 2**).
2. Vortex for 1 min. Heat at 65 °C for 6 min.
3. Vortex for 1 min. Spin down at 18,800× *g* for 5 min.
4. Take the top 450 μL aqueous layer and add to 450 μL of fresh RNA Phenol-Chloroform (pH 6.7).
5. Vortex for 1 min and spin at 21,400× *g* for 5 min.
6. Take the top 400 μL aqueous layer and add to 1 mL of 100% ethanol and 40 μL of 3 M NaOAc pH 5.2 and cool at −80 °C for a minimum of 30 min (*see* **Note 3**).
7. Spin down at maximum speed for 10 min.
8. Remove the supernatant and wash pellets in 500 μL of 70% ethanol. Spin at maximum speed for 5 min.
9. Resuspend the RNA in 20–50 μL of nuclease-free water while on ice (*see* **Note 4**).
10. Determine the resuspended RNA concentration (aim for 5–10 μg/μL).

3.3 DNase Treatment of Total RNAs

The PCR-cDNA Barcoding Kit generates complementary DNA (cDNA) from polyadenylated RNAs. It is important to digest any contaminant DNA to ensure that only RNAs get sequenced (*see* **Note 5**). Use snap lock tubes for following Phenol-Chloroform steps as well as ethanol-resistant markers to label tubes.

1. Take 40 μg of RNA and add 4 μL of 10X DNase buffer, 4 μL of DNase I (8 units). Add nuclease-free water to 40 μL.
2. Mix and incubate at 37 °C for 10 min.
3. Add 40 μL of RNA Phenol-Chloroform (pH 6.7) and vortex for 1 min.
4. Spin at maximum speed for 5 min.
5. Take the top aqueous layer and add to 1 mL of 100% ethanol and 40 μL of 3 M NaOAc pH 5.2. Optional: add 1 μL of GlycoBlue to assist with visualization of the RNA pellet.
6. Cool at −80 °C for 30 min.
7. Remove the supernatant and wash the pellet in 500 μL of 70% ethanol. Spin at maximum speed for 5 min.
8. Remove the supernatant, air-dry the RNA pellet.
9. Resuspend the RNA in 10–20 μL of nuclease-free water while on ice.
10. Measure the resuspended RNA concentration (*see* **Note 6**).

3.4 In Vitro Polyadenylation of RNAs

The PCR-cDNA Barcoding Kit performs complementary strand synthesis and strand switching using kit-supplied oligonucleotides. The oligonucleotides provided with the kit assume that the RNA is polyadenylated. For transcripts which are not polyadenylated, the addition of a poly(A) tail is needed to make the samples compatible with this protocol. A more targeted sequencing approach is possible either using a different kit or by replacing the kit supplied oligonucleotides with custom ones (*see* **Note 7**).

1. Take 1–10 μg of DNase-treated RNA and add 2 μL of 10X Poly (A) Polymerase Reaction Buffer, 2 μL of 10 mM ATP, 1 μL (5 U) of *E. coli* Poly(A) Polymerase, and add nuclease-free water to a final volume of 20 μL.
2. Incubate at 37 °C for 30 min.
3. Proceed directly to ethanol precipitation. Add 1 mL of 100% ethanol and 40 μL of 3 M NaOAc pH 5.2. Optional: add 1 μL of GlycoBlue to assist with visualization of the RNA pellet.
4. Cool at −80 °C for 30 min.
5. Remove the supernatant and wash the pellet in 500 μL of 70% ethanol. Spin at maximum speed for 5 min.
6. Remove the supernatant and air-dry the RNA pellet.
7. Resuspend the RNA in 10–20 μL of nuclease-free water while on ice.
8. Measure the resuspended RNA concentration using a Qubit fluorometer.

3.5 Reverse Transcription and Strand-Switching Using PCR-cDNA Barcoding Kit

The following steps describe how to prepare libraries for Nanopore sequencing (*see* **Notes 8** and **9**). Much of this follows the procedure outlined from Oxford Nanopore's own community protocol. We have included these steps here so the readers can follow along and see some of the changes we made, which we explain in the notes.

1. Start by pipetting up to 200 ng of your poly(A)$^+$ RNA into a PCR tube (*see* **Note 10**). Adjust the volume to 10 μL with nuclease-free water. Next, add 1 μL of cDNA RT Adapter (CRTA) and 1 μL of Annealing Buffer (AB). The total volume is 12 μL. Mix gently by flicking to avoid shearing and spin down.
2. Use a thermocycler to incubate the tube at 60 °C for 5 min, let the tube cool for 10 min at room temperature.
3. To each of the PCR tubes containing you RNA sample(s), add 3.6 μL of NEBNext Quick Ligation Reaction Buffer, 1.4 μL of T4 DNA Ligase (2800 units) and 1 μL of RNaseOUT (40 units). The total volume is now 18 μL. Mix gently by flicking to avoid shearing and spin down. Incubate for 10 min at room temperature.
4. Next, add 1 μL of Lambda Exonuclease and 1 μL of USER (*see* **Note 11**). The total volume is now 20 μL. Mix gently by flicking to avoid shearing and spin down. Set a thermocycler to 37 °C and incubate for 15 min.
5. Transfer each sample to a clean microcentrifuge tube.
6. Resuspend the RNAClean XP beads by vortexing thoroughly.
7. Add 36 μL of RNAClean XP beads to each tube and mix gently by flicking.
8. Incubate gently on a shaker for 5 min at room temperature.
9. Spin down the samples and pellet on a magnet. Keep the tubes on the magnet, and pipette off the supernatant.
10. Keep the tubes on the magnet and wash the beads with 100 μL of Short Fragment Buffer (SFB) without disturbing the pellet. Remove the SFB using a pipette.
11. Repeat **step 10**.
12. Spin down and place the tubes back on the magnet. Pipette off any residual buffer (*see* **Note 12**).
13. Remove the tubes from the magnetic rack and resuspend each pellet in 12 μL of nuclease-free water.
14. Incubate at room temperature for 10 min.
15. Pellet the beads on the magnet until the supernatant is clear and colorless.
16. Remove and retain 12 μL of supernatant into a clean PCR tube for each sample.

17. For each of the PCR tubes from the previous step, add 1 μL of RT Primer (RTP) and 1 μL of 10 mM dNTPs. Mix gently by flicking to avoid shearing and spin down.
18. Let the reaction sit at room temperature for 10 min.
19. Next add the following reagents: 4.5 μL of Maxima H Minus 5× RT Buffer, 1 μL of RNaseOUT (40 units) and 2 μL of Strand Switching Primer II (SSPII). The total volume is now 21.5 μL. Mix gently by flicking and spin down.
20. Set the thermocycler to 42 °C and incubate the reaction for 2 min.
21. Add 1 μL of Maxima H Minus Reverse Transcriptase to each reaction. Mix gently by flicking and spin down. Incubate in the thermocycler using these parameters: 90 min at 42 °C, 5 min at 85 °C, hold at 4 °C (*see* **Note 13**).

3.6 Selecting for Full-Length Transcripts by PCR Using the PCR-cDNA Barcoding Kit

See **Note 14**.

1. Into a PCR tube add the following reagents: 5 μL of reverse-transcribed sample (from the previous section), 0.75 μL of one of the Unique Barcode Primers (BP01-24) per sample, 6.75 μL of nuclease-free water, and 12.5 μL of 2× LongAmp Hot Start Taq Master Mix. The final volume is 25 μL. Mix gently by pipetting.
2. Set the thermocycler as follows: 30 s at 95 °C (initial denaturation), 10–18 cycles (15 s at 95 °C, 15 s at 62 °C, 60 s per kb at 65 °C), 6 min at 65 °C (final extension), hold at 4 °C (*see* **Note 15**).
3. Add 1 μL of Exonuclease I (20 units) directly to each PCR tube (*see* **Note 16**). Mix by pipetting. Incubate the reactions at 37 °C for 15 min, followed by 15 min at 80 °C in the thermal cycler.
4. Transfer each sample to clean 1.5 mL Eppendorf DNA LoBind tubes.
5. Resuspend the AMPure XP beads by vortexing.
6. Add 20 μL of resuspended AMPure XP beads to each reaction and mix gently by flicking the tubes.
7. Incubate gently on a shaker for 5 min at room temperature.
8. Spin down the samples and pellet on a magnet. Keep the tubes on the magnet, and pipette off the supernatant.
9. Keep the tubes on the magnet and wash the beads with 200 μL of 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette.
10. Repeat the previous step.

11. Spin down and place the tubes back on the magnet. Pipette off any residual buffer (*see* **Note 17**).
12. Remove the tubes from the magnetic rack and resuspend each pellet in 12 μL of Elution Buffer (EB).
13. Incubate at room temperature for 10 min.
14. Pellet the beads on the magnet until the supernatant is clear and colorless.
15. Remove and retain 12 μL of supernatant into a clean 1.5 mL Eppendorf DNA LoBind tube.
16. Measure the concentration of each purified sample using a Qubit fluorometer.
17. Pool together equimolar samples of the amplified cDNA barcoded samples to a total of 25 fmols and make the volume up to 11 μL in Elution Buffer (EB).

3.7 Adapter Addition Using PCR-cDNA Barcoding Kit

1. In a fresh 0.2 mL PCR tube, dilute the Rapid Adapter T (RAP T): 1.2 μL of RAP T in 6.8 μL of RAP dilution buffer (RDB). Mix well by pipetting and spin down (*see* **Note 18**).
2. Add 1 μL of the diluted Rapid Adapter T to the amplified cDNA library. Mix well by pipetting and spin down. Incubate the reaction for 5 min at room temperature.

3.8 Priming and Loading the Flow Cell

This step covers how to prepare your Nanopore device for a sequencing run. Once again, we stress that this protocol assumes you are using an R9.4.1 flow cell with a MinION device. Different devices and flow cells will require changes to the priming buffer as well as the volumes to add, please refer to the community protocol if you are using a different set up (*see* **Note 8**).

1. Take the Sequencing Buffer II (SBII), Loading Beads II (LBII), Flush Tether (FLT), and one tube of Flush Buffer (FB). Let these reagents thaw at room temperature. Make sure to vortex and spring down before pipetting. Once thawed, prepare the flow cell priming mix by pipetting 30 μL of FLT directly to the tube of FB, vortex, and spin down.
2. Insert the flow cell into the MinION. Open up Miniknow and perform a flow cell check to ensure the number of pores available is above 800 (*see* **Note 19**).
3. Use a 1 mL pipette to draw around 20–30 μL of buffer from the priming port. Take extreme care not to introduce any air bubbles (*see* **Note 20**). Gently, pipette 800 μL of priming mix into the priming port then wait for 5 min. To assist with guidance this is what Oxford Nanopore recommends to users: "After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove

any bubbles: set a P1000 pipette to 200 µL, insert the tip into the priming port, turn the wheel until the dial shows 220–230 µL, to draw back 20–30 µL, or until you can see a small volume of buffer entering the pipette tip."

4. Next, add the following reagents to 12 µL of your prepared libraries: 37.5 µL of Sequencing Buffer II (SBII) and 25.5 µL of Loading Beads II (LBII). The total volume reaches 75 µL (*see* **Note 21**).
5. Open up the sample port. Pipette 200 µL of priming mix into the priming port.
6. Mix the prepared library gently by pipetting up and down just prior to loading. Add 75 µL of the prepared library to the flow cell into the sample port in a dropwise fashion. Close the sample port cover and then close the priming port.

3.9 Base-Calling, Alignment, and More

Once the flow cell is primed and loaded, it is ready for sequencing. Here we will cover the setup of parameters and then go over a basic alignment workflow.

1. Click on "Start Sequencing" in Miniknow.
2. Enter your preferred experiment name and then continue to kit selection (Fig. 1).
3. Select the appropriate kit (in this case we are going to select PCR-cDNA Barcoding Kit - 24) (Fig. 2).
4. After these steps, reach "Run options" (Fig. 3). Here you can set the amount of time you want the device to be sequencing. An important parameter to set is the minimum read length. This is the minimum length of a read that the sequencer will recognize and output during basecalling. It is important to set it to 20 bp when sequencing very short fragments. The minimum read length does not factor in any barcodes or adapters used for the library prep so the reads may appear longer than the minimum length set.
5. Another option that can be selected here is "Adaptive sampling" which enables users to select or reject target sequences. This can be useful when users only wish to sequence a handful or just a singular transcript. It can also be used to ensure barcodes are sequenced in equal amounts.
6. The raw data output of Nanopore sequencing is stored as fast5 files (now POD5). These files store the signal information generated during sequencing. However, MiniKNOW allows users to basecall their reads in real time thereby saving time. Currently, basecalling is available for 5mC and 5mC + 5hmC modified bases. If users are looking to sequence other base modifications or perhaps apply their own basecalling algorithm

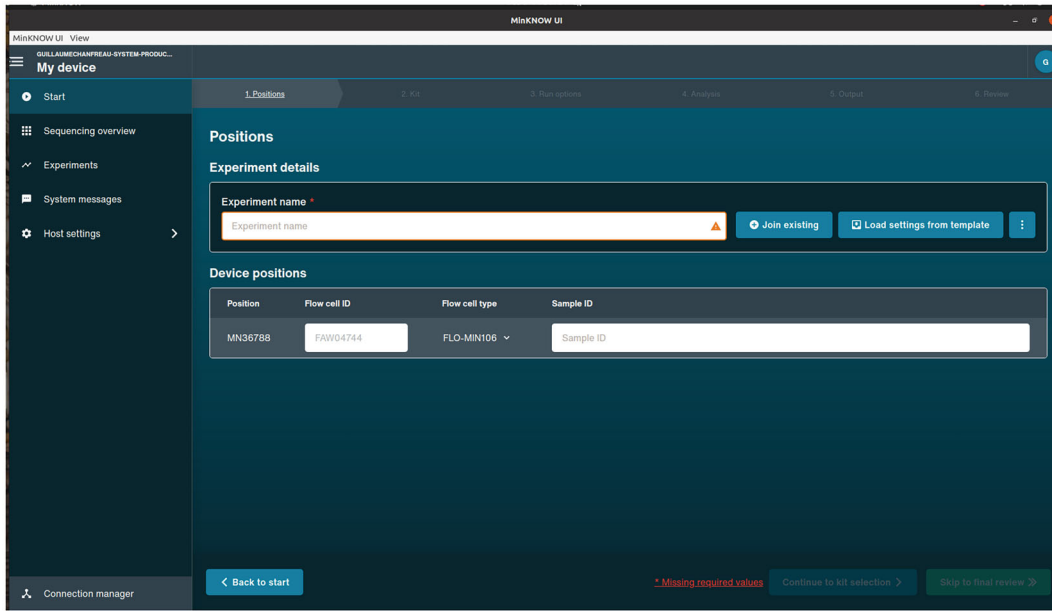

Fig. 1 Entry of the name of the experiment

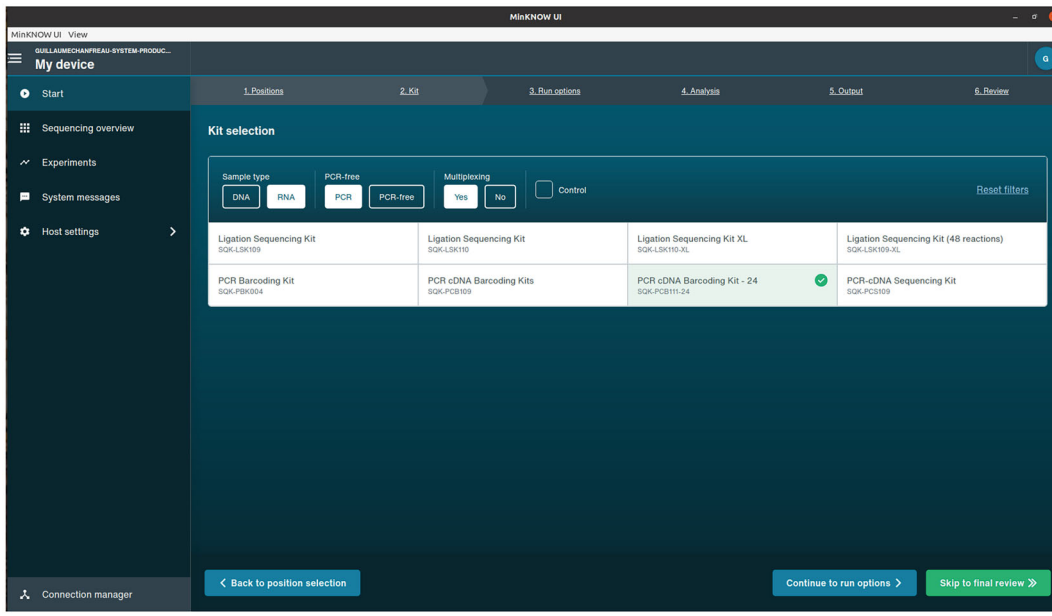

Fig. 2 Miniknow kit selection screen

they can save space and processing time by turning the base-calling option off (Fig. 4).

7. Set the location to save the output and their file types (Fig. 5).

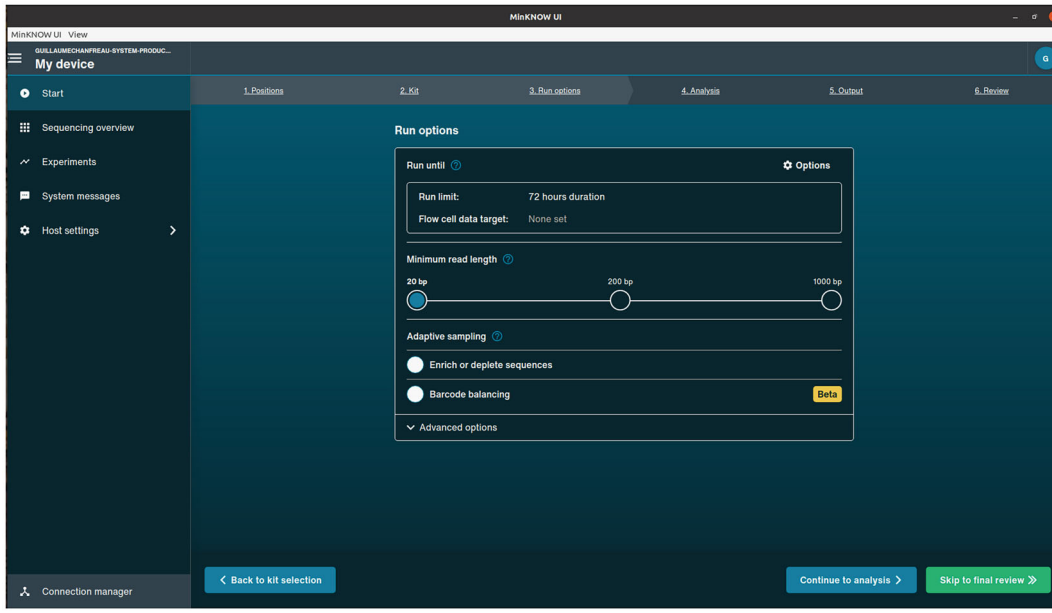

Fig. 3 Input run options

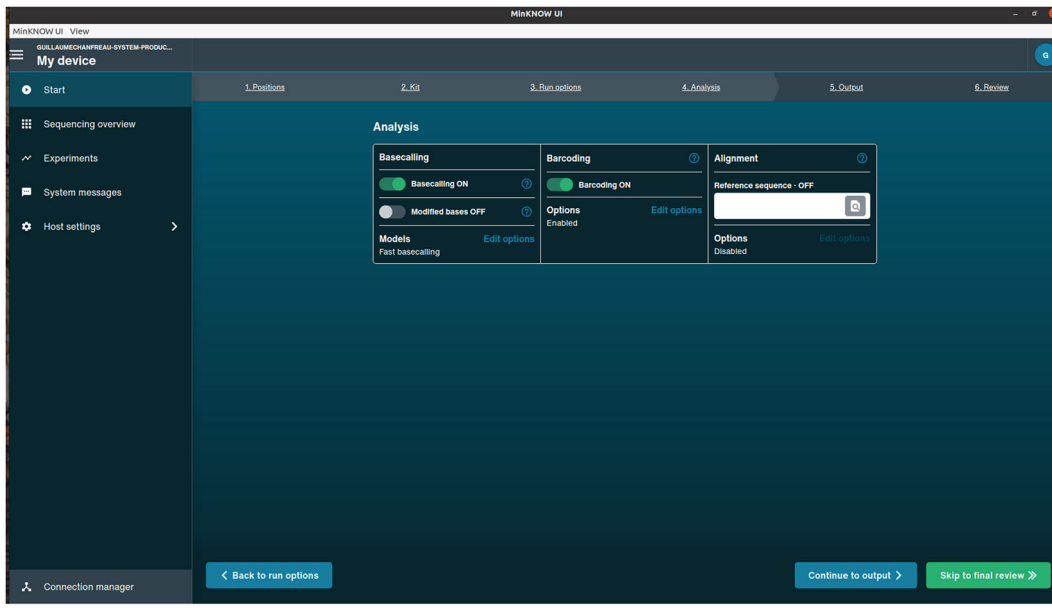

Fig. 4 Miniknow analysis screen. Users can input whether they would like to basecall and barcode during the sequencing run

8. On that same screen under "Advanced options" users can save bulk information that gets added to their fast5 files (Fig. 6). This can be important because we have found that the default MinKNOW configuration can be less than optimal in detecting short reads like tRNAs [6]. Use of alternative MiniKNOW

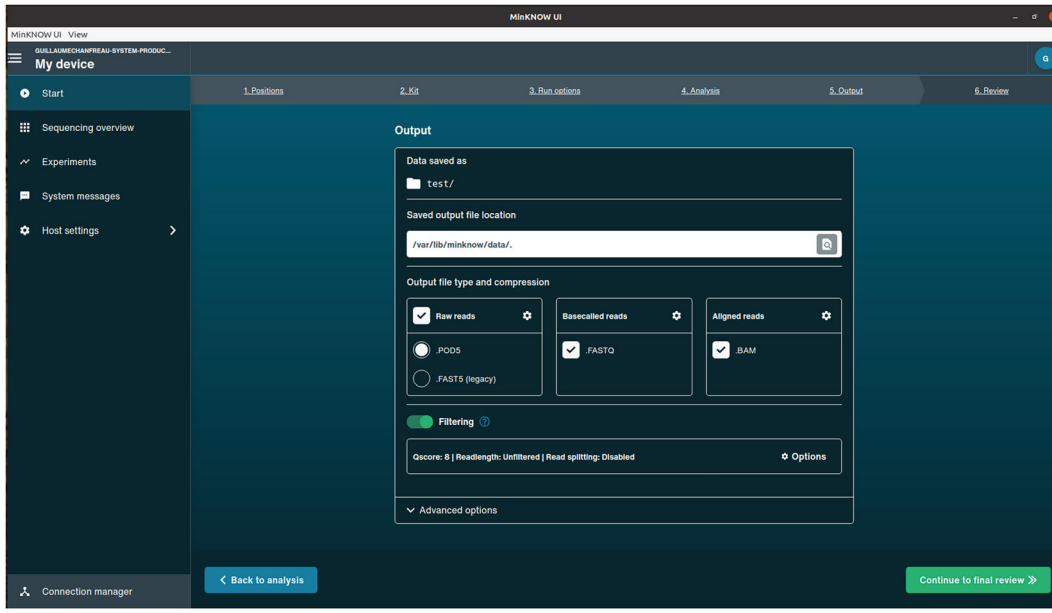

Fig. 5 Output options screen in Miniknow

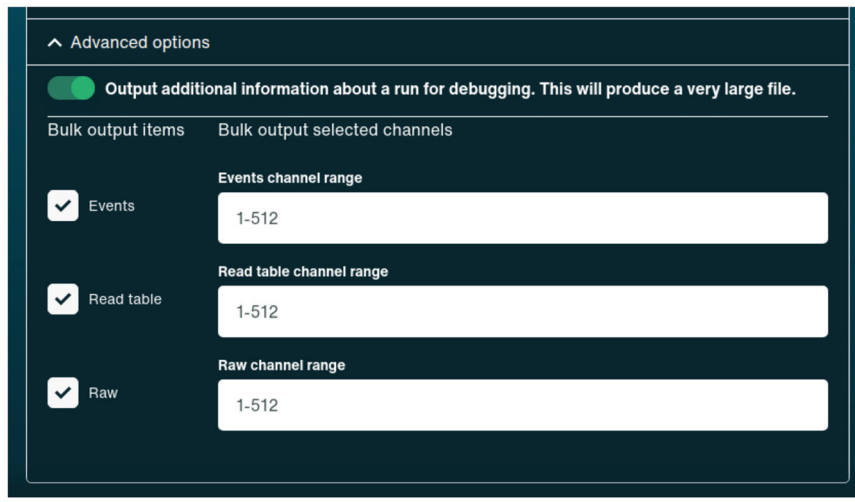

Fig. 6 Advanced output options

configurations can alleviate this problem [6]; the user can do this after the sequencing run is finished by saving that bulk information. Beware, saving these bulk files drastically increases the file sizes. For the data used as an example, the default config set to the lowest minimum read length was sufficient to detect nuclear exosome decay targets.

9. Next, navigate to the review screen and make sure that the parameters have been set up correctly.

10. The following code is based on bash. Once the sequencing run is finished, sequencing reads are aligned to the reference genome. We will begin by concatenating our .fastq.gz files into one using the following command:

    cat '/path/to/fastq.gz' > '/path/to/save/fastq.gz'

    The file that was just created can now be used to align to a reference genome. Our libraries were prepared from total RNAs extracted from an *S. cerevisiae* strain that was then polyadenylated in vitro. This means we are going to align to the *S. cerevisiae* reference genome using the following minimap2 parameters:

    minimap2 -ax splice -G 3000 -uf '/path/to/reference/.fasta' '/path/to/fastq.gz' > '/where/to/save/.bam'

    where *-ax splice* generates CIGAR and output alignments in the SAM format (the "*x splice*" makes the aligner splicing aware), *-G* sets the maximum intron size (in the example, this size is set to 3000 nt), *-uf* will make the aligner attempting to match spliced reads using canonical splicing sites "GT-AG" (the "*f*" tells it to use the transcript strand to search canonical splice sites).

3.10 Visualization of Reads on the Integrative Genomics Viewer (IGV)

Now that the sequencing reads have been aligned, they can be visualized on IGV. This is where users can manually inspect reads and view select parts of the genome.

1. Sort the bam file. On IGV go ahead and select "tools." There will be a drop-down menu.Click on "igvtools" (Fig. 7). This screen will show up. In "command" select sort and browse for the bam file to put in the "input file" section. Afterwards click "run" and you will be notified when the sorting is finished (*see* **Note 22**).
2. Generate the index. We will take the sorted bam file and generate an index for it. Bam is a binary (compressed) format. For some applications, like IGV, the software needs "random access" to the bam file. It needs to be able to find reads without iterating through the entire file. That is what the index is for. On the same igvtools screen in "command" now select the index and browse for the sorted bam file to put in the "input file" section. Afterwards click "run" and you will be notified when the indexing is finished (Fig. 8).
3. The next step is to visualize targets of interest. While a full description of the data obtained in our example is outside of the scope of this chapter, we will briefly describe two examples. In our libraries we are able to detect the SMD site for *BDF2* (Fig. 9). During SMD, the *BDF2* mRNA undergoes the first step of splicing at a 5′ splice site sequence, but instead of completing both steps of splicing, the intermediates are instead

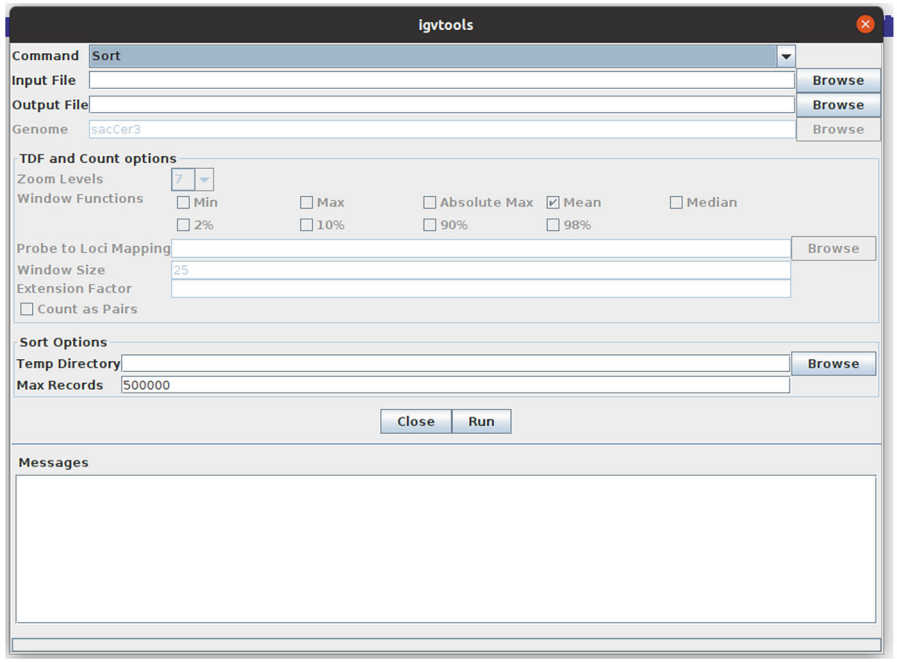

Fig. 7 Sort command option for Igvtools

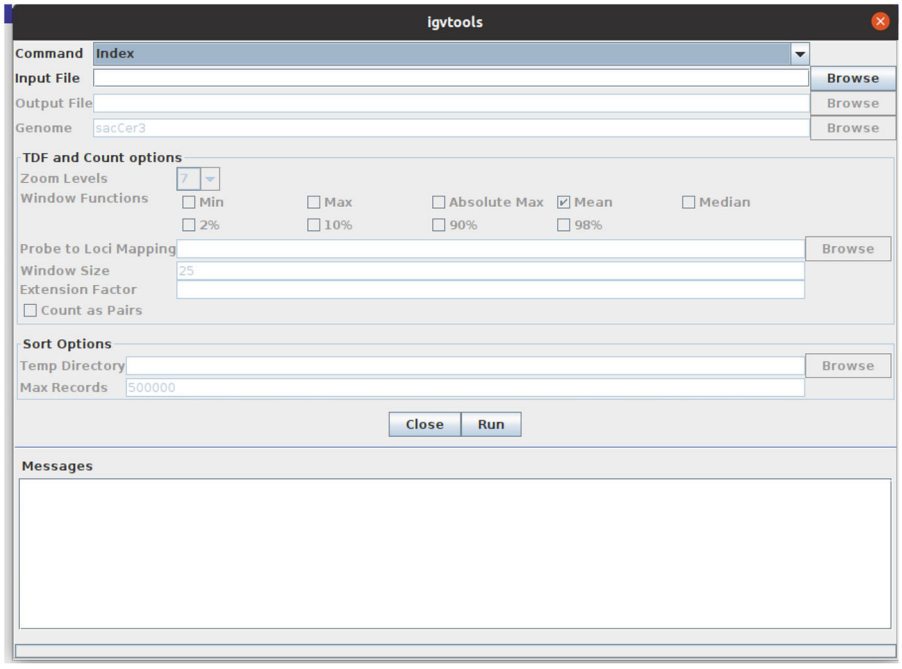

Fig. 8 Index command option for Igvtools

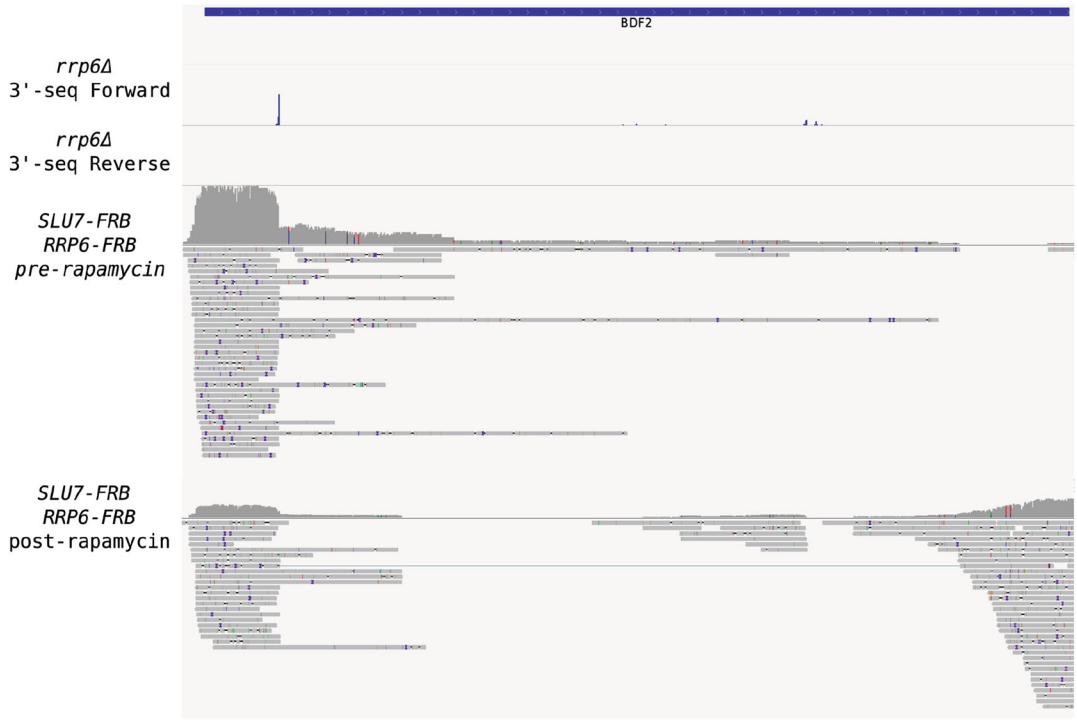

Fig. 9 Detection of SMD targets by Oxford Nanopore RNA sequencing and comparison with 3′-end sequencing data obtained from an *rrp6*Δ deletion strain. Each Nanopore sequencing read is represented by a horizontal gray line. A graph representing the sum of the reads obtained is shown at the top for each sample. 3′-end sequencing reads are represented by peaks, the amplitude of which is proportional to the number of reads obtained. Reads corresponding to the *BDF2* SMD cleavage products are visible on the left side, with their 3′-ends overlapping with the 3′-end sequencing peak obtained from the *rrp6*Δ deletion strain

released and degraded [9]. These SMD related transcripts that have been captured by Nanopore correspond perfectly with the 3′ end sequencing peak which also demonstrates the site of SMD, taken from a strain where *RRP6* was knocked out [10].

It is notable that there does not appear to be a difference in SMD intermediate levels before and after rapamycin treatment. This might be due to confounding effects of simultaneous Rrp6p and Slu7p co-depletion from the nucleus. While Rrp6p functional inactivation is expected to stabilize SMD products, inactivation of the Slu7 splicing factor by anchor away reduces splicing efficiency [11], and the simultaneous co-depletion may functionally offset in terms of accumulation of SMD products.

One of the central roles of the nuclear RNA exosome is 3′ end processing ends of small nucleolar RNAs (snoRNAs) down to their mature 3′ ends. By Nanopore we are able to demonstrate that inactivation of Rrp6p produces an accumulation of unprocessed snoRNAs for example snR64. The precursor of this snoRNA is also subject to upstream RNase III-mediated cleavage by Rnt1p

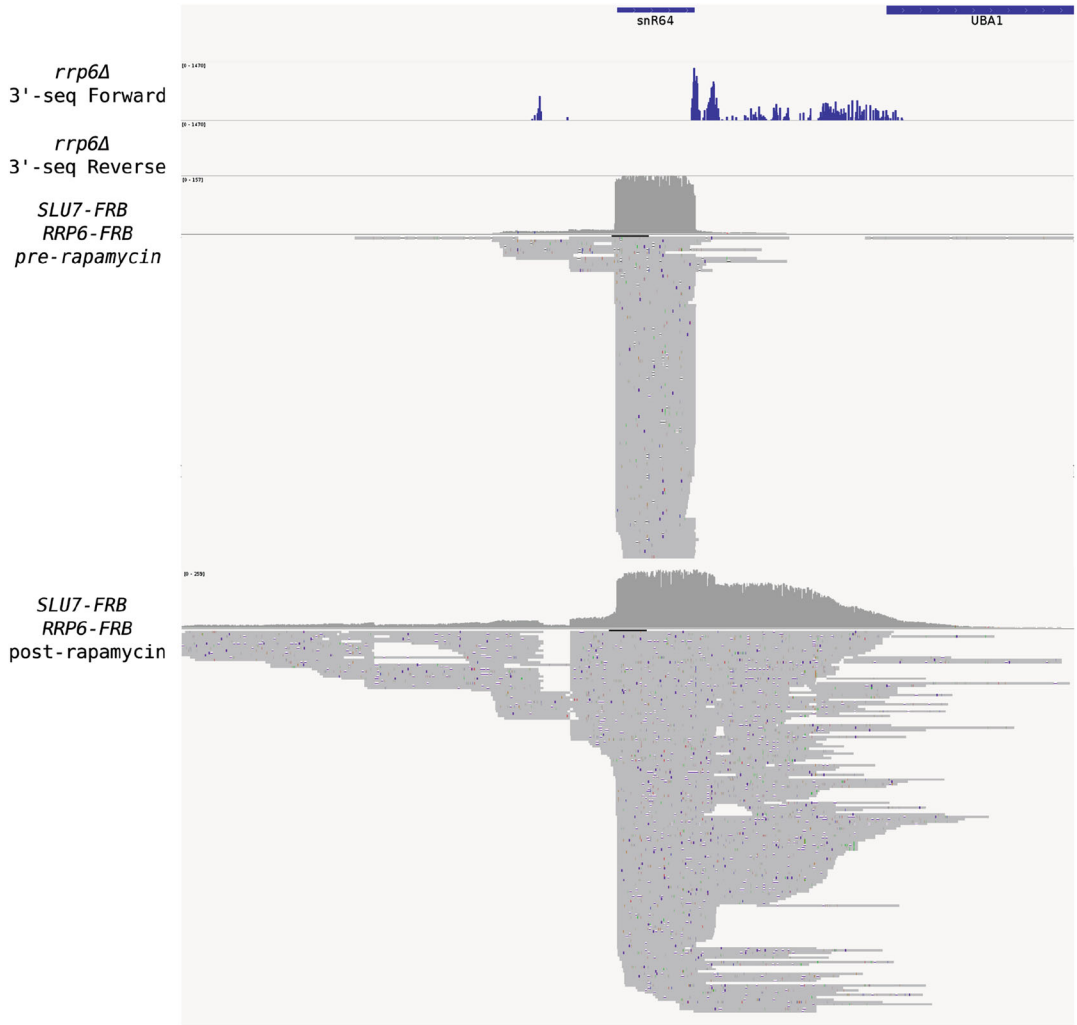

Fig. 10 Detection of unprocessed *snR64* transcripts by Oxford Nanopore RNA sequencing. Legends as in Fig. 9. Accumulation of 3′-extended reads can be detected in the post-rapamycin treatment sequencing reads, due to defective 3′-end processing by the RNA exosome. Sequencing reads accumulating upstream (left) of *snR64* in the post-rapamycin samples represent the RNAs corresponding to the cleavage product by Rnt1p, which are normally degraded by the exosome but accumulate in conditions of Rrp6 depletion from the nucleus. The 3′-ends of these reads match the 3′-end sequencing peak obtained from the *rrp6*Δ deletion strain

[12]. The cleavage fragments are subsequently degraded by nuclear exoribonucleases like Rrp6p. We are able to demonstrate accumulation of these Rnt1p cleavage products upstream from the mature snR64 sequence when *Rrp6p* is anchored away (Fig. 10).

3.11 Future Possibilities

After aligning reads there are many possible analyses that can be performed depending on the goals of the project. It is impossible to cover all of them but we highly encourage readers to check out

Nanoblot: an R-package for visualization of RNA isoforms from long-read RNA-sequencing data for more suggested possibilities [13].

4 Notes

1. Best practices for handling RNAs involve maintaining a dedicated RNase-free workspace. Avoid using pipettes that have previously been used for experiments involving the use of Ribonucleases. The combination of high pH and high temperatures promotes RNA degradation, so using close-to-neutral pH and cold temperatures prevents RNA degradation.
2. Use snap lock tubes for following Phenol-Chloroform steps as well as ethanol resistant markers to label tubes. Phenol/chloroform dissolves latex laboratory gloves, so change gloves if they become soiled with phenol/chloroform.
3. Samples can be stored at −80 °C for several months.
4. Keep samples on ice to prevent RNA degradation.
5. Reaction can be scaled down as necessary if you do not have enough RNAs.
6. The lion's share of total RNA is ribosomal RNA (rRNA). This can be challenging for researchers studying mRNA changes. Normally rRNA depletion is not necessary for Nanopore because of the requirement for RNAs to be polyadenylated. However, if users are using in vitro polyadenylated RNA then rRNA depletion may be required. Depletion of rRNA prior to nanopore sequencing allows users to focus on analyzing high-value portions of the transcriptome. In this demonstration we have chosen to forgo rRNA depletion due to the relatively high sequencing depth that was achieved.
7. Detailed information on the identity of these adapters is supplied by Oxford Nanopore here: https://nanoporetech.com/document/chemistry-technical-document#adapter-sequences.
8. The following steps discuss the library preparation as per Oxford Nanopore's instructions for their PCR-cDNA Barcoding Kit (SQK-PCB111.24). There will be slight differences depending on which nanopore device the user has available (MinION, GridION, or PromethION). We will assume the device is a MinION. Please refer to the community protocol if you are using a different device: https://nanoporetech.com/document/pcr-cdna-barcoding-kit-sqk-pcb111-24.
9. Nanopore is a rapidly evolving technology, one area of intense development is kit chemistry used for the library preparation

and sequencing. As such, these protocols were written following our experience with the PCR-cDNA Barcoding Kit. This kit is now relegated to a legacy product according to Oxford Nanopore's store page and we cannot guarantee it will remain available in the future. Its replacement is the cDNA-PCR Barcoding Kit V14. This version of the kit incorporates the latest Kit 14 chemistry which promises higher modal raw read sequencing accuracy. The steps are still the same as outlined here though users will need to purchase a different kit and a different version of flow cell than the ones discussed here.

10. The kit recommends using 4 ng of poly(A)$^+$ RNA. From our experience, at least 200 ng of poly(A)$^+$ RNA is preferred.
11. Lambda Exonuclease and USER are important because they digest the bottom strand of the ligated CRTA so that the RT Primer (RTP) can be used as a primer for the reverse transcription of the RNA.
12. It is very important to not allow the pellet to dry to the point of cracking.
13. Take your samples forward into the next step. However, at this point it is also possible to store the sample at −20 °C indefinitely.
14. Use only 5 μL of your reverse-transcribed sample. Do not use all of your reverse transcribed cDNA.
15. Oxford Nanopore recommends 14 cycles as a starting point. Optimal cycle number will need to be determined using qPCR analysis. For our purposes, using 14 cycles was adequate.
16. Exonuclease I is added to remove any unincorporated primers.
17. It is imperative to not allow the pellet to dry to the point of cracking.
18. Rapid Adapter T is sensitive to vibrations, do not vortex to mix.
19. Nanopore guarantees new flow cells to have a minimum of 800 available pores for sequencing and will provide replacements if that is not the case. Subsequent washing and reuse will degrade the number of pores. As a rule of thumb, more pores the better. Lower pore count will impact the sequencing depth.
20. Introducing air bubbles into the sensor array will damage pores beyond repair.
21. Mix the Loading Beads II (LBII) tube right before pipetting, these beads settle very quickly.
22. The sort command sorts the bam file based on its genomic position in the reference, as determined by its alignment. Many tools require sorted bam files, it also helps to reduce the file size of the bam file, users should always sort their bam files.

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Chapter 21

Defining the True Native Ends of RNAs at Single-Molecule Level with TERA-Seq

Fadia Ibrahim and Zissimos Mourelatos

Abstract

Turnover of messenger RNAs (mRNAs) is a highly regulated process and serves to control expression of RNA molecules and to eliminate aberrant transcripts. Profiling mRNA decay using short-read sequencing methods that target either the 5′ or 3′ ends of RNAs, overlooks valuable information about the other end, which could provide significant insights into biological aspects and mechanisms of RNA decay. Oxford Nanopore Technology (ONT) is rapidly emerging as a powerful platform for direct sequencing of native, single-RNA molecules. However, as currently designed, the existing ONT platform is unable to sequence the very 5′ ends of RNAs and is limited to polyadenylated molecules. Here, we present a detailed step-by-step experimental protocol for True End-to-end RNA Sequencing (TERA-Seq), a new method that addresses ONT's limitations, allowing accurate representation and characterization of RNAs at the level of single molecules. TERA-Seq describes both poly- and non-polyadenylated RNA molecules and accurately identifies their native ends by ligating uniquely designed adapters to the 5′ ends (5TERA), the 3′ ends (TERA3), or both ends (5TERA3) that are sequenced along with the transcripts.

Key words RNA decay, Messenger RNAs, Native RNA ends, Single-RNA molecule sequencing, Direct RNA sequencing, Oxford Nanopore Technology, ONT, End-to-end sequencing, TERA-Seq

1 Introduction

Messenger RNAs (mRNAs) are capped at their 5′ ends and polyadenylated at their 3′ ends. The decay of mRNAs is a fundamental process for proper cellular function and homeostasis [1, 2]. Exonucleolytic mRNA decay involves shortening of the poly(A) tail by deadenylases [3–6] and removal of the 5′ cap by decapping enzymes [7, 8], leaving the mRNA susceptible to 5′-to-3′ exonucleolysis by XRN1 [4] and by the exosome and Ski complex in the 3′-to-5′ direction [9]. Endonucleases such as AGO2, IRE1, and others [10], and processes such as ribothrypsis [11] may also initiate mRNA decay. Existing 5′ end sequencing methods do not capture the 3′ ends of RNAs, and 3′ end sequencing methods do not discriminate between the 3′ ends of capped or uncapped RNAs.

Maxime Wery (ed.), *mRNA Decay: Methods and Protocols*, Methods in Molecular Biology, vol. 2863,
https://doi.org/10.1007/978-1-0716-4176-7_21,

To better understand mRNAs decay, it is crucial to concurrently capture the native 5′ and 3′ ends of RNAs. Oxford Nanopore Technology (ONT) has a strong potential to illuminate many aspects of RNA biology and metabolism through direct sequencing of single, native RNA molecules of any length preserving their modifications [12–18]. ONT threads single RNA molecules through membrane-piercing nanopores, detecting changes in the current intensity as they traverse the pore. By deconvoluting these electrical signals nucleotide bases and their modifications are identified [14]. The standard nanopore-based library generation protocol selects polyadenylated RNA molecules and involves sequential ligation of DNA adapters to the 3′ ends of RNAs. The first ligation step adds the RTA (Reverse Transcription Adapter) that contains a stretch of thymidine bases to retain RNAs with a 3′ poly(A) tail. This ligation step is followed by an optional reverse transcription step to generate cDNA to improve sequencing output by reducing intramolecular secondary structures of the RNA. The second ligation step adds a sequencing adapter that is equipped with a motor protein to initiate sequencing of RNAs, which proceeds in the 3′-to-5′ direction [12, 18]. However, ONT cannot perform true end-to-end sequencing of RNA molecules due to the inability of the protein pores to sequence the terminal 10–15 nucleotides of the RNA [15, 18, 19]. Additionally, RNA molecules may appear truncated because they are incompletely sequenced as a result of signal artifacts due to stalled pore unblocking, motor protein stalling, or extraneous voltage spikes [16, 18, 20].

Here, we describe a new and straightforward method for True End-to-end RNA Sequencing (TERA-Seq) that is compatible with ONT sequencing. TERA-Seq involves ligation of uniquely designed adapters to the 5′ and 3′ ends of RNA molecules, which can be combined with various treatments to capture mRNA molecules with different 5′ and 3′ ends. We first describe 5TERA-Seq that selects polyadenylated RNAs containing 5′ monophosphate (5P) termini (processing and decay intermediates; Subheading 3.3.1), 5′ cap (full-length or recapped molecules; Subheading 3.3.2), or 5′ cap and 5P termini (full-length/recapped, and processing/decay intermediates; Subheading 3.3.3) by ligating an adapter to their 5′ ends. We then describe TERA3-Seq that captures the 3′ ends of all native transcripts with and without poly(A) tails through ligation of an adapter to their 3′ ends (Subheading 3.3.4). With 5TERA3, we describe the selection of full-length, processing, and decay intermediates from end-to-end, with or without poly(A) tails by attaching both 5′ and 3′ adapters on the same RNA molecule (Subheading 3.3.5) (Fig. 1). Lastly, we describe briefly a modified protocol of ONT for library generation, and for use with a 3′ adapter that allows sequencing of RNAs regardless of the presence of poly(A) tails using the MinION device (Subheading 3.4). Coupling 5TERA, TERA3, and 5TERA3 RNAs can be characterized in

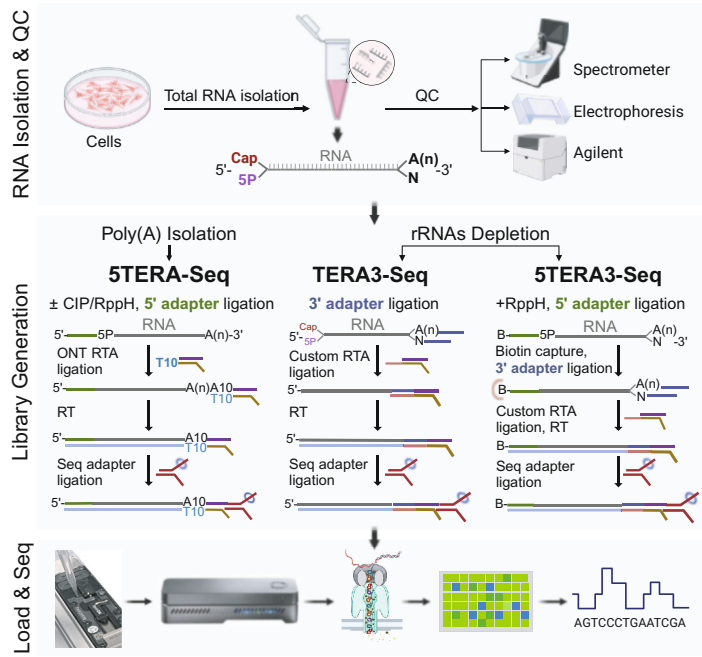

Fig. 1 True End-to-end RNA Sequencing (TERA-Seq). Method schematic for library generation and sequencing. QC, quality control; 5P, 5 monophosphate; A(n), poly(A) tail; N, non-polyadenylated RNAs; rRNAs, ribosomal RNAs; CIP, Calf Intestinal Phosphatase; RppH, RNA 5′ Pyrophosphohydrolase; T, thymidine; ONT, Oxford Nanopore Technology; RTA, Reverse Transcription Adapter; RT, reverse transcription; Seq, sequencing; B, biotin. Schematic at the bottom depicts the ONT sequencing workflow including priming/loading a flow cell, the MinION sequencer, Nanopore sequencing, a representation of sequencing channels (green shades, indicate sequencing/available pores; blue, indicates unavailable pores), and ionic currents generated during sequencing with basecalled sequences. Created with BioRender.com

unprecedented details at the level of single molecules, as we previously demonstrated with the characterization of the protein-coding transcriptome of human HeLa cells [21]. TERA-Seq revealed new insights into mRNA decay and recapitulated key aspects of ribothrypsis [21], which was previously identified with short-read sequencing [11, 22]. TERA-Seq is broadly applicable to characterize RNAs from any source.

2 Materials

2.1 Cell Culture

1. HeLa (human cervical cancer) cells (*see* **Note 1**).
2. Dulbecco's Modified Eagle's Medium (DMEM).
3. Fetal Bovine Serum (FBS).

4. Penicillin/Streptomycin (10,000 U/mL).
5. Trypsin/EDTA 0.05% (w/v).
6. 10x Phosphate-buffered saline (PBS): 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4. Prepare a 1x working solution in water and store at 4 °C.
7. 10 cm petri dishes.
8. 15 mL (RNase-free) centrifuge tubes.

2.2 Oligonucleotides

Use the following RNA/DNA oligonucleotides to generate TERA-Seq libraries (*see* **Note 2**).

1. 5′ adapter (for 5TERA-Seq and 5TERA3-Seq): /5PCBio/rArArUrGrArUrArCrGrGrCrGrArCrCrArCrCrGrArGrArUrCrUrArCrArCrUrCrUrUrUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrU
2. 3′ adapter (for TERA3-Seq and 5TERA3-Seq): /5Phos/rGrUrGrUrCrArGrUrCrArCrUrUrCrCrArGrCGG
3. RTA custom adapter (for TERA3-Seq and 5TERA3-Seq): Top strand: /5Phos/ GGCTTCTTCTTGCTCTTAGGTAG TAGGTTC; Bottom strand: GAGGCGAGCGGTCAATTTT CCTAAGAGCAAGAAGAAGCCCCGCTGGAAGTGACTG ACAC

2.3 Isolation of Total RNA and Quality Control Check

1. TRIzol reagent.
2. RNase Away decontaminating reagent.
3. Nuclease-free water.
4. Acid-Phenol/Chloroform, pH 4.5 (*see* **Note 3**).
5. Chloroform (*see* **Note 3**).
6. RNase-free DNase I.
7. 100% Ethanol, ice cold (*see* **Note 4**).
8. 70% Ethanol in water (v/v), ice cold.
9. 3 M Sodium acetate, pH 5.5.
10. 5 mg/mL Glycogen.
11. 10x Tris-acetate-EDTA (TAE): 0.4 M Tris-acetate, 10 mM EDTA (pH 8.0). Prepare a 1× working solution in water and store at room temperature.
12. Agarose powder.
13. 1 Kb DNA ladder (or equivalent).
14. 1.5 mL (RNase-free) microcentrifuge tubes.
15. Refrigerated microcentrifuge.
16. Nanodrop UV spectrometer (or equivalent).

2.4 Library Generation and Sequencing

1. RNasin Ribonuclease Inhibitor (or equivalent).
2. 10 mM dNTPs solution.
3. T4 RNA Ligase 1.
4. 50% PEG 8000 solution.
5. Quick Calf Intestinal Phosphatase (CIP).
6. RNA 5′ Pyrophosphohydrolase (RppH).
7. 10× Thermopol Buffer.
8. 5× Quick Ligation Reaction Buffer (NEB).
9. 2 M U/mL T4 DNA Ligase.
10. SuperScript III Reverse Transcriptase.
11. Dynabeads Oligo$(dT)_{25}$ (ThermoFisher).
12. Dynabeads MyOne Streptavidin C1 (ThermoFisher).
13. Agencourt RNAClean XP beads (Beckman Coulter).
14. Binding Buffer: 20 mM Tris-HCl (pH 7.5), 1 M LiCl, 2 mM EDTA (pH 8.0). Store at 4 °C (*see* **Note 5**).
15. Washing Buffer B: 10 mM Tris-HCl (pH 7.5), 0.15 M LiCl, 1 mM EDTA (pH 8.0). Store at 4 °C.
16. 1× Annealing Buffer: 10 mM Tris-HCl (pH 8.0), 25 mM NaCl, 0.1 mM EDTA (pH 8.0). Store at 4 °C.
17. 2x Bind and Wash (BW) Buffer: 10 mM Tris-HCl (pH 7.5), 2 M NaCl, 1 mM EDTA (pH 8.0). Store at 4 °C. Prepare a 1× working solution by diluting the 2x stock in nuclease-free water. Store at 4 °C.
18. Formamide Elution Buffer: 95% formamide, 5 mM EDTA (pH 8.0). Store at −20 °C.
19. 70% Ethanol in water (v/v). Store at room temperature.
20. Ribosomal RNAs (rRNAs) depletion kit (*see* **Note 6**).
21. Qubit dsDNA High Sensitivity Assay kit (ThermoFisher).
22. Direct RNA Sequencing kit (ONT, catalog number SQK-RNA002 or equivalent).
23. Flow cells (ONT, catalog number FLO-MIN106 or other compatible cells).
24. MinION sequencer device (ONT, catalog number Mk1B or Mk1C, or equivalent).
25. Qubit Fluorometer.
26. DynaMag-Spin magnet (for 1.5 mL tubes).
27. Thermomixer.
28. Tube rotator.
29. 1.5 mL DNA LoBind tubes.
30. 0.2 mL thin-walled PCR tubes.

3 Methods

Use nuclease-free consumables, reagents, and techniques (*see* **Note 7**). After the isolation of total RNAs, the initial sample preparation steps are different to capture various RNA populations (with or without a 5′ cap or a 3′ poly(A) tail) (Fig. 1). All downstream protocols coverage to the same ONT's protocol steps with minor modifications.

3.1 Cell Culture

1. Seed HeLa cells in DMEM supplemented with 10% FBS and 100 U/mL of penicillin and 100 μg/mL of streptomycin in 10 cm petri dishes under aseptic techniques. Incubate at 37 °C in 5% CO_2 incubator and grow to ~80% confluency before harvest (*see* **Note 8**).
2. For passaging cells, harvest by trypsinization and collect by centrifugation at low speed.

3.2 Isolation of Total RNA and Quality Control Check

1. Harvest cells by aspirating media and washing cells with cold 1x PBS. Aspirate the 1x PBS and collect cells immediately in 1 mL of TRIzol reagent by pipetting to ensure proper lysis of cells. Transfer to a 1.5 mL tube (*see* **Note 9**).
2. Separate RNA by adding 0.2 mL of chloroform, and vortex.
3. Centrifuge at 12,000× g for 15 min at 4 °C.
4. Recover the upper phase. Avoid contaminants from the interphase.
5. Precipitate by adding 2.5 volumes of chilled 100% ethanol, 0.1 volume of 3 M sodium acetate, pH 5.5, and 1 μL of glycogen. Incubate at −80 °C for 2 h.
6. Recover the RNA by centrifugation at 12,000× *g* for 30 min at 4 °C.
7. Discard the supernatant and wash the pellet with chilled 70% ethanol.
8. Centrifuge the RNA at 10,000× *g* for 5 min at 4 °C. Aspirate the supernatant, air-dry, and resuspend the pellet in 25 μL of nuclease-free water.
9. To remove traces of DNA, treat the RNA sample with DNase I (1 U/μg of RNA), and 1x Reaction Buffer in a 50 μL final reaction volume. Incubate at 37 °C for 30 min.
10. Extract the RNA with Acid-Phenol/Chloroform, pH 4.5. Recover and precipitate the RNA as described in **steps 3–8**.
11. Determine the RNA quantity and purity by measuring UV absorption with a spectrometer. The value of the A_{260}/A_{280} ratio is used as an indication for the purity of RNA, acceptable

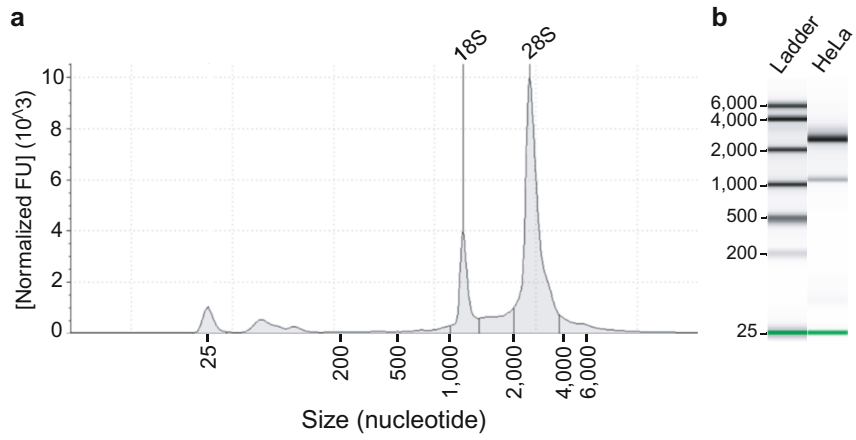

Fig. 2 Quality control check of total RNA. (**a**) Bioanalyzer trace of a representative HeLa total RNA used for TERA-Seq library generation. FU, Fluorescence unit. (**b**) Bioanalyzer profile of HeLa total RNA. Created with BioRender.com

value ranges from 1.8 to 2.0. Determine RNA integrity by running the RNA on a standard agarose gel in 1x TAE buffer, and/or on an Agilent Bioanalyzer (Fig. 2) (*see* **Note 10**).

3.3 Selection of Native 5′ and 3′ Ends of RNAs

To capture the 5′ ends of RNAs (5TERA-Seq), use ~75 μg of total RNAs. To select for different RNA populations (5P, 5′ cap, or 5′ cap and 5P), apply the corresponding enzymatic treatments as described in Subheadings 3.3.1, 3.3.2, or 3.3.3 below (*see* **Note 11**). To capture the 3′ ends (TERA3-Seq) of non- and polyadenylated RNA molecules, use ~100 μg of total RNAs (Subheading 3.3.4). To capture full-length, processing, and decay intermediates of RNA molecules from end-to-end (5TERA3-Seq), with or without poly(A) tails, use ~130 μg of total RNAs and apply the enzymatic treatments described in Subheading 3.3.5 below (Fig. 1) (*see* **Note 12**).

3.3.1 Selection of 5P-Polyadenylated RNAs

1. To enrich for polyadenylated RNAs, adjust the volume of the total RNA to 100 μL with nuclease-free water. Add 100 μL of Binding Buffer and mix by pipetting.
2. Heat the sample at 65 °C for 2 min to disrupt secondary structures. Immediately place on ice.
3. Resuspend the Oligo(dT) beads thoroughly and transfer 100 μL of the beads to a 1.5 mL tube.
4. Place the beads on the magnetic stand and remove the supernatant. Wash the beads by resuspending in 100 μL of Binding Buffer. Remove the supernatant and resuspend the beads in 100 μL of Binding Buffer.

5. Add the total RNA (from **step 2**) to the resuspended beads. Mix by pipetting and allow binding by rotating continuously on a rotator for 5 min at room temperature.
6. Place the tube on the magnetic stand and remove the supernatant.
7. Wash the beads by resuspending in 200 μL of Washing Buffer B. Place the tube on the magnetic stand and remove the buffer. Repeat this step.
8. Ligate the poly(A)-enriched RNAs on beads to the 5′ adapter using T4 RNA Ligase 1 (10 U/μg of RNA), 1x Reaction Buffer, 1 mM ATP, RNasin Ribonuclease Inhibitor (1 U/μL), 12.5% PEG in a 50 μL final reaction volume (*see* **Note 11**). Resuspend the beads in the ligation mixture and then incubate the mixture at 37 °C for 3 h with gentle shaking in a thermomixer.
9. Place the tube on the magnetic stand and remove the supernatant.
10. Wash the beads as described in **step 7**.
11. To elute RNAs off the beads, add 50 μL of nuclease-free water, and mix by pipetting. Incubate the tube at 75 °C for 2 min with gentle shaking in a thermomixer.
12. While waiting, transfer 1.8 volumes of thoroughly resuspended RNAClean XP beads to a 1.5 mL tube. Equilibrate to room temperature.
13. Add the RNA (from **step 11**) to the beads and mix by pipetting. Incubate at room temperature for 5 min with gentle shaking in a mixer.
14. Place the tube on the magnetic stand to allow the beads to settle until the supernatant is clear. Discard the supernatant (*see* **Note 13**).
15. Wash the beads on the magnetic stand by adding 150 μL of 70% ethanol. Carefully discard the ethanol. Leave the beads on the stand and air-dry (*see* **Notes 14** and **15**).
16. Remove the tube from the magnetic stand and thoroughly resuspend the beads in 9.5 μL of nuclease-free water by pipetting. Incubate at room temperature for 5 min in a mixer to allow the release of RNA from the beads. Transfer the RNA to a new 1.5 mL tube.
17. Proceed to Subheading 3.4.

3.3.2 Selection of 5′ Cap-Polyadenylated RNAs

1. Enrich for polyadenylated RNAs as described in **steps 1–7** of Subheading 3.3.1.
2. To remove 5P-containing RNAs, dephosphorylate the poly (A)-enriched RNA on beads in a 50 μL reaction volume using

Quick CIP (1 μL/1–2 pmoles of RNA), 1x CutSmart Buffer (*see* **Note 11**). Incubate at 37 °C for 10 min.

3. Wash the beads three times in 200 μL of Washing Buffer B on the magnetic stand.
4. Remove the 5′ cap of RNA on beads using RppH (1 μL/100 ng of RNA), 1x Thermopol Buffer, in a 200 μL final reaction volume. Resuspend the beads in the decapping mixture and then incubate at 37 °C for 1 h 10 min. Stop the reaction by adding 1 μL of 500 mM EDTA, pH 8.0.
5. Wash the beads as described in **step 3**.
6. Ligate decapped RNAs on beads to the 5′ adapter using T4 RNA Ligase 1 as described in **step 8** of Subheading 3.3.1.
7. Wash the beads as described in **step 3**.
8. Elute RNAs off the oligo(dT) beads, and cleanup and capture RNAs using the RNAClean XP beads as described in **steps 11–16** of Subheading 3.3.1.
9. Proceed to Subheading 3.4.

3.3.3 Selection of Both 5′ Cap- and 5P-Polyadenylated RNAs

1. Enrich for polyadenylated RNAs as described in **steps 1–7** of Subheading 3.3.1.
2. To remove the 5′ cap of poly(A)-enriched RNA on the beads, use RppH as described in **steps 4–5** of Subheading 3.3.2.
3. Ligate RNAs on beads to the 5′ adapter using T4 RNA Ligase 1 as described in **step 8** of Subheading 3.3.1.
4. Wash the beads as described in **steps 9–10** of Subheading 3.3.1.
5. Elute RNAs from the oligo(dT) beads, and cleanup and capture RNAs using the RNAClean XP beads as described in **steps 11–16** of Subheading 3.3.1.
6. Proceed to Subheading 3.4.

3.3.4 Selection of the 3′ Ends of RNAs

1. Anneal the custom RTA adapter by mixing equal concentration of the top strand that contains the RTA sequence and the bottom strand that contains a complementary sequence to the 3′ adapter in 1x Annealing Buffer. Incubate the mixture at 95 °C for 10 min in a thermomixer and gradually cool down to room temperature. Store the annealed adapter in aliquots at −20 °C for later use in Subheading 3.4.
2. Deplete rRNAs from total RNAs using the desired rRNAs depletion kit (*see* **Notes 6** and **12**). In this step rRNAs are depleted using biotinylated oligonucleotides which hybridize with rRNAs, and then the rRNA-oligonucleotides hybrids are removed by binding to Streptavidin beads.

3. Ligate the 3′ adapter to the rRNA-depleted RNAs using T4 RNA Ligase 1, 1x Reaction Buffer, 1 mM ATP, RNasin Ribonuclease Inhibitor (1 U/μL), 7.5% PEG in a 50 μL final reaction volume. Incubate the reaction mixture at 37 °C for 3 h.
4. Cleanup and capture RNAs using RNAClean XP beads as described in **steps 12–16** of Subheading 3.3.1.
5. Proceed to Subheading 3.4.

3.3.5 Selection of Both 5′ and 3′ Ends of RNAs

1. Deplete rRNAs from total RNAs using the desired rRNAs depletion kit (*see* **Notes 6** and **12**).
2. To remove the 5′ cap of RNA, treat the rRNA-depleted RNAs with RppH (1 μL/100 ng of RNA), 1x Thermopol Buffer, in a 200 μL final reaction volume. Incubate the mixture at 37 °C for 1 h 15 min. Stop the reaction by adding 1 μL of 500 mM EDTA, pH 8.0.
3. Cleanup and capture RNAs using the RNAClean XP beads as described in **steps 12–15** of Subheading 3.3.1. Elute the RNA in 20 μL of nuclease-free water.
4. Ligate RNAs to the 5′ adapter using T4 RNA Ligase 1, 1x Reaction Buffer, 1 mM ATP, RNasin Ribonuclease Inhibitor (1 U/μL), 7.5% PEG in a 50 μL final reaction volume (*see* **Note 11**). Incubate the reaction mixture at 37 °C for 3 h.
5. While waiting, prepare the MyOne Streptavidin C1 beads by transferring 50 μL to a 1.5 mL tube.
6. Wash the beads on the magnetic stand by resuspending in 200 μL of 1x BW Buffer. Repeat this step.
7. Resuspend the beads in 50 μL of 2x BW Buffer.
8. To enrich for ligated RNAs, add and mix the RNA from **step 4** with the MyOne Streptavidin C1 beads. Rotate at room temperature for 15 min. Place on the magnetic stand and remove the supernatant.
9. Wash the beads three times with 200 μL of 1x BW Buffer on the magnetic stand.
10. Ligate the RNA to the 3′ adapter on beads using T4 RNA Ligase 1, 1x Reaction Buffer, 1 mM ATP, RNasin Ribonuclease Inhibitor (1 U/μL), 12.5% PEG in a 50 μL reaction (*see* **Note 12**). Resuspend the beads in the ligation mixture and incubate the reaction at 37 °C for 3 h with gentle mixing in a thermomixer.
11. Wash the beads three times with 200 μL of 1x BW Buffer on the magnetic stand.
12. Elute RNAs off the beads by resuspending the beads in 50 μL of Formamide Elution Buffer. Incubate the reaction at 65 °C for 5 min. Transfer the RNA to a new 1.5 mL tube.
13. Cleanup and capture RNAs using RNAClean XP beads as described in **steps 12–16** of Subheading 3.3.1.
14. Proceed to Subheading 3.4.

3.4 Library Generation and Sequencing Using ONT Protocol

Generate the libraries using the SQK RNA002 direct RNA sequencing kit or an equivalent direct RNA sequencing kit as directed by the ONT's protocol (*see* **Note 16**) with minor modifications as described below.

3.4.1 5TERA-Seq Libraries

For all 5TERA-Seq libraries (5P-, 5′ cap-, and 5′ cap- and 5P-polyadenylated RNA molecules), start the library preparation by ligating the ONT's RTA adapter (provided in the ONT kit) using the T4 DNA Ligase and the Quick Ligation Reaction Buffer per ONT protocol's instruction with the following modification; incubate the ligation mixture at 30 °C for 15 min. Synthesize the first strand cDNA using SuperScript III reverse transcriptase per ONT protocol's instruction. Cleanup and capture the RNA-cDNA using the RNAClean XP beads. Elute the RNAs off the beads in 20 μL of nuclease-free water. Ligate the ONT's sequencing adapter (provided in the ONT kit) with the following modification; incubate the ligation mixture at 30 °C for 15 min. Cleanup and capture the ligated RNA-cDNA using the RNAClean XP beads as directed by the ONT's protocol. Elute the RNAs off the beads in 21 μL of Elution Buffer (provided in the ONT kit).

3.4.2 TERA3-Seq and 5TERA3 Libraries

For the generation of all TERA3 and 5TERA3 libraries, use the ligated custom RTA adapter (**step 1** of Subheading 3.3.4) instead of the ONT's RTA adapter. Ligate the adapter using the T4 DNA Ligase and the Quick Ligation Reaction Buffer per ONT protocol's instruction with the following modification; incubate the ligation mixture at 30 °C for 15 min. Synthesize the first strand cDNA using SuperScript III reverse transcriptase, and cleanup and capture the RNA-cDNA using the RNAClean XP beads per ONT protocol's instruction. Elute the RNAs off the beads in 20 μL of nuclease-free water. Ligate the sequencing adapter with the following modification; incubate the ligation mixture at 30 °C for 15 min. Cleanup and capture the ligated RNA-cDNA using the RNAClean XP beads as directed by the ONT's protocol. Elute the RNAs off the beads in 21 μL of Elution Buffer.

3.4.3 Sequencing of the Libraries

Use 1 μL of each library for quantitation using Qubit dsDNA High Sensitivity Assay kit per manufacturer's instructions. If the sample is ready for sequencing, prime the MinION flow cell by thawing the priming buffers (provided in the ONT kit) on ice and as directed by the ONT's protocol. Sequence the libraries on a MinION device (Mk1B or Mk1C) using a flow cell that is compatible with the used direct RNA sequencing kit. Use the standard MinKNOW settings recommended by ONT and run each library for 72 h.

4 Notes

1. Mycoplasma contamination test should be carried out routinely using Mycoplasma detection kit provided from Promega, Lonza, or other commercial suppliers.

2. Oligonucleotides can be obtained from several commercial sources, including Integrated DNA Technologies or Sigma, and are purified using RNase-free HPLC or PAGE. The following are the abbreviations for the listed oligonucleotides; PCBio, photocleavable biotin; 5Phos, 5′ phosphate group; r, ribonucleoside. The sequences are listed in the 5′-to-3′ direction.
3. Phenol and chloroform are highly corrosive and can cause severe burns. Wear protective equipment (solvent-resistant gloves, protective clothing, and safety glasses) when handling phenol and chloroform. Work in a chemical hood and dispose of waste in accordance with institutional regulations.
4. Ethanol is highly flammable and volatile. All manipulation should be carried out under a fume hood.
5. Solutions are prepared from analytical grade chemicals and molecular biology grade (nuclease-free) water. Filter-sterilize reagents and store at the indicated temperature.
6. Ribosomal RNAs (rRNAs) depletion kits such as RiboMinus, NEBNext, and RiboCop are provided from many commercial suppliers including ThermoFisher, NEB, and Lexogen. If the rRNAs-depletion kit does not deplete abundant rRNA species (~85% depletion), the rest of the untargeted rRNAs can be extracted from the researcher's own data to design biotinylated oligonucleotides that target these contaminants. We previously designed a set of biotinylated DNA oligonucleotides based on abundant rRNAs observed in our sequencing runs of samples derived from HeLa cells [22], and using published sequences [23].
7. All collection tubes, tubes containing RNAs, and reaction mixtures should be chilled on ice. For library generation, 0.2 mL PCR tubes should be used for all adapter ligation steps. Non-sticky nuclease-free 1.5 mL LoBind tubes should be used for all beads-purification steps. LoBind tubes are available from several commercial supplies including Eppendorf and USA Scientific.
8. For all TERA-Seq libraries, 1–2 petri dishes should be enough to generate one biological library of each TERA-Seq.
9. RNA samples are recommended to be processed immediately. For long-term storage, RNA can be stored in TRIzol at −80 °C for later usage. The protocol can be safely stopped here.
10. RNA can be stored at −80 °C for later usage. Avoid repeated freeze–thaw cycles of RNA samples.
11. It is recommended to perform a pilot experiment and measure the concentration of the recovered polyadenylated RNAs to calculate the approximate molar concentrations required for

some enzymatic reactions including dephosphorylation and ligation of RNAs to the 5′ adapter (Subheadings 3.3.1, 3.3.2, and 3.3.3). Ligate the adapter by following a 1:1 to 1:2 molar ratio of RNA: adapter.

12. After rRNAs depletion, it is recommended to measure the concentration of RNAs to calculate the approximate molar concentrations required for ligating the rRNA-depleted RNAs to the 3′ and/or 5′ adapters (Subheadings 3.3.4 and 3.3.5). Ligate the adapters by following a 1:1 to 1:2 molar ratio of RNA: adapter.
13. Avoid aspirating beads as this will reduce the yield.
14. The ethanol wash should be performed with the tube on the magnetic stand without suspending the beads, and by rotating the tube on the stand.
15. It is important to remove all ethanol from the bottom of the tube, as it will affect downstream processes. Do not over-dry the beads.
16. Access to ONT's protocols, sequencing kits, devices, compatible flow cells, and updates on direct RNA sequencing kit chemistry are available to ONT community members at https://community.nanoporetech.com/docs/prepare/library_prep_protocols.

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Authors Contribution F.I. and Z.M. designed, F.I. developed and optimized TERA-Seq and modified the Nanopore direct RNA sequencing protocol. F.I. wrote the manuscript with edits from Z.M.

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Chapter 22

Global Profiling and Analysis of 5′ Monophosphorylated mRNA Decay Intermediates

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Abstract

During RNA turnover, the action of endo- and exo-ribonucleases can yield RNA decay intermediates with specific 5′ ends. These RNA decay intermediates have been demonstrated to be the outcome of decapping, microRNA-directed endo-cleavage, or the protected fragments of ribosomes and exon-junction complexes. Therefore, global analysis of RNA decay intermediates can facilitate studies of many RNA decay pathways. In this chapter, we describe a high-throughput sequencing protocol named parallel analysis of RNA ends (PARE), which allows genome-wide profiling of 5′ monophosphorylated mRNA decay intermediates from plants or other eukaryotes. Also, we present the tools and scripts necessary for the proper analysis of RNA degradome data obtained from the PARE method. Details and modifications of library construction procedures and bioinformatic analyses to optimize sequencing quality and cope with emerging sequencing platforms and findings are highlighted.

Key words 5′-3′ RNA decay, Endonucleolytic cleavage, Degradome-seq, 5′P end, miRNA target, Ribosome footprint, EJC footprint

1 Introduction

RNA degradation is crucial for RNA maturation, RNA surveillance, and precise control of gene expression. Exoribonucleases degrade RNA by removing terminal nucleotides while endoribonucleases cut RNA in the middle. Compared to intact eukaryotic mRNAs, RNA decay intermediates are uncapped and/or deadenylated. Although RNA decay intermediates are thought to be transient, their termini may reflect the site cleaved by endoribonucleases or stalling exoribonucleases. Therefore, analysis of RNA decay intermediates can facilitate the studies of diverse posttranscriptional gene controls such as microRNA-guided cleavage, uORF-mediated

Authors Ai-Ping Chen and Wan-Chieh Chen have equally contributed to this chapter.

Maxime Wery (ed.), *mRNA Decay: Methods and Protocols*, Methods in Molecular Biology, vol. 2863,
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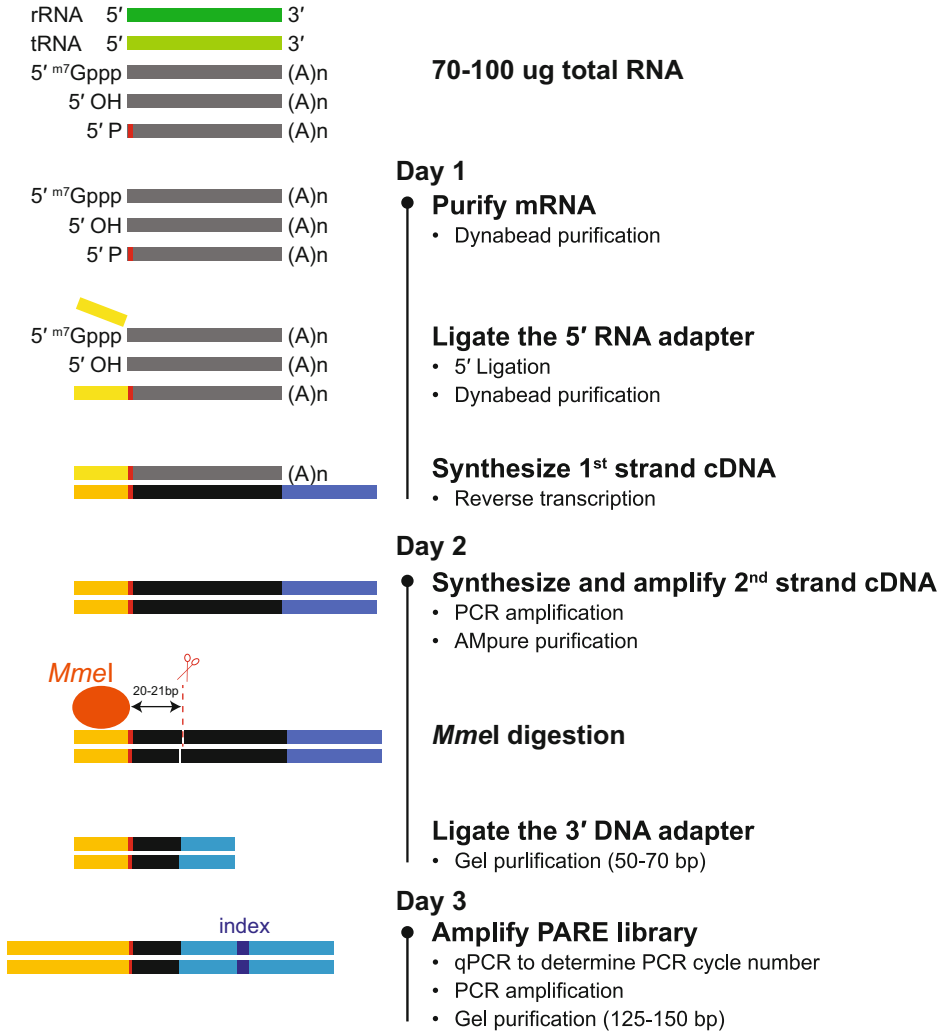

Fig. 1 Workflow of PARE library preparation. The procedures are usually divided into 3 days as illustrated

translational control, and nonsense-mediated decay. With the advancements in sequencing technologies, several methods have been developed for genome-wide profiling of 5′ monophosphorylated mRNA decay intermediates (RNA degradomes) [1–3]. These methods are mostly based on the principles of 5′ RNA ligase-mediated rapid amplification of cDNA ends (5′ RLM-RACE) and next-generation sequencing library construction. However, compared to routine RNA-seq workflows, the construction of RNA degradome libraries can be challenging and impeded by many technical issues. In this chapter, we present a protocol modified from the parallel analysis of RNA ends (PARE) method published by Zhai et al. [4] with step-by-step instructions (Fig. 1). The details of quality checks at multiple points, size selection with gel electrophoresis, and optimization of PCR cycle number in library

amplification presented in this protocol can help to apply this approach to profile the RNA degradomes of other species and yield high-quality data. The computational analysis of PARE data is different from that of regular transcriptome data as PARE reads are only 20–21 nt long and the counts are assigned to the position aligned with the first base of reads. Hence, we cover the bioinformatic tools and special settings for proper preprocessing, mapping, and quantification of *Arabidopsis* PARE data. Also, we demonstrate the use of a genome browser to view PARE data and provide the scripts for producing metagene plots. The experimental and bioinformatic procedures described in this chapter, with some minor modifications, are likely to be applicable to the analysis of RNA degradomes beyond plants and accelerate the studies of many RNA decay pathways.

2 Materials

2.1 Consumables and Reagents

1. 1.5 mL RNase/DNase-free tubes.
2. 1.5 mL DNA LoBind tube.
3. 0.2 mL thin-walled PCR tubes.
4. DNase/RNase-free distilled water.
5. 1 M Tris-HCl, pH 7.5.
6. 1 M Tris-HCI, pH 8.0.
7. Ethanol (EtOH 99.9%), GR grade.
8. Isopropanol (2-Propanol ≥99.8%), ACS grade.
9. 3 M Sodium acetate (NaOAC), pH 5.2.
10. Agilent RNA 6000 Nano Kit and Pico Kit.
11. Dynabeads mRNA Purification Kit (Thermo Fisher Scientific).
12. T4 RNA ligase (Thermo Fisher Scientific).
13. RNaseOUT recombinant ribonuclease inhibitor (Thermo Fisher Scientific).
14. SuperScript III reverse transcriptase (Thermo Fisher Scientific).
15. Phusion Hot Start II polymerase, 5× GC buffer and 100% DMSO.
16. *Mme*I (NEB).
17. T4 DNA ligase (NEB).
18. Novex™ TBE gels 8%, 10 well (Thermo Fisher Scientific).
19. TBE buffer.
20. Blue/Orange loading dye.
21. SYBR Gold Nucleic Acid Gel Stain.

22. SYBR Green I Nucleic Acid Gel Stain—10,000× concentrate in DMSO.
23. 25-bp DNA Step Ladder.
24. O′RangeRuler 10-bp DNA Ladder (Thermo Fisher Scientific).
25. Single-edge razor blade.
26. Corning® Costar® Spin-X® centrifuge tube filters, 0.45 μm.
27. Linear acrylamide.
28. 0.2 mL 8-tube PCR strips without caps, low profile, clear.
29. 0.2 mL flat PCR tube 8-cap strips, optical, ultraclear.
30. Agencourt AMPure XP (Beckman-Coulter).
31. Qubit dsDNA HS Assay Kits (Invitrogen).
32. Fragment Analyzer DNA/NGS Kits (Agilent Technologies).

2.2 Oligonucleotides

1. Sequences and purification methods of the oligonucleotides are described in Table 1. The index primers of the PARE library can be replaced by Illumina TruSeq small RNA PCR Primer Index 1–48.

2.3 Apparatus

1. Nanodrop spectrophotometer.
2. Agilent 2100 Bioanalyzer.
3. PCR machine.
4. Dry bath incubator.
5. MagRack 6 (Cytiva).
6. NEBNext magnetic separation rack.
7. XCell SureLock™ Mini-Cell (Thermo Fisher Scientific).
8. Orbital shaker.
9. Microcentrifuge pestle.
10. Refrigerated centrifuge.
11. CFX96 Touch Real-Time PCR detection system (Bio-Rad, discontinued).
12. Qubit-2.0 fluorometer (Thermo Fisher Scientific).
13. LED Transilluminator, Blue 470 nm.
14. 5200 Fragment Analyzer System (Agilent Technologies).
15. Eppendorf concentrator.

2.4 Bioinformatics

The recommended requirements for computers for PARE data analysis are 64-bit Linux OS, 16-core CPU, and 32 GB of RAM. Also, the following tools are required and can be downloaded from the URLs indicated in parentheses:

Table 1
Adapters and sequencing primers

Name	Sequence (5′ to 3′)[a]	Length	Purification
PARE 5′ RNA adapter	rGrUrUrCrArGrArGrUrUrCrUrArCrArGrUrCrCrGrArC	22	RNase free HPLC
PARE RT-primer	CGAGCACAGAATTAATACGAC TTTTTTTTTTTTTTTTT	39	HPLC
PARE cDNA PCR primer F	GTTCAGAGTTCTACAGTCCGAC	22	HPLC
PARE cDNA PCR primer R	CGAGCACAGAATTAATACGACT	22	HPLC
PARE 3′ DNA adapter top	TGGAATTCTCGGGTGCCAAGG	21	HPLC
PARE 3′ DNA adapter bottom	CCTTGGCACCCGAGAATTCCANN	23	Desalting
PARE final PCR PE primer F	AATGATACGGCGACCACCGAGATCTACACG TTCAGAGTTCTACAGTCCGA	50	HPLC
PARE final PCR primer 2R	CAAGCAGAAGACGGCATACGAGAT**ACATCG**GTGAC TGGAGTTCCTTGGCACCCGAGAATTCCA	63	HPLC
PARE final PCR primer 3R	CAAGCAGAAGACGGCATACGAGAT**GCCTAA**GTGAC TGGAGTTCCTTGGCACCCGAGAATTCCA	63	HPLC
PARE final PCR primer 4R	CAAGCAGAAGACGGCATACGAGAT**TGGTCA**GTGAC TGGAGTTCCTTGGCACCCGAGAATTCCA	63	HPLC
PARE final PCR primer 5R	CAAGCAGAAGACGGCATACGAGAT**CACTGT**GTGAC TGGAGTTCCTTGGCACCCGAGAATTCCA	63	HPLC
PARE PE Seq primer	CCACCGAGATCTACACGTTCAGAGTTCTACAG TCCGAC	38	HPLC

[a]Bold letters indicate barcodes

1. SRA Toolkit and Entrez Direct (https://www.ncbi.nlm.nih.gov/home/tools/).
2. SAMtools and HTSlib (https://www.htslib.org/).
3. GFF utilities (https://ccb.jhu.edu/software/stringtie/gff.shtml).
4. Bowtie (https://bowtie-bio.sourceforge.net/).
5. STAR (https://github.com/alexdobin/STAR).
6. Cutadapt (https://cutadapt.readthedocs.io/).
7. deepTools (https://github.com/deeptools/deepTools).
8. JBrowse 2 desktop (https://jbrowse.org/jb2/).
9. Guitar of R/Bioconductor Package (https://bioconductor.org/packages/release/bioc/html/Guitar.html).

3 Methods

3.1 Purification of mRNA from Total RNA

1. Prepare 70–100 μg of high-quality total RNA (*see* **Note 1**) and adjust the sample volume to 100 μL with nuclease-free water in a PCR tube. Incubate the RNA sample at 65 °C for 2 min in a PCR machine and keep it on ice immediately to disrupt RNA secondary structures.
2. Vortex the Dynabeads vigorously for 1 min. Transfer 200 μL of Dynabeads to a 1.5 mL microcentrifuge tube. Place the tube on a magnetic stand at room temperature for 30 s or until the solution is clear. Discard all of the supernatant carefully without disturbing the beads.
3. Remove the tube from the magnetic stand. Add 200 μL of Binding Buffer from the Dynabeads kit to wash the beads by pipetting.
4. Place the tube on the magnetic stand for 30 s or until the solution is clear, and discard the supernatant again.
5. Remove the tube from the magnetic stand. Add 100 μL of Binding Buffer and resuspend the beads by pipetting.
6. Mix the resuspended beads with the total RNA sample from **step 1** by pipetting. Place the sample tube at room temperature for 10 min and vortex shortly in the middle of incubation to allow poly(A) RNA (mRNA) to anneal to the beads. Place the tube on the magnetic stand for 1 min or until the solution is clear, and discard the supernatant.
7. Remove the tube from the magnetic stand and wash the beads with 200 μL of Washing Buffer B from the Dynabeads kit by pipetting. Place the tube on the magnetic stand for 1 min and discard the supernatant. Repeat this step once.
8. Remove the tube from the magnetic stand. Add 16 μL of 10 mM Tris-HCl, pH 7.5 (elution buffer) to resuspend the beads by pipetting. Incubate the sample at 65 °C for 2 min in a dry bath incubator to release mRNA from the beads. Place the tube on the magnetic stand immediately and transfer 15 μL of the supernatant (mRNA) to a PCR tube. Keep the mRNA on ice before proceeding to the next step. The profile of the RNA after the first mRNA purification might be checked using a Bioanalyzer (*see* **Note 2**) (Fig. 2).

3.2 Ligation of the 5′ RNA Adapter

1. Prepare the ligation reaction as followed (total volume of 20 μL): 15 μL of RNA, 1 μL of 5′ RNA adapter (200 μM), 2 μL of 10× RNA ligase buffer, 1.5 μL of T4 RNA ligase (5 U/μL), and 0.5 μL of RNaseOUT (40 U/μL). Mix the reaction thoroughly by pipetting. Incubate the ligation reaction at 37 °C for 2 h.

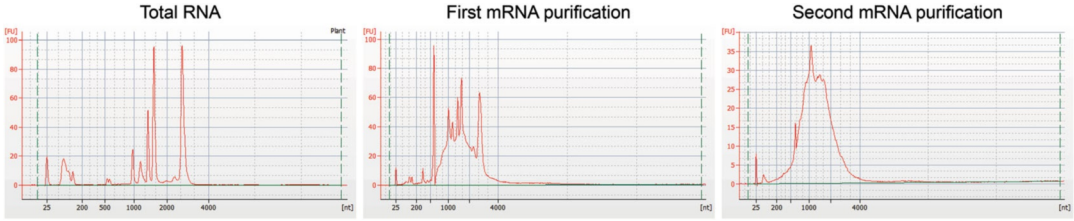

Fig. 2 Bioanalyzer profiles of *Arabidopsis* high-quality total RNA, and the RNA after the 1st and 2nd mRNA purification

2. Add 80 μL of nuclease-free water to the ligation mixture and heat at 65 °C for 10 min in a PCR machine to terminate the reaction. Then move the ligation mixture to ice immediately.

3.3 Unligated 5′ Adapter Removal

1. Add the ligation mixture to 100 μL of Dynabeads/Binding buffer suspension that is prepared using the same procedure as described in **steps 2–5** of Subheading 3.1. Mix it thoroughly by pipetting. Place the tube at room temperature for 10 min and vortex briefly in the middle of incubation.
2. Place the tube on the magnetic stand for 1 min or until the solution is clear, and discard the supernatant. Wash the beads twice using the same procedure as described in **step 7** of Subheading 3.1.
3. Remove the tube from the magnetic stand. Add 27.5 μL of 10 mM Tris-HCl, pH 7.5 to resuspend the beads to elute the mRNA and transfer the supernatant (5′ adapter-ligated mRNA) into a PCR tube. Keep the tube on ice before proceeding to the next step. The profile of the RNA after the 2nd mRNA purification can be checked using a Bioanalyzer (*see* **Note 3**) (Fig. 2).

3.4 First-Strand cDNA Synthesis

1. Mix 27.5 μL of the purified 5′ adapter-ligated mRNA with 2 μL of 100 μM PARE RT primer. Denature the sample at 65 °C for 2 min and then transfer the sample to 25 °C or room temperature immediately.
2. Prepare a reverse transcription (RT) reaction as followed: 29.5 μL of 5′ ligated RNA + RT primer, 10 μL of 5× FS buffer, 2 μL of 10 mM dNTP mix, 2.5 μL of 0.1 M DTT, 2 μL of RNaseOUT (40 U/μL), and 2 μL of SuperScript III (200 U/μL). Mix it thoroughly by pipetting.
3. Incubate the RT reaction at 48 °C for 90 min, then add again 2 μL of SuperScript III. Incubate again at 48 °C for 90 min, then at 72 °C for 10 min, and keep at 4 °C before cDNA amplification (*see* **Note 4**).

3.5 Synthesis and Amplification of Second-Strand cDNA

1. Prepare the second-strand cDNA synthesis reaction as follows: 25 μL of first strand cDNA, 7.25 μL of H_2O, 1.5 μL of 100% DMSO, 10 μL of 5× GC buffer, 1.25 μL of 10 mM dNTP mix, 2 μL of PARE cDNA primer F (10 μM), 2 μL of PARE cDNA primer R (10 μM), and 1 μL of Phusion Pol. Mix it thoroughly by pipetting.
2. Perform the polymerase chain reaction (PCR) using the following program: 60 s at 98 °C (initial denaturation); 7 cycles of 30 s at 98 °C, 30 s at 58 °C, 5 min at 72 °C; 7 min at 72 °C (final extension), hold at 4 °C.

3.6 Purification of Double-Stranded cDNA

1. Add 90 μL of AMPure XP beads (*see* **Note 5**) to 50 μL of the amplified cDNA sample (volumetric ratio of beads: DNA = 1.8:1). Mix the mixture thoroughly by pipetting and then keep it at room temperature for 15 min.
2. Place the tube on the magnetic stand for 2 min and discard the supernatant.
3. Keep the tube on the magnetic stand and add 200 μL of freshly prepared 80% EtOH. After 30 s without disturbing the beads, discard the supernatant. Repeat this step once.
4. Remove any remaining EtOH and dry the pellet for 2–5 min at room temperature (*see* **Note 6**).
5. Add 16.5 μL of nuclease-free water to the beads and then remove the tube from the magnetic stand. Fully resuspend the beads by pipetting and keep the tube at room temperature for 5 min. Place the tube on the magnetic stand for 2 min and transfer 16 μL of supernatant (double-stranded cDNA) to a PCR tube.

3.7 Mme*I* Digestion

1. Prepare the *Mme*I digestion reaction as followed: 16 μL of double-stranded cDNA, 2 μL of CutSmart buffer, 2 μL of *Mme*I (2 U/μL). Mix the reaction thoroughly by pipetting. Incubate the mixture at 37 °C for 120 min.
2. Stop the reaction by heating at 65 °C for 10 min in a PCR machine. Keep the mixture at room temperature before ligation.

3.8 3′ DNA Adapter Ligation

1. Prepare the 3′ DNA adapter by mixing an equal volume of 200 μM PARE 3′ DNA adapter top and bottom. The minimum volume for this reaction is 30 μL. Heat the mixed oligonucleotides to 100 °C for 5 min and gradually cool down at 0.1 °C per second until the mixture reaches 25 °C. The annealed 3′ DNA adapter can be stored at −20 °C for up to four freeze–thaw cycles.
2. Prepare the ligation reaction as followed: 20 μL of digested cDNA, 4.5 μL of H_2O, 2 μL of 3′ adapter (100 μM), 3 μL of

10× T4 DNA ligase buffer, 0.5 μL of T4 DNA ligase (2000 U/μL). Mix thoroughly by pipetting. Incubate the ligation reaction at 25 °C for 1 h.

3.9 Gel-Selection and Concentration of the Ligated dsDNA Product

1. Use an 8% TBE gel for selection of the ligated dsDNA product. Prepare dsDNA samples by adding 6 μL of Blue/Orange Loading Dye to the 30 μL of ligated dsDNA. Prepare 6 μL of each ladder by mixing 2.5 μL of 25-bp DNA step ladder or 10-bp DNA ladder with 2.5 μL of H_2O and 1 μL of Blue/Orange Loading Dye. Each sample is accompanied by two ladders. To avoid sample contamination, a 10-well gel is only used for two samples (Fig. 3). Run the gel with 0.5× TBE buffer at 100 V for 10 min first and then increase the voltage to 200 V and run for 35 min.
2. With the slot side facing up, disassemble the gel cassette (Fig. 3). Keep the gel foot to determine the front side of the gel. Cut each gel in half with the gel knife between lanes 5 and 6, and transfer each half to an individual container to minimize sample contamination (*see* **Note 7**). Cover the container with aluminum foil to avoid light exposure, and perform gel staining (2 μL of SYBR Gold in 40 mL of deionized water) at room temperature on an orbital shaker set at 30 rpm for 10–20 min.
3. Transfer the stained gel with a piece of plastic wrap to a gel imaging system to visualize the samples and markers, and record the image. The expected size of a successful product after *Mme*I digestion and ligation of adapters on both ends is 63 bp [5′ adapter (22 bp) + *Mme*I digested fragment (20 bp) + 3′ adapter (21 bp)]. However, the products of this size are likely invisible but there is one distinct nonspecific band at 70 bp (Fig. 3). On a blue light transilluminator, excise a gel slice from the sample lane spanning between the lower edge of 70 bp and the upper edge of the 50 bp band of the 10-bp Ladder with a razor blade while avoiding to include the nonspecific band (Fig. 3).

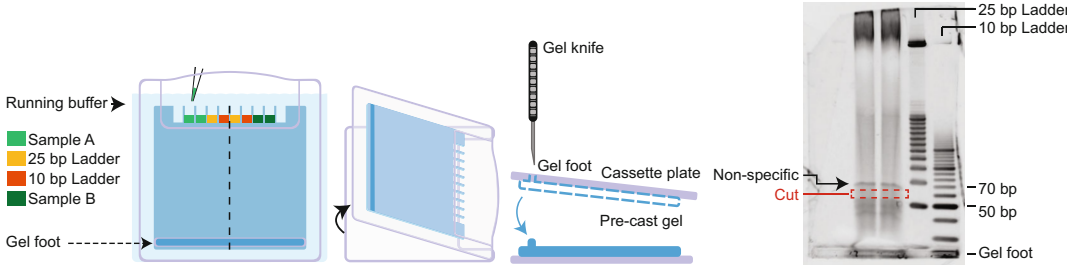

Fig. 3 Gel electrophoresis and size selection of the ligated dsDNA. DNA samples and markers are loaded on a gel with the order illustrated. Using a gel knife, open the gel cassette from the bottom to avoid contamination. After electrophoresis, the desired DNA fragments are not visible. The red dashed box marks the cutting area

4. Transfer the gel slice to a 1.5 mL microcentrifuge tube, spin down the tube to ensure that the gel slice is at the bottom of the tube. Store the tube at −20 °C for 5 min to freeze the gel slice. Grind the gel slice into smaller pieces with a microcentrifuge-pestle (*see* **Note 8**). Add 500 μL of 10 mM Tris-HCl, pH 8.0 and let the buffer flow through the pestle to collect as much as possible of the gel particles.
5. Incubate the gel-containing tube at 70 °C for 10 min. Vortex and spin down the tube three times during incubation. Then centrifuge the tube at 16000× *g* for 1 min, transfer the gel mixture to a Spin-X centrifuge tube filter, and centrifuge at 16000× *g* for 10 min to collect the flow-through.
6. Transfer the flow-through to a DNA LoBind tube and adjust the sample volume to 500 μL with nuclease-free water. Add 50 μL of 3 M Sodium acetate (NaOAc) pH 5.2 and 2.2 μL of linear acrylamide (5 mg/mL), and fully resuspend the mixture before adding 550 μL of isopropanol. Invert the tube for several times, spin down and keep the tube at −80 °C overnight.
7. Centrifuge the tube at 16000× *g* for 50 min at 4 °C to precipitate DNA. Remove 1000 μL of the supernatant carefully and leave approximately 100 μL behind to avoid disturbing the pellet.
8. Wash the pellet with 1 mL of 80% ice-cold ethanol and flick the tube several times to dislodge the pellet from the tube wall. Centrifuge the sample at 16000× *g* for 20 min at 4 °C and drain off the supernatant without disturbing the pellet. Centrifuge the sample at 16000× *g* for 1 min at 4 °C and remove the remaining ethanol carefully with a P10 tip.
9. Dry the pellet in a concentrator at room temperature for 5 min.
10. Add 34 μL of nuclease-free water and entirely resuspend the DNA by flicking the tube. The DNA can be kept at −20 °C for long-term storage.

3.10 Determination of the PCR Cycle Number and DNA Input by qPCR

1. Use 1 μL of the gel-purified dsDNA from **step 10** of Subheading 3.9 as a template and prepare the qPCR master mix as followed: 11.3 μL of H_2O, 0.8 μL of 25× SYBR Green I, 0.6 μL of 100% DMSO, 4 μL of 5× GC buffer, 0.5 μL of 10 mM dNTP mix, 0.8 μL of PARE final PCR PE primer F (10 μM), 0.8 μL of PARE final PCR primer 2R (10 μM), and 0.2 μL of Phusion Pol. Mix the reaction thoroughly by pipetting (*see* **Note 9**).
2. Perform the qPCR using the following program: 30 s at 98 °C (initial denaturation); 35 cycles of 10 s at 98 °C, 30 s at 60 °C, 20 s at 72 °C; 10 s at 95 °C followed by a melting curve

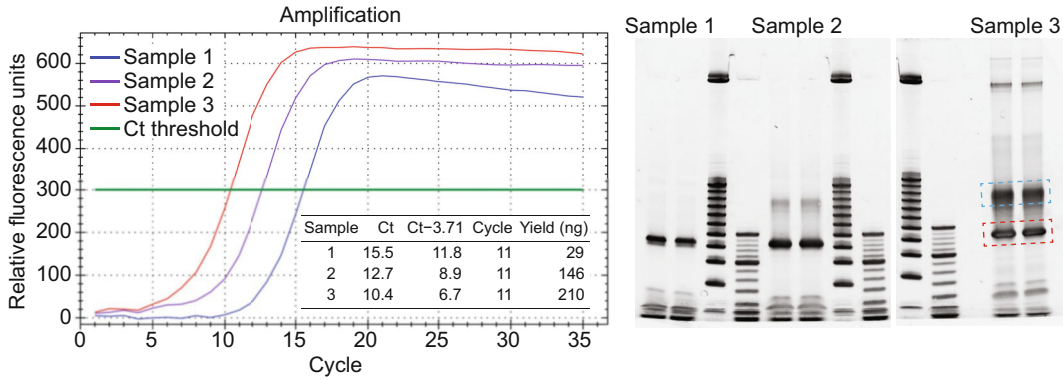

Fig. 4 An example of determining the PCR cycle number for library amplification. An appropriate PCR cycle number for final library amplification of each sample can be calculated using the Ct value obtained from a qPCR test. The table presents the Ct values (Ct), the appropriate PCR cycle numbers (Ct-3.71), and the final gel-purified yield of three samples. The gel image shows the 11-cycle PCR products of three samples. The red dashed box marks the products of the correct size, and the blue dashed box marks the incorrect products due to over-amplification

protocol starting at 65 °C and increasing by 0.5 °C every 0.5 s until 95 °C.

3. Use the Cycle threshold (Ct) value to determine the PCR cycle number (X) for final library amplification or adjust the amount of DNA input (Fig. 4) (*see* **Note 10**).

3.11 PCR Amplification of the PARE Library

1. Adjust the volume of each gel-purified dsDNA from **step 10** of Subheading 3.9 to 16.4 μL by adding nuclease-free water or using a concentrator.
2. Add 1 μL of 10 μM PARE Final PCR primer (index primer) to each dsDNA sample.
3. Prepare the PCR master mix on ice as followed: 0.75 μL of 100% DMSO, 5 μL of 5× GC buffer, 0.6 μL of 10 mM dNTP mix, 1 μL of final PE PCR primer F (10 μM), and 0.25 μL of Phusion Pol. Add 7.6 μL of the master mix to each sample and pipet several times. Perform the PCR as followed, using the cycle number (X) determined in **step 3** of Subheading 3.10: 30 s at 98 °C (initial denaturation); (X) cycles of 10 s at 98 °C, 30 s at 60 °C, 20 s at 72 °C; 5 min at 72 °C (final extension); hold at 4 °C.

3.12 Gel-Selection and Concentration of the PARE Library

1. Use an 8% TBE gel for selection and concentration of the PARE library. At this step, a 10-well gel can be used for three barcoded samples as illustrated in Fig. 5.
2. Add 5 μL of Blue/Orange Loading Dye to the 25 μL of the final PCR products. Prepare the ladders by mixing in separate tubes 2.5 μL of each ladder (25-bp DNA step ladder and 10-bp

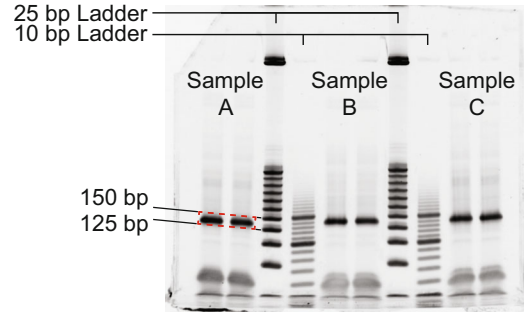

Fig. 5 Size selection of the PCR-amplified PARE library from a TBE gel. The red dashed box marks the cutting area

DNA ladder) with 2.5 μL of H_2O and 1 μL of Blue/Orange Loading Dye.

3. Load the samples, run the gel with 0.5× TBE at 100 V for 10 min first, and later increase the voltage to 145 V to run for 60 min.
4. Disassemble the gel cassette, perform gel staining and gel visualization using the same procedure as described in **steps 2–3** of Subheading 3.9. Here, staining the gels in individual containers is unnecessary as each library has a unique index.
5. The expected size of the final library product is 134 bp [5′ adapter (51 bp) + insert (20 bp) + 3′ index adapter (63 bp)]. A clear and prominent band of this size should be observed (Fig. 5). On the transilluminator, excise a gel slice of the sample lane spanning between the lower edge of the 150 bp band and the upper edge of the 125 bp band (25-bp Ladder) with a razor blade.
6. Perform gel elution and isopropanol precipitation using the same procedure as described in **steps 4–9** of Subheading 3.9.
7. Add 20 μL of 10 mM Tris-HCl, pH 8.0 to the pellet and fully resuspend it by flicking the tube. The PARE library can be kept at −20 °C for long-term storage.

3.13 Analysis of the Quantity and Quality of the PARE Library

1. Use 1 μL of the PARE library to determine the concentration using a Qubit fluorometer or other fluorescence-based methods. Typically, the yield of PARE libraries ranges between 20 and 150 ng.
2. Use 2 μL of the diluted library (0.5–1 ng/ μL) to check the fragment size profile using the Fragment Analyzer systems with the DNF-477 HS NGS Fragment Analysis Kit (1–1500 bp). The Fragment Analyzer profile of a high-quality PARE library constructed with the primers described in this protocol should have a single and prominent peak around 139 bp (Fig. 6).

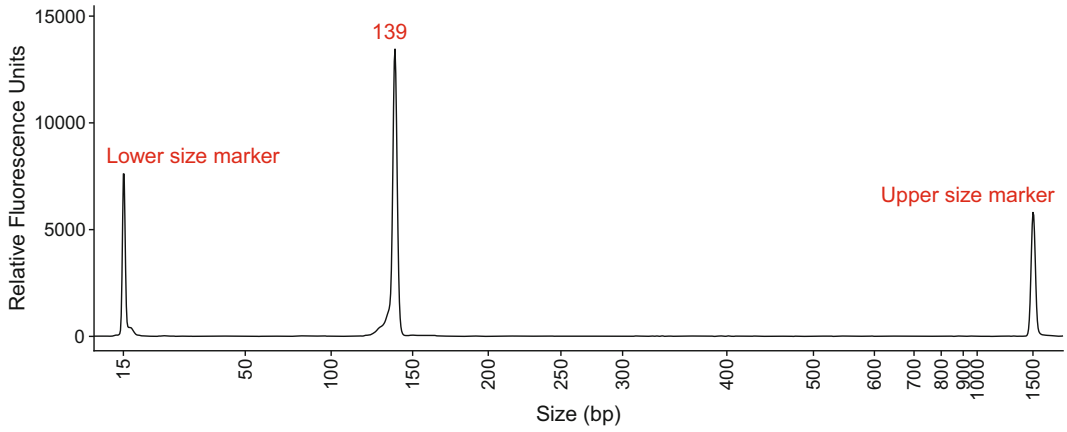

Fig. 6 Size distribution of DNA fragments in an *Arabidopsis* PARE library determined by Fragment Analyzer. The library displays a single peak of 139 bp

3.14 PARE Library Sequencing

1. Use nuclease-free water to adjust the concentration of the library to 2 nM or the one your sequencing service provider requires.
2. Due to the presence of an *Mme*I site within the 5′ adapter used in PARE library construction, a customized Read 1 primer (PARE PE Seq Primer; *see* Table 1) is essential for sequencing, which must accompany your PARE libraries submitted to your sequencing service providers. Previously, PARE libraries, as described by Zhai et al. [4], were only compatible with Illumina SR (Single Read) flow cells. However, in our modified protocol, two primer sequences (PARE Final PCR PE primer and PARE PE Seq Primer; *see* Table 1) have been modified to enable sequencing of PARE libraries on both Illumina SR and PE (Paired End) flow cells, such as those found in HiSeq2000/2500 or NextSeq500/550 platforms. The sequencing read length needs to exceed 50 nt, and we recommend a minimum of 40 million reads per library. While samples with different barcodes can be sequenced within the same lane of a flow cell, it is crucial to note that libraries using different Read 1 primers cannot share the same lane.

3.15 PARE Data Analysis

Computational analysis of PARE data includes preprocessing of raw data (adapter trimming and quality control), mapping of clean reads (genome and transcriptome alignments), count quantification (assignment and normalization), visualization of RNA decay profiles (plot of PARE counts along transcripts or specific sites), and global analysis/comparison (metagene plots). The parameters or steps specifically required for PARE data analysis are highlighted below and the complete scripts can be found at https://github.com/LabHMChenABRC/PARE-analysis.

3.15.1 Acquisition of Published PARE Datasets

Published PARE datasets can be downloaded from the NCBI Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra) using "fasterq-dump" program from SRA-Toolkit [5]. Download a test PARE dataset consisting of three *Arabidopsis* samples of WT, *xrn4-6* and *fry1-6* mutants (SRR7652708, SRR7652710, SRR7652712) in fastq.gz format using the following script:

```
For Run in SRR7652708 SRR7652710 SRR7652712
do
 fasterq-dump $Run
 bgzip $Run.fastq
done
```

3.15.2 Preparation of the Reference Sequences and Indexes

The *Arabidopsis* reference genome sequence (TAIR10_chr_all.fas.gz) and annotation (TAIR10_GFF3_genes.gff) can be downloaded from The *Arabidopsis* Information Resource (TAIR) website at https://www.arabidopsis.org/download/overview. The sequences of *Arabidopsis* noncoding RNAs in fasta format (TAIR10.ncRNAs.fasta) are the annotated ncRNAs extracted from the genome sequence using gffread with the gff file, plus an unannotated rRNA fragment (geneBank ID:X52320.1) downloaded from the NCBI by efetch of Entrez Direct [6, 7]. *Arabidopsis* mitochondria and chloroplast genome sequences in fasta format (TAIR10.chrCM.fasta) can be extracted from the genome by samtools faidx [8]. These sequence files are indexed before mapping using the following commands of Bowtie [9] or STAR [10].

1. Build a Bowtie index of *Arabidopsis* noncoding RNAs (TAIR10.ncRNA):

   ```
   > bowtie-build TAIR10.ncRNAs.fasta ./BowtieIndex/TAIR10.ncRNA
   ```

2. Build a Bowtie index of *Arabidopsis* mitochondria and chloroplast genomes (TAIR10.chrCM):

   ```
   > bowtie-build TAIR10.chrCM.fasta ./BowtieIndex/TAIR10.chrCM
   ```

3. Build a STAR index of *Arabidopsis* genome sequence (TAIR10) with the annotated file (TAIR10_GFF3_genes.gtf):

   ```
   > gffread -T -o TAIR10_GFF3_genes.gtf TAIR10_GFF3_genes.gff
   > STAR \
   --runMode genomeGenerate \
   --genomeDir ./STARIndex/TAIR10 \
   --genomeFastaFiles TAIR10_chr_all.fasta \
   --sjdbGTFfile TAIR10_GFF3_genes.gtf
   ```

3.15.3 Preprocessing of Raw Data and Mapping

Raw reads (PARE.raw.fastq.gz) derived from the PARE library construction are 20 or 21 bp of inserts followed by the 3′ adapter sequence. Use Cutadapt [11] to trim the 3′ adapter sequence, select 20–21 bp trimmed reads with low total sequencing error rates into a trimmed file (trimmed.adapter.fastq.gz), and save the reads longer than 21 bp reads to the overlength file (overlength.fastq.gz). Lastly, trimmed reads mapped to organelle genomes or noncoding RNAs are filtered out using bowtie and mapped the remaining reads to the reference sequence by STAR. In *Arabidopsis*, PARE reads that are perfectly mapped to noncoding RNAs and organelle genomes normally account for less than 4% and 14% of total processed reads, respectively. After filtering these reads, a minimum of 90% of the remaining reads can be mapped to the nuclear genome.

1. Remove the adapter and filter read quality (expected errors) by Cutadapt:

```
> cutadapt \
--adapter TGGAATTCTCGGGTGCCAAGG \
--minimum-length 20 \
--maximum-length 21 \
--max-expected-errors 1 \
--too-long-output overlength.fastq.gz \
-o trimmed.adapter.fastq.gz \
PARE.raw.fastq.gz     >PARE.trimmed.adapter.
report.txt
```

2. For a PARE dataset generated through a two-channel SBS sequencing technology (*see* **Note 11**), the first 20-nt sequences of overlength reads (overlength.fastq.gz) are extracted and combined with the adapter-trimmed file from **step 1** to become the read file (PARE.trimmed.fastq.gz) for mapping:

```
> cutadapt \
--length 20 \
--max-expected-errors 1 \
-o shorten.fastq.gz \
overlength.fastq.gz     >shorten.overlength.
report.txt
> cat trimmed.fastq.gz shorten.fastq.gz >PARE.
trimmed.fastq.gz
```

3. Remove the reads perfectly matched to noncoding RNAs (TAIR10.ncRNA) by Bowtie:

```
> bowtie -q -k 1 -v 0 \
--un PARE.remove.ncRNA.fastq.gz \
--norc \
-x ./BowtieIndex/TAIR10.ncRNA \
PARE.trimmed.fastq.gz /dev/null
```

4. Remove the reads perfectly matched to mitochondria or chloroplast genomes (TAIR10.chrCM) by Bowtie:

```
> bowtie -q -k 1 -v 0 \
--un PARE.remaining.fastq.gz \
-x ./BowtieIndex/TAIR10.chrCM \
PARE.remove.ncRNA.fastq.gz /dev/null
```

5. Map the remaining reads (PARE.remaining.fastq.gz) to the *Arabidopsis* nuclear genome and allow spliced alignments across annotated splice junctions by STAR with the parameter settings suitable for PARE reads (*see* **Notes 12–17**). Genome and transcriptome alignment files are generated in bam format separately.

```
> STAR \
--readFilesIn PARE.remaining.fastq.gz \
--outFileNamePrefix PARE_ \
--genomeDir ./STARIndex/TAIR10 \
--outMultimapperOrder Random \
--alignEndsType EndToEnd \
--outFilterMismatchNmax 0 \
--outFilterMultimapNmax 10 \
--alignIntronMax 100000 \
--alignSJoverhangMin 100 \
--alignSJDBoverhangMin 1 \
--outSAMtype BAM SortedByCoordinate \
--quantMode TranscriptomeSAM
```

3.15.4 Quantification

After mapping, counts of PARE reads are assigned to the coordinate of a transcript or genome that is aligned with the first base of reads (*see* **Note 18**). The total count of a coordinate is normalized to tags per 40 million (TP40M), which then is saved in bigwig format for display on genome browsers. Two bigwig files in genomic coordinates are created to display the normalized abundance of PARE reads on the forward (Watson) and reverse (Crick) strands separately. The bigwig file in transcript coordinates only contains the forward alignments from the transcript bam file. Steps to create a forward bigwig file using deepTools modules bamCoverage [12] (*see* **Note 19**) are shown below:

1. Obtain the total count of primary alignments (mapping_cnt) in the genomic bam file (PARE_Aligned.sortedByCoord.out.bam) using SAMtools:

```
> mapping_cnt=$(samtools view -c -F 256 PARE_Aligned.sortedByCoord.out.bam)
```

2. Calculate the scale factor of TP40M (scale_factor) for normalization:

```
> scale_factor=$(perl -e "printf('%.2f',40*1000000/$mapping_cnt)")
```

3. Index the bam file to run bamCoverage:

```
samtools index PARE.Aligned.sortedByCoord.out.bam
```

4. Create a bigwig file with genomic forward alignments (PARE.genomic.plus.bw) using bamCoverage with the scale factor.

```
> bamCoverage \
-b PARE.Aligned.sortedByCoord.out.bam \
--outFileFormat bigwig \
--Offset 1 \
--binSize 1 \
--samFlagExclude 256 \
--scaleFactor $scale_factor \
--filterRNAstrand reverse -o PARE.genomic.plus.bw
```

3.15.5 Visualization of PARE Data with a Genome Browser

View the distribution of normalized abundance of PARE reads across the genome or transcripts using a genome browser. Load bigwig files in genomic or transcript coordinates to JBrowse2, which can be installed on a desktop or server [13]. Steps to create the required files and launch JBrowse2 desktop version are described below.

1. Prepare the genomic fasta index (TAIR10_chr_all.fasta.fai) for JBrowse2 using samtools:

```
> samtools faidx TAIR10_chr_all.fasta
```

2. Prepare the Tabix gff files (TAIR10_GFF3_genes.sorted.gff.gz and TAIR10_GFF3_genes.gff.gz.tbi) for JBrowse2 using tabix program of HTSlib [8].

```
> (grep ^"#" TAIR10_GFF3_genes.gff; grep -v ^"#" TAIR10_GFF3_genes.gff | grep -v "^$" | grep "\t" | sort -k1,1 -k4,4n) | bgzip >TAIR10_GFF3_genes.sorted.gff.gz
> tabix -p gff TAIR10_GFF3_genes.sorted.gff.gz
```

3. Open the Jbrowse2 desktop, and press "open sequence" with genomic fasta (TAIR10_chr_all.fasta) and fai files (TAIR10_chr_all.fasta.fai). Then launch the linear genome view and click "open track selector". Add tracks with Tabix gff files and genomic bigwig files (PARE.genomic.plus.bw and PARE.genomic.minus.bw) on the right panel. Figure 7 shows a screenshot of JBrowse2 displaying PARE data of two *Arabidopsis* genes in genomic coordinates.
4. Run Jbrowse2 desktop with transcriptome fasta (TAIR10.transcript.fasta), fai (TAIR10.transcript.fasta.fai), and bigwig files (PARE.transcriptome.bw) as described in **step 3** to display PARE data in transcript coordinates. Figure 7 shows a screenshot of JBrowse2 displaying PARE data of a miR156 target. The transcriptome fasta can be created using gffread:

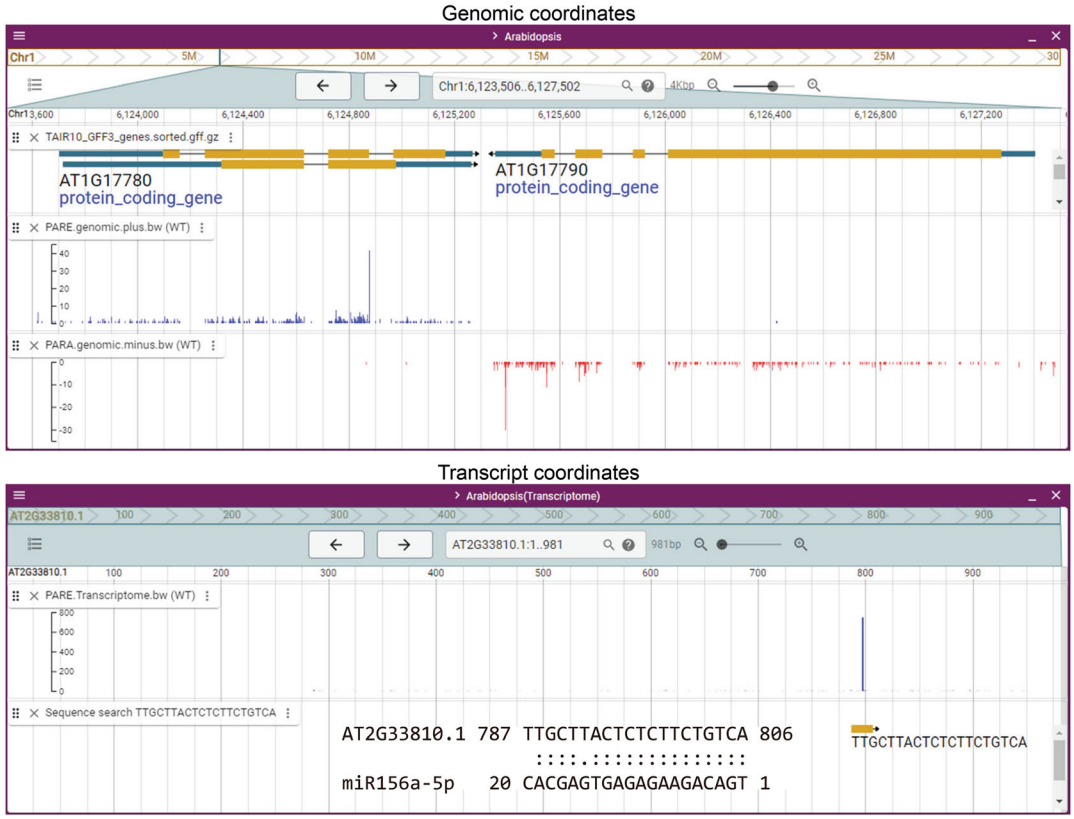

Fig. 7 Display of PARE data in Jbrowse2. The Jbrowse2 display of PARE data of two *Arabidopsis* genes in genomic coordinates. Counts of PARE reads are assigned to the coordinate aligned with the first base of PARE read, and the distribution of PARE counts is shown below the gene model. PARE reads mapped to the forward and reverse strands of the reference genome are displayed in the plus (red peaks) and minus (blue peaks) tracks, respectively. The Jbrowse2 display of PARE data of an *Arabidopsis* miR156 target, *SPL2* (At2g33810), in transcript coordinates. A prominent PARE peak located in the middle of the miRNA target site (the search sequence) supports the endo-cleavage directed by miR156

```
> gffread -w TAIR10.transcript.fasta -g
TAIR10_chr_all.fasta TAIR10_GFF3_genes.gff
```

3.15.6 Validation of miRNA-Guided Cleavage Sites Using PARE Data

The computationally predicted miRNA targets can be validated by viewing the PARE data around the predicted endo-cleavage site (between the target bases opposite to the 10–11 bases of miRNAs) using JBrowse2 in transcript coordinates.

1. Obtain the alignment between miRNAs and targets using plant miRNA target prediction tools or miRNA target databases (e.g., psRNAtarget, and TarDB) [14, 15].
2. Display PARE data in transcript coordinates using JBrowse2. Use the "sequence search" function in linear genome view to search the target sequence paired with the miRNA. Check

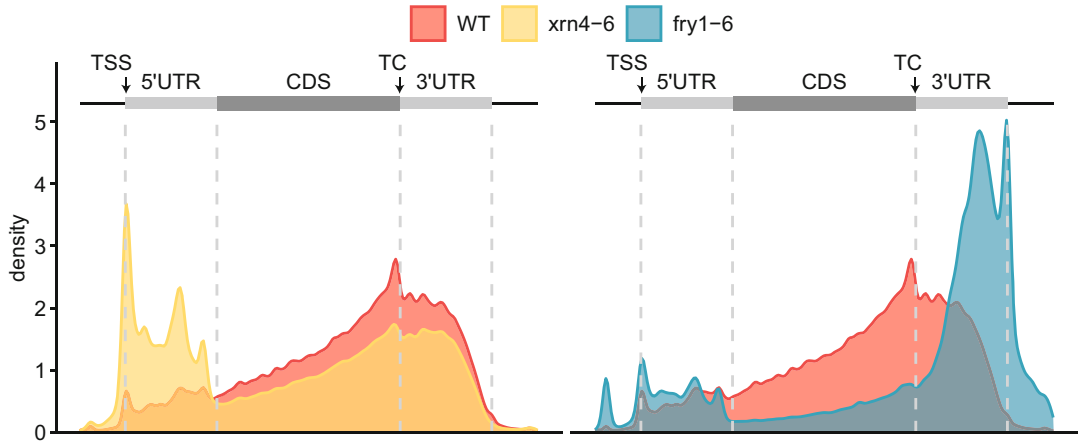

Fig. 8 Metagene plots of PARE data of *Arabidopsis* coding transcripts from wild type, *xrn4-6* and *fry1-6*. The global profiles of 5′ monophosphorylated mRNA decay intermediates of *Arabidopsis* wild type (WT) and two mutants with impairments in 5′-3′ RNA decay (*xrn4-6* and *fry1-6*) can be compared using metagene plots. In WT, a metagene plot of PARE data exhibits a strong 3′ bias, accompanied by local enrichment near the transcription start site (TSS) and the termination codon (TC). The enrichment observed at the TSS corresponds to decapped 5′ ends, which are notably increased in the *xrn4-6* and *fry1-6* mutants. Conversely, the TC enrichment corresponds to ribosome-protected fragments, which show decreased abundance in the *xrn4-6* and *fry1-6* mutants. Furthermore, in the *fry1-6* mutant, there is a notable accumulation of decay intermediates within the 3′ UTR, comprising cleaved 5′ termini at the polyadenylation site

whether there is a prominent PARE peak in the middle of the predicted target site. Figure. 7 shows a screenshot of JBrowse2 displaying the PARE data at the miR156 target site of *Arabidopsis SPL2* (AT2G33810.1).

3.15.7 Metagene Plots for Comparisons of RNA Decay Profiles

Metagene plots of PARE data are crucial to visualize and compare the global distribution of the 5′ monophosphorylated ends of RNA decay intermediates between genotypes or treatments [16]. Metagene plots of PARE data can be generated using a custom R script GuitarPlotFast.R, which was modified from a R package Guitar v.2.10.0 for efficient computing [17]. The R script and demo files can be downloaded from https://github.com/LabHMChenABRC/PARE-analysis. Metagene plots of *Arabidopsis* PARE data of three genotypes are shown in Fig. 8.

4 Notes

1. To capture the events of RNA degradation happening in living cells but not during RNA isolation, high-quality RNA is crucial for profiling the RNA degradome. The quality of RNA samples should be checked by Bioanalyzer with RNA 6000 Nano Kit. A Bioanalyzer profile of high-quality *Arabidopsis* total RNA is shown in Fig. 2. RNA is generally considered of high quality

when RNA integrity number (RIN) value is ≥8. However, chloroplast rRNA in plant tissues may lower RIN values.

2. Take 0.5 μL of the mRNA left on the beads and mix it with 2 μL of nuclease-free water for the QC using a Bioanalyzer with the RNA 6000 Pico Kit. The profile of *Arabidopsis* RNA after the first mRNA purification is shown in Fig. 2. This QC is optional but recommended.
3. Take 1 μL of the 5′ ligated mRNA left on the beads for the QC of the 2nd mRNA purification using the Agilent 2100 Bioanalyzer with the RNA 6000 Pico Kit. The profile of *Arabidopsis* RNA after the 2nd mRNA purification is shown in Fig. 2. This QC is optional but highly recommended.
4. To enhance the reverse transcription efficiency, 2 μL of SuperScript III is added again after 90 min incubation at 48 °C.
5. Place the AMpure XP beads at room temperature for at least 30 min and vortex the beads until they are fully resuspended before use.
6. The drying time for AMPure XP beads is influenced by both humidity and temperature. Typically, the most common drying time ranges from 1 to 2 min. However, under humidity levels exceeding 70%, it may take more than 5 min.
7. A gel staining container can be reused. To avoid cross-contamination from other samples, soak the container in bleach solution overnight, wash it with tap water, and rinse it with deionized water before use.
8. Ensuring an optimal granule size of gels after grinding is crucial. If the grind size is too fine, the gel particles may clog the filter in the subsequent step, resulting in failure to pass the sample through the spin column. Conversely, if the granule size is too large, it can significantly reduce elution efficiency and introduce bias to the library.
9. The qPCR experiment must incorporate suitable positive and negative controls. Previously prepared PARE libraries can serve as good positive controls because of available information on their Ct values, actual PCR cycles, and library yield.
10. Set the Ct threshold at half of the peak RFU and determine the Ct for each sample. The volume of the qPCR test is 0.8-fold the volume of the final library amplification. Therefore, in the test, 1 μL of the gel purified dsDNA is about 1/13 ($16.4 \times 0.8 \approx 13.1$) input of the final PCR reaction. Since the logarithm base 2 of 1/13.1 is −3.71, an appropriate PCR cycle number of the final library is the Ct value of the qPCR test minus 3.71. If the Ct values vary among samples, select an appropriate cycle number close to every sample or adjust the

input of the PCR template to generate similar yields for each sample. An example to determine the PCR cycle number is illustrated in Fig. 4. Based on the Ct values obtained from a qPCR test, the appropriate PCR cycle number for Samples 1–3 is 11.8, 8.9 and 6.7, respectively. Using 11 PCR cycles, the amplification of Sample 1 library is slightly low while Sample 2 is slightly high and Sample 3 library is overamplified.

11. PARE libraries sequenced by Illumina two-channel SBS sequencing platforms such as NextSeq and NovaSeq can aggravate the problem of poor-quality calls in the 3′ adapter sequence due to low diversity. This impairs the success of 3′ adapter trimming which might be as low as 1–20%.
12. To correctly determine the 5′ position of PARE read alignment, use "`--alignEndsType EndToEnd`" to force a read to align to reference without soft-clipping at both ends.
13. STAR aligns multiple-mapping reads randomly with "`--outMultimapperOrder Random`". Use "`--outFilterMultimapNmax 10`" to discard reads with more than 10 hits on the reference sequence to prevent low-confidence alignments. If the gene of interest belongs to a big family, the cutoff of multiple hits can be adjusted based on the number of homologs to retain multiple-mapping reads.
14. Use "`--alignSJDBoverhangMin 1`" to correctly align short reads (i.e., 20-21 nt) to annotated spliced junctions.
15. To prevent STAR from reporting low-confidence alignments over unannotated splice junctions, give a number bigger than the maximum read length for "`--alignSJoverhangMin`".
16. Use "`--alignIntronMax`" to control the maximum intron size and define the size of the seed stitching window which affects the discovery of annotated junctions. The suitable values for these two parameters are dependent on species.
17. Use "`--quantMode TranscriptomeSAM`" to make STAR produce an additional bam file in transcript coordinates.
18. PARE reads are the 5′ end tag of RNA decay intermediates; therefore, the first position of forward alignments indicates the site where endonucleolytic cleavage occurs or exoribonuclease activity stops.
19. Run bamCoverage with "`–filterRNAstrand forward`" to keep the reads mapped to the reverse strand.

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Part IX

Beyond mRNA Decay

Chapter 23

SelectRepair Knockout: Efficient PTC-Free Gene Knockout Through Selectable Homology-Directed DNA Repair

Michael A. Cortázar and Sujatha Jagannathan

Abstract

Generating nonessential gene knockouts using CRISPR/Cas9 technology is becoming increasingly common in biological research. In a typical workflow, the Cas9 endonuclease is used to induce a DNA double-strand break that relies on nonhomologous end-joining (NHEJ) to introduce a premature termination codon (PTC) in the target gene. The goal is to isolate clones in which the gene produces PTC-containing mRNA transcripts that are degraded via nonsense-mediated mRNA decay (NMD) to cause loss of gene function. Unfortunately, this approach is laborious, and not all PTCs trigger NMD. More importantly, mounting evidence suggest that PTC mutations can also result in a transcriptional adaptation response that can mask the effects of a PTC-mediated gene knockout. In this chapter, we present a PTC-free gene knockout strategy that implements homology-directed DNA repair (HDR) with selectable markers to substantially reduce the complexity of the screening and validation of genome edits in cells containing more than one gene copy as in the case of the commonly used hypotriploid HEK293 cell line. We describe how to obtain a complete knockout of the Ligase IV protein (LIG4) and provide considerations for the application of this SelectRepair Knockout method to other genes.

Key words CRISPR/Cas9 gene editing, Knockout, Homology-directed repair, Nonsense-mediated mRNA decay, NMD

1 Introduction

The unprecedented ability of CRISPR/Cas9 technology to edit genomes has revolutionized genome engineering and enabled the generation of many gene knockouts. The simplest way to generate a gene knockout with this technology involves the expression of the Cas9 endonuclease and a single guide RNA (sgRNA) that form a complex and can generate a DNA double-strand break directed by the sequence specificity encoded in the sgRNA [1]. This is followed by the isolation of single cell-derived clones in which the target gene has acquired a PTC mutation through nonhomologous end joining (NHEJ) [2]. PTCs have been thought of as an effective tool to achieve loss of gene function given that they are expected to not

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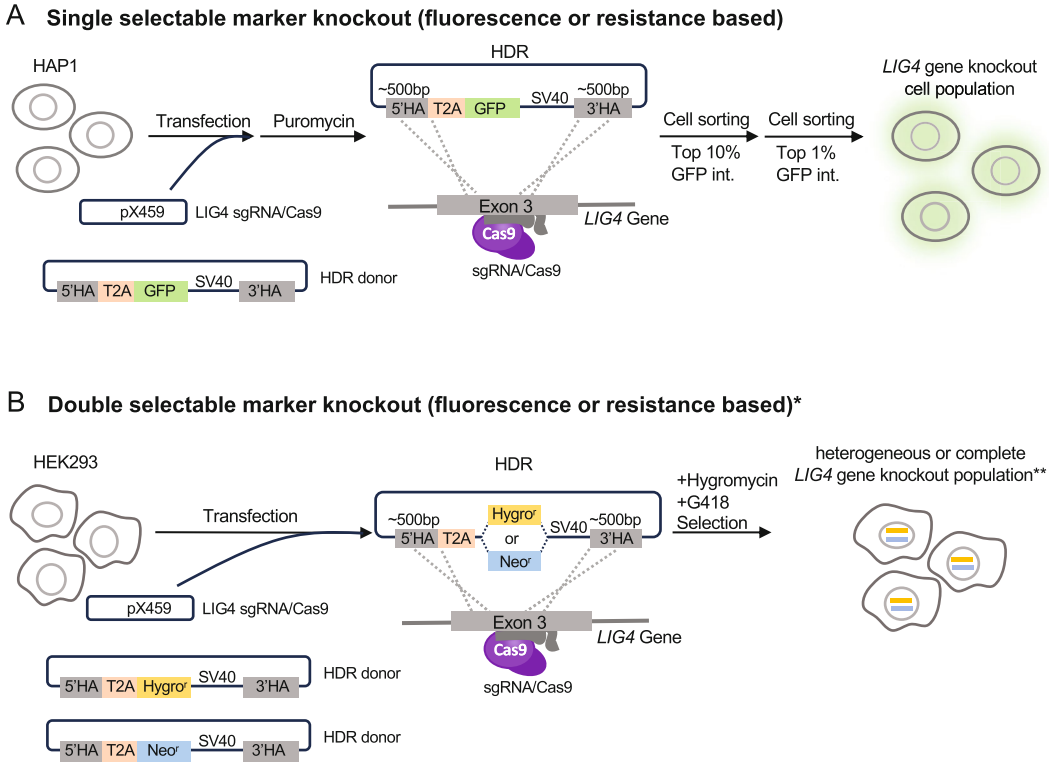
only cause protein truncation but are also known to activate the highly conserved RNA quality control pathway, nonsense-mediated mRNA decay (NMD), which causes degradation of the mRNA [3].

While generally true, nonsense codons within a transcript do not always cause loss of the mRNA [4], or the expected defects from loss of gene function [5]. This phenomenon is best illustrated by the observation that CRISPR-mediated "knockout" of many individual genes in zebrafish does not result in a phenotype that corresponds to knocking down the same gene [6]. The explanation for this "missing phenotype" is a transcriptional compensation response triggered by the NMD-undergoing PTC-containing mRNA that results in transcriptional upregulation of a wild-type allele or genes related in sequence [7–9]. Moreover, different mechanisms can allow the escape of a nonsense-containing transcript from NMD, including alternative splicing, translation reinitiation, or stop codon readthrough [10]. As a result, PTC-dependent gene knockout is not always a reliable strategy to induce loss of gene function. Deletion of the promoter region, or a gene segment encoding an essential protein domain, with the use of two Cas9/sgRNA complexes could be a good alternative [11]. Nevertheless, the potential use of a cryptic transcription start site or creation of a PTC by use of a cryptic splicing event near the edit site, which are difficult to detect and rule out, is still a concern [12]. Furthermore, relying on two DNA breaks on the same allele for deletion of the entire gene raises technical complexity. Or in the case of the strategy called MACHETE, used to delete chromosomal segments, it requires two consecutive genome editing events, and it is not designed to enrich cells in which all alleles have been modified [13].

An important technical consideration is that isolation and screening of single cell-derived clones to identify a complete knockout is time-consuming and laborious when depending on NHEJ. The error-prone nature of NHEJ affords integration of different types of insertions and deletions (indels) but not all indels result in frameshifts or nonsense mutations, typically requiring substantial screening of diploid or polyploid cells to identify clones in which all alleles contain a PTC. On the other hand, homology-directed DNA repair (HDR) has been previously used to incorporate defined sequences at the DNA break, "knock-in" [14], and to facilitate the detection of the knockout alleles [15]. These approaches, however, are still limited by the low frequency of HDR events relative to NHEJ [16]. A CRISPR-trap approach that enriches edited sites through the use of selectable markers has been used for gene knockout, although this requires the presence of an intron upstream of the translation start site of the target gene [17]. Here, we present SelectRepair Knockout, a PTC-free approach to generate gene knockouts that relies on HDR to introduce selectable markers in-frame with the open reading frame



*the resistance-based approach offers reduced technical complexity compared to a double fluorescence cell sorting approach.

**depends on selectable marker HDR integration specificity and cell ploidy.

Fig. 1 Schematic representation of stable selectable marker insertion via homology-directed DNA repair to establish gene knockouts. (**a**) Single selectable marker can be used for the haploid HAP1 cells. (**b**) Double selectable markers are more amenable to diploid or polyploid cell lines. Given the positive selection of cells in G-418 and hygromycin, no puromycin selection is needed in the double selectable marker approach

(ORF) encoded by the target gene to disrupt gene function. The selectable marker is followed by an SV40 cleavage and polyadenylation (SV40 pA) signal to terminate transcript synthesis immediately downstream (Fig. 1). Interruption of the endogenous ORF with the selectable marker, relatively close to the translation start site, is designed to interfere with and prevent full-length synthesis of the target gene's encoded protein. While the frequency of DNA repair through HDR is low compared to NHEJ, the use of multiple selectable markers allows for ample enrichment of cell clones where all wild-type alleles of the target gene have been successfully knocked out. We applied SelectRepair to enrich several complete *LIG4* gene knockout clones, even in the hypotriploid HEK293 human cell line, from a screening of less than ten colonies. In the case of the near-haploid human HAP1 cell line, our approach can

eliminate the detection of the wild-type allele and protein product in the whole cell population without the need to isolate single clones. The high success rate of this approach stems from the prerequisite that the selectable marker must successfully interfere with the ORF encoded by the target gene and use its corresponding translation start site for its own expression. It follows that this approach is not recommended to knock out genes not expressed at the time of genome editing or for those with limited expression levels. Importantly, we additionally provide a simple PCR-gel electrophoresis strategy for screening of colonies that circumvents subcloning or the need for Sanger sequencing, as well as considerations that might raise the odds for success when applying this method to other genes.

2 Materials

Prepare all DNA reagents using nuclease-free water. Chemical stock solutions should be prepared in deionized water. Prepare 1X dilutions of stock solutions as needed. The plasmids constructed in the context of this chapter have been deposited at AddGene and can be obtained using the following IDs: 225357 (LIG4 HAL-T2A-GFP-SV40pA-HAR), 225358 (LIG4 HAL-T2A-HYG-SV40pA-HAR), 225359 (LIG4 HAL-T2A-NEO-SV40pA-HAR), 225363 (pX549-LIG4-Exon3).

2.1 Construction of the HDR Donor Template and sgRNA/Cas9-Encoding Plasmids

1. BbsI restriction site ligation-competent, complementary protospacer-sequence DNA oligos (*see* Subheading 3.1).
   LIG4 sgRNA F: 5′-CACCGCATAATGTCACTACAGAT C-3′.
   LIG4 sgRNA R: 5′-AAACGATCTGTAGTGACATTATG C-3′.
2. sgRNA/Cas9 expression vector (i.e. pX459).
3. BbsI restriction enzyme.
4. T4 Polynucleotide Kinase (PNK).
5. T4 DNA ligase.
6. Competent *E. coli* cells.
7. SOC medium.
8. LB agar plates containing carbenicillin at 100 μg/mL.
9. LB medium.
10. Plasmid DNA extraction kit (for endotoxin-free plasmid DNA).
11. NucleoSpin Gel and PCR Clean-up kit.

12. Homology-directed DNA repair donor template plasmid (*see* Subheading 3.1 and **Note 1**).
13. Taq DNA polymerase with its buffer.
14. LKO.1 5′ primer: 5′- GACTATCATATGCTTACCGT-3′.

2.2 Cell Culture

1. HEK293 cell line.
2. HAP1 cell line.
3. Dulbecco's modified Eagles medium (DMEM).
4. Iscove's Modified Dulbecco's Medium (IMDM).
5. 10% fetal bovine serum (FBS).
6. TrypLE™ Express Enzyme (1X), phenol red.
7. Phosphate buffered saline (DPBS), no calcium, no magnesium.
8. Scienceware® Sterile Cloning Cylinders, Bel-Art, Small, I.D. x H = 4.7 x 8 mm.
9. Puromycin dihydrochloride, Ultra Pure Grade.
10. G-418 Sulfate.

2.3 Transfection

1. Lipofectamine 2000 transfection reagent.
2. Opti-MEM I Reduced Serum Medium.

2.4 Screening of Gene Knockout by Genomic DNA PCR-Gel Electrophoresis

1. Genomic DNA extraction kit (e.g. DNeasy Blood & Tissue Kit).
2. LIG4 screening PCR primers.
   LIG4 F: 5′-ACTTGAGCAAAAGTGGCTTATACGGATG-3′.
   LIG4 R: 5′-GTGCTCAATATCTGCAATAGCAGCTAGC-3′.
3. DNA Polymerase (e.g. KAPA HiFi DNA polymerase).
4. Agarose, Molecular-biology grade.
5. GelGreen Nucleic Acid Stain: 10,000X in water.
6. 6X Orange DNA Loading dye.
7. GeneRuler 1 kb plus DNA ladder.
8. 10X TBE buffer.

2.5 Protein Extraction and Sample Preparation

1. Whole cell lysis buffer: 20 mM HEPES pH 7.9, 0.1 mM EDTA, 10% glycerol, 150 mM Potassium acetate, 1.5 mM $MgCl_2$, 0.5% NP40.
2. Halt™ Protease and Phosphatase Inhibitor Single-Use Cocktail, EDTA-free.
3. Benzonase nuclease.
4. Bovine Serum Albumin (BSA).
5. BCA protein assay kit.

6. NuPAGE LDS Sample Buffer (4X).
7. 1 M DTT.
8. Heating block.

2.6 Western Blot

1. NuPAGE 4–12% Bis-Tris Protein Gels.
2. Gel electrophoresis system.
3. Novex NuPAGE MOPS SDS Running Buffer (20X).
4. Protein molecular weight ladder.
5. NuPAGE 20X Transfer buffer.
6. Nitrocellulose membrane.
7. Transfer system, e.g. Mini Blot Module.
8. Intercept® (PBS) Blocking buffer.
9. DNA ligase IV Recombinant Rabbit Monoclonal Antibody.
10. Secondary antibody (e.g. IRDye 800CW Goat anti-Rabbit IgG Secondary Antibody).
11. Tween® 20.
12. Wash buffer: 1X Phosphate buffered saline solution, 0.1% Tween 20.
13. Imager.

3 Methods

The target gene copy number or ploidy of the cell line is an important consideration in choosing the method for gene knockout. As shown below (Fig. 1), for haploid cell lines such as HAP1, a single selectable maker is sufficient to isolate a complete knockout cell population. For diploid or polyploid cell lines, using multiple selection markers increases the efficiency of obtaining complete knockouts.

3.1 Design of the Protospacer Sequence and of the HDR Donor Template Plasmids

1. Select the genomic Cas9-based, 20-nucleotide protospacer target sequence of the gene candidate relatively close to the translation start site but downstream of the first exon (*see* **Note 2**). Design the sgRNA protospacer sequence using online tools such as the sgRNA Scorer 2.0 online tool (https://crispr.med.harvard.edu/sgRNAScorerV2/) [18] and/or follow guides of RNA features that influence on-target activity [19]. To target the ligase IV gene, we used the previously designed protospacer sequence 5′- GCATAATGTCACTACA GATC-3′ [20] within exon 3 of the *LIG4* gene.
2. Using the protospacer sequence, design a pair of complementary forward and reverse DNA oligos in which the forward oligo contains the protospacer sequence. If the protospacer sequence does not start with a "G" nucleotide, *see* **Note 3**.

Append the "CACC" nucleotides to the 5′ end of the LIG4 sgRNA F forward oligonucleotide (5′-CACC GCATAATGT CACTACAGATC-3′) and "AAAC" nucleotides to the 5′ end of the LIG4 sgRNA R reverse oligonucleotide (5′-AAAC GAT CTGTAGTGACATTATGC-3′) to create BbsI restriction site ligation-competent 5′ overhangs.

3. Design the HDR donor template plasmid by creating 500–800 bp left and right homology arms (HA) starting at the codon expected to be targeted for double-strand DNA break. The DNA break occurs between nucleotides 3 and 4 upstream of the PAM sequence (*see* **Note 4**). Include this codon position as the 3′ end boundary of the left arm and replace its identity with any of the four alanine codons to reduce targeting of the HDR donor template by the Cas9 nuclease. Use the codon immediately downstream of the DNA break as the start boundary of the right homology arm. *See* Table 1 for sequence information corresponding to the left and right homology arms to target the *LIG4* gene.
4. Add the self-cleaving 2A peptide coding sequence (*see* Table 1) downstream of the left HA sequence, which will maintain the

Table 1
LIG4-specific HDR donor plasmid sequences

Name	Sequence (5′ to 3′)
LIG4 left homology arm (HA_L)	acagttattaaatgtagaggttgaaaagatacatcatttagagcattgatctttttttta gtaaacattaataaacatttattatgatctcagtgatc tttgtaagatctttacattaaatatttagttgaatcttatcttttacttaaagttattctctttttgcttt actagttaaacgagaagattcatcaccgctttgatggctgcctcacaaacttcacaaact gttgcatctcacgttccttttgcagatttgtgttcaactttagaacgaatacagaaaagtaaaggacgtg cagaaaaaatcagacacttcagggaatttttagattcttggagaaaatttcatgatgctcttcataagaa ccacaaagatgtcacagactctttttatccagcaatgagactaattcttcctcagctagaaagagagagaat ggcctatggaattaaagaaactatgcttgctaagctttatattgagttgcttaatttacctagagatgga aaagatgccctcaaacttttaaactacagaacacccactggaactcatggagatgctggagactttgca atgattgcatattttgtgttgaagccaagatgtttacagaaaggaagtttaaccatacagcaagtaaacgac cttttagactcaattgccagcaataattctgctaaaagaaaagacctaataaaaaagagccttcttcaact tataactcagagttcagcacttgagcaaaagtggcttatacggatgatcataaaggatttaaagcttg gtgttagtcagcaaactatcttttctgtttttcataatgatgctgctgagttgcataatgtcactacagcc underlined: protospacer sequenced, followed by the GAT>GCC conversion
LIG4 right homology arm (HA_R)	atctggaaaaagtctgtaggcaactgcatgatccttctgtaggactcagtgatatttctatcactttattttctg catttaaaccaatgctagctgctattgcagatattgagcacattgagaaggatatgaaacatcagagtt tctacatagaaaccaagctagatggtgaacgtatgcaaatgcacaaagatggagatgtatataaatact tctctcgaaatggatataactacactgatcagtttggtgcttctcctactgaaggttctcttaccccattca ttcataatgcattcaaagcagatatacaaatctgtattcttgatggtgagatgatggcctataatcctaata cacaaactttcatgcaaaagggaactaagtttgatattaaaagaatggtagaggattctgatctgcaaa cttgttattgtgtttttgatgtattgatggttaataataaaaagctagggcatgagactctgagaaaga ggtatgagattcttagtagtatttttacaccaattccaggtagaatagaaatagtgcagaaaacacaag

(continued)

Table 1
(continued)

Name	Sequence (5′ to 3′)
	ctcatactaagaatgaagtaattgatgcattgaatgaagcaatagataaaagagaagagggaattatgg taaaacaacctctatccatctacaagccagacaaaagaggtgaagggtggttaaaaattaaaccagagt atgtcagtggactaatggatgaattggacattttaattgttggaggatattggggtaaaggatcacg gggtggaatgatgtctcattttctgtgtgcagtagcagagaagccccctcctggtgagaagccatc tgtgtttcatactctctctcgtgttgggtctggctgcaccatgaaagaactgtatgatctgggtttga aattggccaagtattggaagccttttcatagaaaagctccaccaagcagcattttatgtggaacaga gaagccagaagtatacattgaaccttgtaa
self-cleaving 2A peptide coding sequence	aagcttgagggcagaggaagtcttctaacatgcggtgacgtggaggagaatcccggccctgctag cggtagcggcagcggtagc
GFP-SV40pA	agcgggggcgaggagctgttcgccggcatcgtgcccgtgctgatcgagctggacggcgacgtg cacggccacaagttcagcgtgcgcggcgagggcgagggcgacgccgactacggcaagct ggagatcaagttcatctgcaccaccggcaagctgcccgtgccctggcccaccctggtgaccac cctctgctacggcatccagtgcttcgcccgctaccccgagcacatgaagatgaacgacttcttcaa gagcgccatgcccgagggctacatccaggagcgcaccatccagttccaggacgacggcaa gtacaagacccgcggcgaggtgaagttcgagggcgacaccctggtgaaccgcatcgagctg aagggcaaggacttcaaggaggacggcaacatcctgggccacaagctggagtacagcttca acagccacaacgtgtacatccgccccgacaaggccaacaacggcctggaggctaacttcaa gacccgccacaacatcgagggcggcggcgtgcagctggccgaccactaccagaccaacgt gcccctgggcgacggccccgtgctgatccccatcaaccactacctgagcactcagaccaagat cagcaaggaccgcaacgaggcccgcgaccacatggtgctcctggagtccttcagcgcctgct gccacacccacggcatggacgagctgtacaggtaactcgagggatccagacatgataagatacatt gatgagtttggacaaaccacaactagaatgca gtgaaaaaaatgctttatttgtgaaatttgtgatgctattgctttatttgtaaccattataagctgcaataaaca agttaacaacaacaattgcattcattttatgtttcaggttcaggggggaggtgtgggaggttttttaaagca agtaaaacctctacaaatgtggtatggctgattatgatcagtcgac
Hygromycin-SV40pA	aaaaagcctgaactcaccgcgacgtctgtcgagaagtttctgatcgaaaagttcgacagcgtctccg acctgatgcagctctcggagggcgaagaatctcgtgctttcagcttcgatgtaggagggcgtggat atgtcctgcgggtaaatagctgcgccgatggtttctacaaagatcgttatgtttatcggcactttgcatcg gccgcgctcccgattccggaagtgcttgacattggggaattcagcgagagcctgacctattgcatc tcccgccgtgcacagggtgtcacgttgcaagacctgcctgaaaccgaactgcccgctgttctgca gccggtcgcggaggccatggatgcgatcgctgcggccgatcttagccagacgagcgggttcg gcccattcggaccgcaaggaatcggtcaatacactacatggcgtgatttcatatgcgcgattgctg atccccatgtgtatcactggcaaactgtgatggacgacaccgtcagtgcgtccgtcgcgcaggct ctcgatgagctgatgctttgggccgaggactgccccgaagtccggcacctcgtgcacgcggattt cggctccaacaatgtcctgacggacaatggccgcataacagcggtcattgactggagcgaggc gatgttcggggattcccaatacgaggtcgccaacatcttcttctggaggccgtggttggcttgtatgg agcagcagacgcgctacttcgagcggaggcatccggagcttgcaggatcgccgcggctccg ggcgtatatgctccgcattggtcttgaccaactctatcagagcttggttgacggcaatttcgatgatgc agcttgggcgcagggtcgatgcgacgcaatcgtccgatccggagccgggactgtcgggcgta cacaaatcgcccgcagaagcgcggccgtctggaccgatggctgtgtagaagtactcgccgata gtggaaaccgacgccccagcactcgtccgagggcaaaggaatagctcgagggatccagacatgata agatacattgatgagtttggacaaaccaca actagaatgcagtgaaaaaaatgctttatttgtgaaatttgtgatgctattgctttatttgtaaccattataagc tgcaataaacaagttaacaacaacaattgcattcattttatgtttcaggttcaggggggaggtgtgggag gttttttaaagcaagtaaaacctctacaaatgtggtatggctgatt atgatcagtcgac Underlined: stop codon

(continued)

Table 1
(continued)

Name	Sequence (5′ to 3′)
Neomycin-SV40pA	attgaacaagatggattgcacgcaggttctccggccgcttgggtggagaggctattcggctatgactg ggcacaacagacaatcggctgctctgatgccgccgtgttccggctgtcagcgcaggggcgcc cggttctttttgtcaagaccgacctgtccggtgccctgaatgaactgcaagacgaggcagcgcgg ctatcgtggctggccacgacgggcgttccttgcgcagctgtgctcgacgttgtcactgaagcggg aagggactggctgctattgggcgaagtgccggggcaggatctcctgtcatctcaccttgctcctgc cgagaaagtatccatcatggctgatgcaatgcggcggctgcatacgcttgatccggctacctgcc cattcgaccaccaagcgaaacatcgcatcgagcgagcacgtactcggatggaagccggtcttg tcgatcaggatgatctggacgaagagcatcaggggctcgcgccagccgaactgttcgccagg ctcaaggcgagcatgcccgacggcgaggatctcgtcgtgacccatggcgatgcctgcttgccg aatatcatggtggaaaatggccgcttttctggattcatcgactgtggccggctgggtgtggcggacc gctatcaggacatagcgttggctacccgtgatattgctgaagagcttggcggcgaatgggctgac cgcttcctcgtgctttacggtatcgccgctcccgattcgcagcgcatcgccttctatcgccttcttgacga gttcttctgactcgagggatccagacatgataagatacattgatgagtttggacaaaccacaactagaat gcagtgaaaaaaatgctttatttgtgaaatttgtga tgctattgctttatttgtaaccattataagctgcaataaacaagttaacaacaacaattgcattcattttatgttt caggttcaggggggaggtgtgggaggttttttaaagcaagtaaaacctctacaaatgtggtatggctgatt atgatcagtcgac Underlined: stop codon
LIG4 HA_L-T2A-GFP-SV40pA-HA_R	acagttattaaatgtagaggttgaaaagatacatcatttagagcattgatctttttttttagtaaacattaataaa catttattatgatctcagtgatctttgtaagatctttacattaaatatttagttgaatcttatcttttacttaaagttattc tctttttgctttactagttaaacgagaagattcatcaccgctttgatggctgcctcacaaacttcacaaact gttgcatctcacgttccttttgcagatttgtgttcaactttagaacgaatacagaaaagtaaaggacgtg cagaaaaaatcagacacttcagggaatttttagattcttggagaaaatttcatgatgctcttcataagaa ccacaaagatgtcacagactctttttatccagcaatgagactaattcttcctcagctagaaagagaga gaatggcctatggaattaaagaaactatgcttgctaagctttatattgagttgcttaatttacctagagatg gaaaagatgccctcaaacttttaaactacagaacacccactggaactcatggagatgctggaga ctttgcaatgattgcatattttgtgttgaagccaagatgtttacagaaaggaagtttaaccatacagcaa gtaaacgacctttagactcaattgccagcaataattctgctaaaagaaaagacctaataaaaaaga gccttcttcaacttataactcagagttcagcacttgagcaaaagtggcttatacggatgatcataaagg atttaaagcttggtgttagtcagcaaactatcttttctgtttttcataatgatgctgctgagttgcataatgtcac tacagccaagcttgagggcagaggaagtcttctaacatgcggtgacgtggaggagaatcccgg ccctgctagcggtagcggcagcggtagcagcgggggcgaggagctgttcgccggcatcgtg cccgtgctgatcgagctggacggcgacgtgcacggccacaagttcagcgtgcgcggcgagg gcgagggcgacgccgactacggcaagctggagatcaagttcatctgcaccaccggcaagct gcccgtgccctggcccaccctggtgaccaccctctgctacggcatccagtgcttcgcccgctac cccgagcacatgaagatgaacgacttcttcaagagcgccatgcccgagggctacatccagga gcgcaccatccagttccaggacgacggcaagtacaagacccgcggcgaggtgaagttcgag ggcgacaccctggtgaaccgcatcgagctgaagggcaaggacttcaaggaggacggcaac atcctgggccacaagctggagtacagcttcaacagccacaacgtgtacatccgccccgacaag gccaacaacggcctggaggctaacttcaagacccgccacaacatcgagggcggcggcgtg cagctggccgaccactaccagaccaacgtgcccctgggcgacggccccgtgctgatccccat caaccactacctgagcactcagaccaagatcagcaaggaccgcaacgaggcccgcgacca catggtgctcctggagtccttcagcgcctgctgccacacccacggcatggacgagctgtacagg taactcgagggatccagacatgataagatacattgatgagtttggacaaaccacaactagaatgca gtgaaaaaaatgctttatttgtgaaatttgtgatgctattgctttatttgtaaccattataagctgcaataaaca agttaacaacaacaattgcattcattttatgtttcaggttcaggggggaggtgtgggaggttttttaaagca agtaaaacctctacaaatgtggtatggctgattatgatcagtcgacatctggaaaaagtctgtaggca actgcatgatccttctgtaggactcagtgatatttctatcactttattttctgcatttaaaccaatgctagctgc

(continued)

Table 1
(continued)

Name	Sequence (5′ to 3′)
	tattgcagatattgagcacattgagaaggatatgaaacatcagagtttctacatagaaaccaagcta gatggtgaacgtatgcaaatgcacaaagatggagatgtatataaatacttctctcgaaatggatataa ctacactgatcagtttggtgcttctcctactgaaggttctcttaccccattcattcataatgcattcaaagca gatatacaaatctgtattcttgatggtgagatgatggcctataatcctaatacacaaactttcatgcaaaa gggaactaagtttgatattaaaagaatggtagaggattctgatctgcaaacttgttattgtgtttttgatgtatt gatggttaataataaaaagctagggcatgagactctgagaaagaggtatgagattcttagtagtatttttt acaccaattccaggtagaatagaaatagtgcagaaaacacaagctcatactaagaatgaagtaat tgatgcattgaatgaagcaatagataaaagagaagaggggaattatggtaaaacaacctctatccat ctacaagccagacaaaagaggtgaagggtggttaaaaattaaaccagagtatgtcagtggacta atggatgaattggacattttaattgttggaggatattggggtaaaggatcacggggtggaatgatgtct cattttctgtgtgcagtagcagagaagccccctcctggtgagaagccatctgtgtttcatactctctctc gtgttgggtctggctgcaccatgaaagaactgtatgatctgggtttgaaattggccaagtattggaag ccttttcatagaaaagctccaccaagcagcattttatgtggaacagagaagccagaagtatacattg aaccttgtaa
LIG4 HA_L-T2A-HYG-SV40pA-HA_R	acagttattaaatgtagaggttgaaaagatacatcatttagagcattgatctttttttttagtaaacattaataaa catttattatgatctcagtgatctttgtaagatctttacattaaatatttagttgaatcttatcttttacttaaagttattc tctttttgctttactagttaaacgagaagattcatcaccgctttgatggctgcctcacaaacttcacaaact gttgcatctcacgttccttttgcagatttgtgttcaactttagaacgaatacagaaaagtaaaggacgtg cagaaaaaatcagacacttcagggaatttttagattcttggagaaaatttcatgatgctcttcataagaa ccacaaagatgtcacagactctttttatccagcaatgagactaattcttcctcagctagaaagagaga gaatggcctatggaattaaagaaactatgcttgctaagctttatattgagttgcttaatttacctagagatg gaaaagatgccctcaaacttttaaactacagaacacccactggaactcatggagatgctggaga ctttgcaatgattgcatattttgtgttgaagccaagatgtttacagaaaggaagtttaaccatacagcaa gtaaacgacctttagactcaattgccagcaataattctgctaaaagaaaagacctaataaaaaaga gccttcttcaacttataactcagagttcagcacttgagcaaaagtggcttatacggatgatcataaagg atttaaagcttggtgttagtcagcaaactatcttttctgtttttcataatgatgctgctgagttgcataatgtcac tacagccaagcttgagggcagaggaagtcttctaacatgcggtgacgtggaggagaatcccgg ccctgctagcggtagcggcagcggtagcaaaaagcctgaactcaccgcgacgtctgtcgaga agtttctgatcgaaaagttcgacagcgtctccgacctgatgcagctctcggagggcgaagaatctc gtgctttcagcttcgatgtaggagggcgtggatatgtcctgcgggtaaatagctgcgccgatggtttct acaaagatcgttatgtttatcggcactttgcatcggccgcgctcccgattccggaagtgcttgacattg gggaattcagcgagagcctgacctattgcatctcccgccgtgcacagggtgtcacgttgcaaga cctgcctgaaaccgaactgcccgctgttctgcagccggtcgcggaggccatggatgcgatcgct gcggccgatcttagccagacgagcgggttcggcccattcggaccgcaaggaatcggtcaatac actacatggcgtgatttcatatgcgcgattgctgatccccatgtgtatcactggcaaactgtgatggac gacaccgtcagtgcgtccgtcgcgcaggctctcgatgagctgatgctttgggccgaggactgcc ccgaagtccggcacctcgtgcacgcggatttcggctccaacaatgtcctgacggacaatggcc gcataacagcggtcattgactggagcgaggcgatgttcggggattcccaatacgaggtcgccaa catcttcttctggaggccgtggttggcttgtatggagcagcagacgcgctacttcgagcggaggca tccggagcttgcaggatcgccgcggctccgggcgtatatgctccgcattggtcttgaccaactctat cagagcttggttgacggcaatttcgatgatgcagcttgggcgcagggtcgatgcgacgcaatcgt ccgatccggagccgggactgtcgggcgtacacaaatcgcccgcagaagcgcggccgtctgg accgatggctgtgtagaagtactcgccgatagtggaaaccgacgccccagcactcgtccgagg gcaaaggaatagctcgagggatccagacatgataagatacattgatgagtttggacaaaccaca actagaatgcagtgaaaaaaatgctttatttgtgaaatttgtgatgctattgctttatttgtaaccattataagc tgcaataaacaagttaacaacaacaattgcattcattttatgtttcaggttcaggggggaggtgtgggag gtttttttaaagcaagtaaaacctctacaaatgtggtatggctgattatgatcagtcgacatctggaaaaa gtctgtaggcaactgcatgatccttctgtaggactcagtgatatttctatcactttatttttctgcatttaaacca

(continued)

Table 1
(continued)

Name	Sequence (5′ to 3′)
	atgctagctgctattgcagatattgagcacattgagaaggatatgaaacatcagagtttctacataga aaccaagctagatggtgaacgtatgcaaatgcacaaagatggagatgtatataaatacttctctcga aatggatataactacactgatcagtttggtgcttctcctactgaaggttctcttaccccattcattcataatg cattcaaagcagatatacaaatctgtattcttgatggtgagatgatggcctataatcctaatacacaaa ctttcatgcaaaagggaactaagtttgatattaaaagaatggtagaggattctgatctgcaaacttgttat tgtgtttttgatgtattgatggttaataataaaaagctagggcatgagactctgagaaagaggtatgaga ttcttagtagtatttttacaccaattccaggtagaatagaaatagtgcagaaaacacaagctcatactaa gaatgaagtaattgatgcattgaatgaagcaatagataaaagagaagagggaattatggtaaaac aacctctatccatctacaagccagacaaaagaggtgaagggtggttaaaaattaaaccagagtat gtcagtggactaatggatgaattggacattttaattgttggaggatattggggtaaaggatcacggggt ggaatgatgtctcattttctgtgtgcagtagcagagaagccccctcctggtgagaagccatctgtgttt catactctctctcgtgttgggtctggctgcaccatgaaagaactgtatgatctgggtttgaaattggcca agtattggaagccttttcatagaaaagctccaccaagcagcattttatgtggaacagagaagccag aagtatacattgaaccttgtaa
LIG4 HA_L-T2A-NEO-SV40pA-HA_R	acagttattaaatgtagaggttgaaaagatacatcatttagagcattgatctttttttttagtaaacattaataaa catttattatgatctcagtgatctttgtaagatctttacattaaatatttagttgaatcttatcttttacttaaagttattc tctttttgctttactagttaaacgagaagattcatcaccgctttgatggctgcctcacaaacttcacaaact gttgcatctcacgttccttttgcagatttgtgttcaactttagaacgaatacagaaaagtaaaggacgtg cagaaaaaatcagacacttcagggaatttttagattcttggagaaaatttcatgatgctcttcataagaa ccacaaagatgtcacagactctttttatccagcaatgagactaattcttcctcagctagaaagagaga gaatggcctatggaattaaagaaactatgcttgctaagctttatattgagttgcttaatttacctagagatg gaaaagatgccctcaaacttttaaactacagaacacccactggaactcatggagatgctggaga ctttgcaatgattgcatattttgtgttgaagccaagatgtttacagaaaggaagtttaaccatacagcaa gtaaacgacctttagactcaattgccagcaataattctgctaaaagaaaagacctaataaaaaaga gccttcttcaacttataactcagagttcagcacttgagcaaaagtggcttatacggatgatcataaagg atttaaagcttggtgttagtcagcaaactatcttttctgtttttcataatgatgctgctgagttgcataatgtcac tacagccaagcttgagggcagaggaagtcttctaacatgcggtgacgtggaggagaatcccgg ccctgctagcggtagcggcagcggtagcattgaacaagatggattgcacgcaggttctccggcc gcttgggtggagaggctattcggctatgactgggcacaacagacaatcggctgctctgatgccgc cgtgttccggctgtcagcgcaggggcgcccggttctttttgtcaagaccgacctgtccggtgccctg aatgaactgcaagacgaggcagcgcggctatcgtggctggccacgacgggcgttccttgcgc agctgtgctcgacgttgtcactgaagcgggaagggactggctgctattgggcgaagtgccgggg caggatctcctgtcatctcaccttgctcctgccgagaaagtatccatcatggctgatgcaatgcggc ggctgcatacgcttgatccggctacctgcccattcgaccaccaagcgaaacatcgcatcgagcg agcacgtactcggatggaagccggtcttgtcgatcaggatgatctggacgaagagcatcaggg gctcgcgccagccgaactgttcgccaggctcaaggcgagcatgcccgacggcgaggatctc gtcgtgacccatggcgatgcctgcttgccgaatatcatggtggaaaatggccgcttttctggattcatc gactgtggccggctgggtgtggcggaccgctatcaggacatagcgttggctacccgtgatattgct gaagagcttggcggcgaatgggctgaccgcttcctcgtgctttacggtatcgccgctcccgattcg cagcgcatcgccttctatcgccttcttgacgagttcttctgactcgagggatccagacatgataagat acattgatgagtttggacaaaccacaactagaatgcagtgaaaaaaatgctttatttgtgaaatttgtga tgctattgctttatttgtaaccattataagctgcaataaacaagttaacaacaacaattgcattcattttatgttt caggttcaggggaggtgtgggaggttttttaaagcaagtaaaacctctacaaatgtggtatggctg attatgatcagtcgacatctggaaaaagtctgtaggcaactgcatgatccttctgtaggactcagtgat atttctatcactttattttctgcatttaaaccaatgctagctgctattgcagatattgagcacattgagaagga tatgaaacatcagagtttctacatagaaaccaagctagatggtgaacgtatgcaaatgcacaaaga tggagatgtatataaatacttctctcgaaatggatataactacactgatcagtttggtgcttctcctactga aggttctcttaccccattcattcataatgcattcaaagcagatatacaaatctgtattcttgatggtgagat gatggcctataatcctaatacacaaactttcatgcaaaagggaactaagtttgatattaaaagaatggt

(continued)

Table 1
(continued)

Name	Sequence (5′ to 3′)
	agaggattctgatctgcaaacttgttattgtgtttttgatgtattgatggttaataataaaaagctagggcatg agactctgagaaagaggtatgagattcttagtagtatttttacaccaattccaggtagaatagaaatagt gcagaaaacacaagctcatactaagaatgaagtaattgatgcattgaatgaagcaatagataaaa gagaagagggaattatggtaaaacaacctctatccatctacaagccagacaaaagaggtgaag ggtggttaaaaattaaaccagagtatgtcagtggactaatggatgaattggacattttaattgttggagg atattggggtaaaggatcacggggtggaatgatgtctcattttctgtgtgcagtagcagagaagccc cctcctggtgagaagccatctgtgtttcatactctctctcgtgttgggtctggctgcaccatgaaagaa ctgtatgatctgggtttgaaattggccaagtattggaagccttttcatagaaaagctccaccaagcag cattttatgtggaacagagaagccagaagtatacattgaaccttgtaa

open reading frame downstream of the alanine codon and cause release of the selectable marker from the N-terminal endogenous protein sequence.

5. Add the selectable marker sequence downstream of the self-cleaving 2A peptide coding sequence (i.e. GFP, hygromycin, or neomycin). *See* Table 1 for selectable markers sequence information.
6. Finally, add the right homology arm sequence downstream of the selectable marker sequence.
7. Clone the HDR donor template into a vector of choice and validate the plasmid DNA sequence for transfection into mammalian cells (*see* **Note 1**).

3.2 Cloning of sgRNA/Cas9-Encoding pX459 Plasmids

1. Digest 10 μg of the pX459 plasmid in a total volume of 30 μL in 1X CutSmart buffer using 1 μL of BbsI HF restriction enzyme. Incubate the reaction at 37 °C for 2 h. Purify the digested product using the NucleoSpin Gel and PCR Clean-up kit.
2. On ice, add 6.5 μL of nuclease-free water, 1 μL of 100 μM LIG4 sgRNA F oligonucleotide, 1 μL of 100 μM LIG4 sgRNA R oligonucleotide, 1 μL of 10X T4 DNA ligase buffer, and 0.5 μL of T4 PNK into a PCR tube. Mix by gently pipetting up and down. Place the PCR tube in a thermal cycler and run the following program: 37 °C for 30 min, 95 °C for 5 min, ramp down to 25 °C at a rate of 5 °C/min.
3. Create a 200X dilution of the annealed primers solution and add 1 μL from this dilution into a new PCR tube. Add 10 ng of BbsI-digested pX459 plasmid, 1 μL of 10X T4 DNA ligase buffer and bring to 9.5 μL with nuclease-free water. Add 0.5 μL of T4 DNA ligase and mix. Incubate the reaction at room temperature for 30 min. Heat inactivate the reaction by incubating at 65 °C for 10 min.

4. Mix 1 μL of the ligation reaction with 20 μL of competent *E. coli* cells previously thawed on ice. Incubate for 20 min on ice. Heat shock at 42 °C for 45 s. Add 500 μL of SOC medium and mix by pipetting up and down. Plate 150 μL of transformed bacteria onto LB agar plates containing 100 μg/mL carbenicillin. No recovery incubation time in SOC is required before plating. Place the plates in a 37 °C incubator overnight to allow colonies to grow.
5. Screen bacterial colonies to confirm the identity of the acquired plasmid. Pick colonies with a pipette tip and resuspend cells in 300 μL of LB medium containing 100 μg/mL carbenicillin. From this resuspension, use 1 μL as a template for a 50 μL PCR reaction using standard Taq DNA polymerase following the manufacturer's protocol. For specific amplification of the protospacer-containing plasmid, we chose the commonly used forward primer LKO.1 5′ that binds at the human U6 promoter (Weinberg Lab) and the reverse LIG4 sgRNA R primer. Perform a 1.5% agarose gel electrophoresis to confirm a 98 bp amplicon from successful clones. Inoculate an overnight 100 mL LB culture containing 100 μg/mL carbenicillin using the bacterial cell suspension from the positive clones and isolate the plasmid using a plasmid DNA extraction kit (e.g. the NucleoBond® Xtra Midi EF kit). Finally, confirm the correct integration of the protospacer sequence into the pX459 plasmid (LIG4 pX459) by Sanger sequencing using the LKO.1 5′ primer.

3.3 Homology-Directed Repair for Single Selectable Marker Knockout Strategy

The single selectable marker knockout strategy (Fig. 1a) is recommended for the mostly haploid HAP1 cell line, or for cell lines where the use of additional selectable markers might be prohibited (i.e. the cells already express several selectable markers; *see* **Note 5**). Below, we describe a protocol using the GFP selectable marker in HAP1 cells, but this approach could, in principle, be substituted with a different selectable marker, such as a different fluorescence- or drug resistance-based selectable marker as we describe below for HEK293 cells.

3.3.1 Transfection of HAP1 Cells

1. The day before, seed HAP1 cells in one well of a 6-well plate such that they are at approximately 70% confluency the next day at the time of transfection in a total of 3 mL of cell medium.
2. Prepare Lipofectamine 2000-based transfection complexes by mixing 1 μg of LIG4 pX459 plasmid and 1 μg of HDR donor plasmid in 120 μL of Opti-MEM medium to create the DNA solution.
3. In a separate tube, mix 4 μL of Lipofectamine 2000 reagent with 120 μL of Opti-MEM medium to create the Lipofectamine solution.

4. Add the plasmid solution to the Lipofectamine solution. Mix the solutions by pipetting up and down.
5. Incubate the mix for 15 min and then add dropwise to the cell medium containing the cells.
6. The next day, dissociate all cells from the plate and transfer them to a new 10 cm dish to expand cells.

3.3.2 Enrichment of the Gene Knockout Population

1. After 5 days post-transfection, use Fluorescence-Activated Cell Sorting (FACS) to isolate a fraction of cells expressing GFP corresponding to the top 10% GFP intensity from the bulk of transfected cells.
2. Culture the isolated GFP+ cell population until they stably grow and perform a second round of FACS to isolate a fraction of cells expressing GFP corresponding to the top 1% GFP intensity to obtain the final LIG4 knockout population of HAP1 cells (LIG4-KOp HAP1). This approach generates a population of cells with a complete LIG4 protein knockout as described below (*see* Subheading 3.7). This protocol may be continued with the isolation of single-cell derived clones if a knockout is not reached at this stage.

3.4 Homology-Directed Repair for Double Selectable Marker Knockout Strategy

The double selectable marker knockout strategy (Fig. 1b) is recommended for diploid cells. Selection of cells with two selectable markers enriches for cells with at least two edited alleles of the target gene. The selectable markers may be fluorescence- or drug-resistance-based, though we recommend using a drug resistance-based approach for this strategy (*see* **Note 6**). This approach might target more than two copies of the target gene within cells, but it could also be possible to deliver additional selectable markers if more than two copies of the target gene are known to be present in the genome of the cell line of choice. Here we describe this approach for the hypotriploid human HEK293 cell line.

3.4.1 Transfection of HEK293 Cells

1. The day before, seed HEK293 cells in one well of a 6-well plate such that they are at approximately 70% confluency the next day at the time of transfection in a total of 3 mL of cell medium.
2. Prepare Lipofectamine 2000 based transfection complexes by mixing 1 μg of LIG4 pX459 plasmid and 500 ng of the Hygromycin HDR donor plasmid and 500 ng of the Neomycin HDR donor plasmid in 120 μL of Opti-MEM medium to create the DNA solution.
3. In a separate tube, mix 4 μL of Lipofectamine 2000 reagent with 120 μL of Opti-MEM medium to create the Lipofectamine solution.
4. Add the plasmid solution to the Lipofectamine solution. Mix solutions by pipetting up and down.

5. Incubate the mix for 15 min and then add dropwise to the cell medium containing the cells.
6. The next day, dissociate all cells from the plate and transfer them to two 10 cm dishes to expand cells.

3.4.2 Positive Selection of the Knockout Population

1. After 48 h post-transfection, supplement the cell medium with 100 μg/mL of hygromycin and 200 μg/mL of G-418 (*see* **Note 7**).
2. Select cells in these conditions for at least 1 week, including at least one splitting event of the cell population into additional plates to confirm cell growth of the *LIG4* gene knockout HEK293 cell population (LIG4-KOp HEK293). Use non-transfected parent cells as controls to confirm death of unedited cells in the presence of hygromycin and G-418.

3.5 Isolation and Expansion of Single-Cell HEK293 Knockout Clones

1. Use a fraction of the cell population in hygromycin and G-418 to seed colonies in 15 cm culture dishes. Measure cell density and seed approximately 30 cells per dish for a total of ten dishes and incubate cells until colonies have formed (*see* **Note 8**).
2. Identify cell colonies and draw a circle around them on the bottom of the dish with a marking pen. Remove the cell medium and wash the plate with 10 mL of DPBS. Using sterile medium forceps, pick up a sterile cloning cylinder. Set the cylinder over the colony as to encircle (*see* **Note 9**). Add 100 μL of TrypLE Express dissociation reagent to the cloning cylinder. Incubate for 5 min without moving or disturbing the plate. Gently pipette up and down to resuspend the cells. Transfer the cells to a well of a 24-well plate and add the proper volume of cell medium.

3.6 Screening of Gene Knockout Clones by Genomic DNA PCR and Gel Electrophoresis

For PCR screening of successfully edited alleles from genomic DNA, design primers flanking the site of the DNA break such that amplification of the wild-type allele generates a PCR product between 200 and 400 bp (Fig. 2a). This allows for amplification across the selectable marker when properly integrated at the DNA break, and detection of a mobility shift of the PCR product in the gel electrophoresis.

1. Remove the cell medium from cells growing in 6-well plates and wash once with DPBS. Add 600 μL of RLT buffer and transfer the cell lysate to a 1.5 mL tube for extraction of gDNA using a genomic DNA extraction kit.
2. Perform a 50 μL PCR using the KAPA HiFi Enzyme and follow the manufacturer's protocol using 10 ng of gDNA with the LIG4 F and LIG4 R oligonucleotides each at a final 0.3 μM concentration, 1 min extension time and 32 PCR cycles. Using 1 min extension time ensures amplification of the GFP/hygromycin/neomycin cassettes.

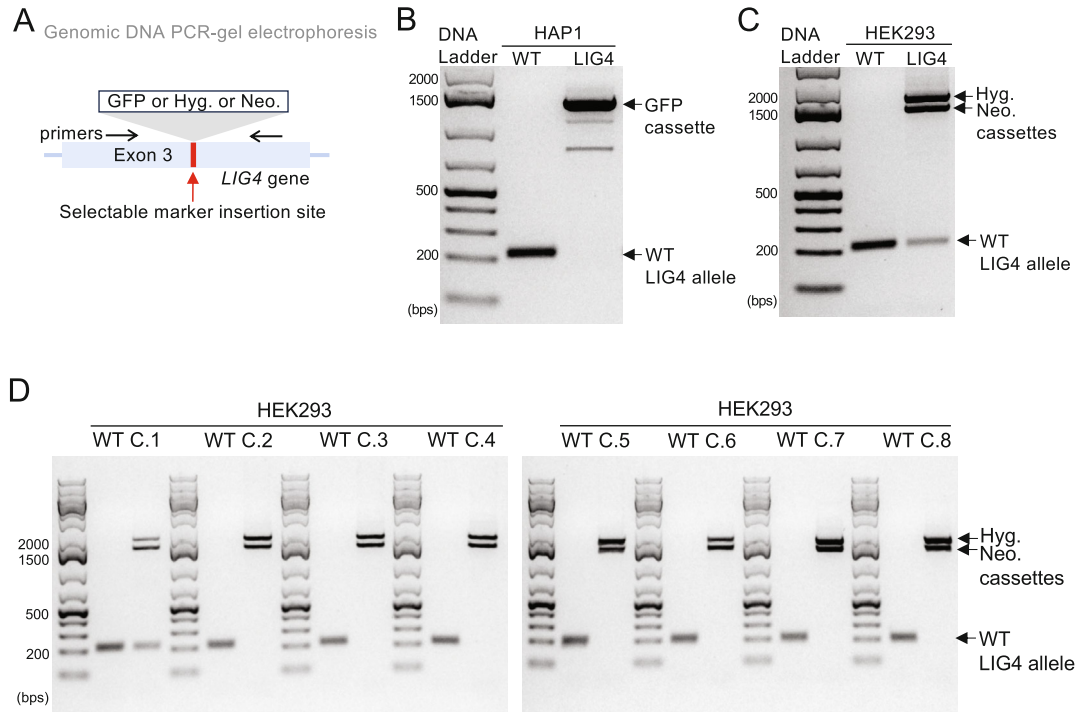


Fig. 2 Characterization of the LIG4 knockout by genomic DNA PCR and gel electrophoresis. (**a**) PCR primer design. (**b, c**) Genomic DNA PCR of the *LIG4* gene locus in the population of LIG4-KOp HAP1 (**b**) and LIG4-KOp HEK293 cells (**c**). (**d**) Genomic DNA PCR of the *LIG4* gene locus in the single cell-derived clones (C.1 to C.8), LIG4-KO HEK293 cells

3. Load 3 μL of each PCR reaction product into a 1% agarose TBE gel, including the GeneRuler 1 kb plus DNA ladder, and run the gel electrophoresis for 1 h at 120 V. From the population of LIG4-KOp HAP1 cells, the band corresponding to the wild-type *LIG4* allele was lost. Instead, the appearance of a novel band of higher molecular weight can be seen, which corresponds to the integrated GFP cassette (Fig. 2b). Similarly, the population of LIG4-KOp HEK293 cells yield two novel bands corresponding to the integration of the hygromycin and neomycin cassettes (Fig. 2c). While the wild-type *LIG4* band is still present, its intensity is substantially reduced, which suggests that a high number of *LIG4* alleles have been targeted in the cell population (Fig. 2c). Indeed, seven single cell-derived clones from this population completely lost the wild-type band from a screening of eight clones (Fig. 2d).

3.7 Confirmation of Protein Knockout by Immunoblotting

1. Remove the cell medium from the cells growing in 10 cm dishes and place them on ice. Wash once with 10 mL of DPBS and add 500 μL of whole cell lysis buffer supplemented with protease and phosphatase inhibitors. Scrape and transfer

the cells into a new 1.5 mL tube and place it on ice for 5 min. Add 1 μL of benzonase enzyme, mix by pipetting up and down, and incubate at 4 °C for 40 min.

2. Centrifuge the protein lysates at maximum speed on a tabletop centrifuge for 10 min. Transfer the supernatant into a new 1.5 mL tube avoiding carrying over any formed pellet.
3. Prepare a set of BCA assay protein standards by serially diluting the BSA stock using water as the diluent. We typically use 0, 25, 125, 250, 500, 750, 1000, 1500, and 2000 μg/mL of BSA as the final standard concentrations. Prepare the BCA Working Reagent by mixing 50 parts of Reagent A with 1 part of Reagent B. Use the following formula to determine the total volume of working reagent required: (# standards + # unknowns) × (# replicates) × (volume of working reagent per sample [200 μL]) = total volume working reagent required. Dilute protein samples 1:5 with water to a final volume of 10 μL. Pipette 10 μL of each standard or diluted protein sample into a microplate well. Add 200 μL of the working reagent to each well and mix the plate thoroughly on a plate shaker for 30 s. Cover the plate and incubate at 37 °C for 30 min. Cool the plate to room temperature and measure the absorbance at 562 nm on a plate reader. Plot the average blank-corrected 562 nm measurement for each standard versus its concentration in μg/mL and use the resulting standard curve to determine the protein concentration of the samples.
4. Based on the BCA assay results, aliquot 20 μg of each protein sample into 1.5 mL tubes. To prepare the protein samples for gel loading, add the appropriate volume of 4X NuPAGE LDS Sample Buffer containing 50 mM DTT and heat at 90 °C for 10 min.
5. Conduct SDS-PAGE using the NuPAGE 4–12% Bis-Tris Protein Gel. Prepare 1X NuPAGE MOPS SDS Running Buffer by diluting 50 mL of the 20X stock solution with 950 mL of deionized water. Prepare the pre-cast mini gel by gently removing the gel comb, peeling the tape off the slot at the lower portion of the cassette, and rinsing the wells thoroughly with 1X Running Buffer. Place the gel cassette in the electrophoresis tank and fill both the top and bottom chambers of the tank with 1X running buffer. Load the protein samples and the molecular weight ladder. Place the lid on the electrophoresis tank and run the gel at ~100–200 V.
6. Soak two pieces of sponge, two filter papers, and the nitrocellulose membrane in 1X transfer buffer.
7. After the electrophoresis is complete, carefully disassemble the gel cassette and remove the plate to which the gel is not attached. Place a wet filter paper on the gel, and using the gel

knife, release the gel from the plate and place it onto the filter paper. Trim the edges of the gel with the gel knife. Place the wet nitrocellulose membrane onto the gel and ensure there are no air bubble between the gel and the membrane (a roller may be used for this purpose). Add the other wet filter paper onto the membrane to create the transfer sandwich.

8. To assemble the transfer, place the cathode core (−) of the Mini Blot Module on a flat surface and add 5–10 mL of 1X Transfer Buffer to the core. Place a presoaked sponge on the cathode core, then the transfer sandwich, and finally the second presoaked sponge, being careful to avoid introducing air bubbles. Place the anode core (+) of the Mini Blot Module on top of the assembled blot sandwich and press the two module halves together. Place the blot module into a chamber of the electrophoresis tank with the cathode core (−) facing outwards towards you. The final setup should be: Cathode (−) sponge-filter paper-gel-membrane-filter paper-sponge-anode (+). Completely submerge the blot sandwich in 1X transfer buffer. Place the lid on the electrophoresis tank and transfer at 15 V for 60 min. The transfer may also be conducted at a lower voltage overnight in the cold room.
9. After the transfer is complete, disassemble the blot module and carefully move the membrane with tweezers to a gel box containing Intercept Blocking buffer. Block for 1 h at room temperature in a rocker.
10. Add the primary DNA ligase IV Recombinant Rabbit Monoclonal Antibody, or the appropriate primary antibody, to the blocking buffer at the concentration recommended for western blotting (1:1000 dilution for the DNA ligase IV Recombinant Rabbit Monoclonal Antibody) and rock for 1 h at room temperature or overnight in the cold room. Wash with PBS containing 0.1% Tween three times for 5 min each with rocking.
11. Add the secondary antibody diluted in the blocking buffer and incubate for 30 min to 1 h at room temperature with rocking. Wash with PBS containing 0.1% Tween three times for 5 min each with rocking.
12. Conduct a final wash in PBS without Tween and promptly image in the imager with the settings appropriate for the secondary antibody conjugate. Consistent with the genomic DNA PCR gel electrophoresis result, the band corresponding to the ligase IV protein product is completely lost in the population of LIG4-KOp HAP1 cells (Fig. 3a), and substantially reduced in the population of LIG4-KOp HEK293 cells (Fig. 3b).

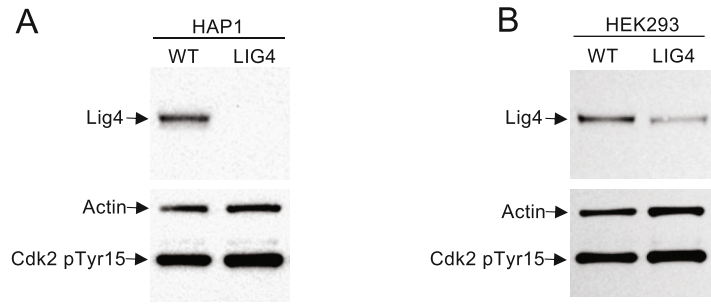


Fig. 3 Characterization of the LIG4 protein knockout by Western blot. (**a, b**) Western blot for LIG4 protein confirms complete knockout in LIG4-KOp HAP1 cells (**a**) and reduced levels in LIG4-KOp HEK293 cells (**b**) compared to the corresponding wild-type (WT) cells. Loading control is provided by an anti-Actin and anti-phospho-Cdk2 (Tyr15) antibody cocktail

4 Notes

1. The HDR donor template to target the *LIG4* gene in this study was synthesized by Twist Bioscience as a "clonal gene" plasmid using the "pTwist Amp High Copy" vector (*see* Table 1 for final HDR donor sequence) and propagated using the NucleoBond® Xtra Midi EF kit to ensure minimal endotoxin contamination.
2. Including the endogenous first exon–exon junction that may recruit the exon–exon junction complex (EJC) is recommended. The EJC is an anchor for various molecular transactions of mRNAs [21], which may enhance expression of the selectable cassette.
3. If the protospacer sequence does not start with a "G" nucleotide, append a "G" nucleotide to the 5′ end for a total of 21 nucleotides. The human U6 promoter prefers a "G" nucleotide at the transcription start site for increased expression. If adding the "G" nucleotide, ensure both forward and reverse oligos are complementary for the entire 21 nucleotides.
4. Using the DNA break as the starting boundary for creation of homology regions increases the complementarity of the cleaved target DNA region with the donor template for HDR. Regardless of where the start and end boundaries of the homology arms are defined, ensure that the protospacer sequence is not fully contained in the final HDR donor template to avoid targeting by the Cas9 endonuclease.
5. Fluorescent markers are also a good alternative when expression of drug-based selectable markers is not desirable in downstream experiments (e.g. they might be needed for selection of cells carrying additional modifications in the knockout

background). Please note that G-418 and/or hygromycin treatment does not need to be sustained after positive selection of the knockout cell population.

6. The drug-based double selectable marker knockout strategy offers a simpler positive selection of cells compared to a double FACS selection strategy. Isolation of cells with expression of two fluorescent reporters via FACS may additionally require incorporating additional conditions such as compensation controls. Nevertheless, relying on fluorescent selectable markers might still be a good alternative if the drug-based selectable markers are already in use in the cell line of choice.
7. The cells might tolerate different concentrations of hygromycin and G-418 depending on the expression level of the target gene. We recommend including a higher concentration condition (200 μg/mL of hygromycin and 400 μg/mL of G-418) in one of the two dishes generated after transfection and choosing the condition with the highest drug concentration if cell survival is obtained in both conditions.
8. Generally, not all cells are viable and form colonies. This number of cells is expected to result in a few colonies per plate, which reduces the potential for cross-contamination. Place the cells in the incubator for at least 2 weeks, or until large colonies can be identified. Make sure to provide fresh cell medium as recommended for your cell line of choice.
9. Although not absolutely necessary, you may use sterile silicone grease at the bottom of the cloning cylinder to seal the cylinder against the bottom of the dish.

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