Chapter 2

Determining mRNA Decay Rates Using RNA Approach to Equilibrium Sequencing (RATE-Seq)

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Abstract

RATE-seq is a *4-t*hio*u*racil (4-tU)-based method that enables the in vivo measurement of transcriptome-wide RNA degradation rates. 4-tU is an analog of uracil that is rapidly incorporated into newly synthesized RNA and facilitates the conjugation of a biotinylated molecule containing a reactive thiol group. The biotinylated RNA can then be fractionated from the unlabeled RNA with streptavidin magnetic beads. By adding 4-tU to a culture of cells growing in steady-state conditions, fractionating the labeled population of RNA at multiple time points following 4-tU addition, and quantifying the abundance of newly transcribed RNAs using RNAseq, it is possible to estimate the degradation rates of all transcripts in a single experiment. The analysis of the RATE-seq data entails normalization of RNAseq libraries to thiolated RNA spike-ins and nonlinear model fitting to estimate the degradation rate constant for each RNA species.

Key words 4-thiouracil, RNA degradation, RNA turnover, Metabolic labeling, RNA stability

1 Introduction

Measuring RNA degradation rates poses two main challenges: (1) minimizing perturbation of cellular physiology and (2) efficiently assaying thousands of transcripts to assess transcriptome-wide dynamics. Historically, most methods for measuring RNA decay rates involved halting transcription, through chemical or genetic inhibition of RNA polymerase, and tracking the decrease in RNA levels over time [1–3]. However, such methods are typically deleterious to cells and thereby are potentially confounded by stress-induced changes in RNA metabolism and gene expression. More recently, the metabolic label 4-thiouracil (4-tU), a uracil analogue that is rapidly incorporated into newly synthesized RNA, has been used in two different ways to study transcriptome-wide degradation rates: using either a pulse-chase or a pulse-in experimental design [4–6]. In a pulse-chase experiment, 4-tU is added to a growing cell population for several generations. The 4-tU label is then chased with unlabeled uracil and the rate of

Shireen R. Lamandé (ed.), *mRNA Decay: Methods and Protocols*, Methods in Molecular Biology, vol. 1720,
https://doi.org/10.1007/978-1-4939-7540-2_2,

decrease in labeled RNA is used to estimate degradation kinetics. In a pulse-in experiment, the rate with which RNA is labeled and reaches an equilibrium value is used to estimate transcript degradation rates. Pulse-chase methods are potentially susceptible to effects of nucleotide recycling, which may result in the reincorporation of the 4-tU label from degraded transcripts into newly synthesized RNAs. An aberrantly "slow chase" can result, leading to an underestimation of mRNA decay rates. By contrast, in pulse-in methods this problem is circumvented by quantifying the incorporation kinetics of 4-tU.

*R*NA *A*pproach to *E*quilibrium *S*equencing (RATE-seq) combines a pulse-in design with time course sampling and RNA sequencing (RNAseq) to estimate transcriptome-wide degradation rates. Briefly, multiple RNA samples are collected at defined time points after 4-tU is pulsed into a culture of cells growing in steady-state conditions. Following RNA purification, a cocktail of synthetic thiolated spike-ins is added to each time point sample in a fixed ratio of spike-in to RNA quantity. After conjugation to a biotin-containing reactive molecule (either biotin-HPDP or biotin-MTS [7]), the labeled RNA is fractionated using streptavidin magnetic beads, cDNA libraries are generated, and the entire pool of cDNA is processed using standard RNAseq protocols [4].

The underlying principle of RATE-seq is that the abundance of any mRNA species can be modeled by the mathematical relationship: $d[\mathrm{mRNA}]/dt = k - \alpha \cdot [\mathrm{mRNA}]$, where [mRNA] is the concentration of a particular mRNA, k is the rate of transcript synthesis, α is the exponential decay constant, and t is time. On the basis of this model, the abundance of any mRNA at time (t) is given by the relationship $[\mathrm{mRNA}](t) = [\mathrm{mRNA}]_{ss} \cdot (1 - e^{\alpha \cdot t})$, where $[\mathrm{mRNA}]_{ss}$ is the concentration of a given mRNA in steady-state conditions [8, 9]. When the labeled fraction is considered independent of the unlabeled fraction, the same relationship holds for labeled RNA: $[\mathrm{mRNA}_{\mathrm{labeled}}](t) = [\mathrm{mRNA}_{\mathrm{labeled}}]_{ss} \cdot (1 - e^{\alpha \cdot t})$. A feature of this model is that the time an RNA takes to reach its equilibrium value is a function only of α. In general, an RNA that reaches its equilibrium value quickly has a fast degradation rate (i.e., a larger value of α) whereas an RNA that reaches its equilibrium value slowly has a slow degradation rate (i.e., a smaller value of α). RATE-seq aims to determine the value of the exponential degradation rate constant, α, by fitting this model to each mRNA using spike-in-normalized RNAseq counts of the labeled mRNAs over time. Here, we describe the experimental procedures for generating RATE-seq data and provide guidelines for the analysis of data using normalization of RNAseq data to exogenous thiolated spike-ins and nonlinear model fitting. Our protocol focuses on 4-tU labeling and fractionation. A protocol for RNAseq library preparation has not been provided as any standard RNAseq protocol can be employed. RATE-seq has been developed in yeast cells, but is amenable to any cell type that transports and incorporates 4-tU.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cmat 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Follow all waste disposal regulations when disposing of waste materials.

2.1 Sampling

1. 4-tU labeling reagent: Dissolve 4-tU in DMSO so that the final concentration is 500 μM in the cell culture.
2. 25mm, 0.45 micron circular filters.
3. Liquid nitrogen.
4. Filtration device (vacuum flask, vacuum source, glass micro-analysis filter holder assembly).
5. 2 mL eppendorf tubes.

2.2 RNA Extraction

1. Lysis buffer: 0.01 M EDTA, 0.5% SDS, 0.01 M Tris pH 7.5, RNase-free H_2O.
2. Acid phenol.
3. Heavy phase lock gel (PLG) tubes.
4. Chloroform.
5. Sodium acetate: 3 M sodium acetate. pH with glacial acetic acid to 5.2.
6. 95% ethanol.
7. 70% ethanol.
8. Qubit RNA HS kit and Qubit fluorometer.

2.3 Making In Vitro Spike-ins

1. *Escherichia coli* strains with plasmids DGP 104, 105, 106 (*see* **Note 1**).
2. LB media: 10 g Bacto tryptone, 5 g yeast extract, and 10 g NaCl in 1 L of water.
3. EcoRI restriction enzyme.
4. 10× EcoRI compatible buffer.
5. Qiagen Miniprep Kit.
   (a) 100 mM DTT.
6. Promega Riboprobe in vitro Transcription System.
   (a) rNTP.
   (b) SP6 RNA Polymerase.
   (c) Transcription optimized 5× buffer.
7. Qiagen PCR Cleanup Kit.
8. 10 mM 4-thiouridine.
9. Qubit DNA HS.

2.4 Fractionation of Labeled RNA

1. 1 M Tris–HCl, pH 7.4.
2. 0.5 M EDTA.
3. 1 M HEPES.
4. Biotinylation reagent: Dissolve 10 mg of Biotin MTS in 100 mL of Dimethylformamide.
5. 75% ethanol.
6. Chloroform–Isoamyl alcohol (24:1).
7. Isopropoanol.
8. 5 M NaCl.
9. Glycogen.
10. RiboZero Kit.
11. Magnetic stand.
12. Streptavidin magnetic beads.
13. 5% Beta-mercaptoethanol.
14. 1.5 mL eppendorf tubes.
15. Streptavidin bead buffer: 1 M NaCl, 10 mM EDTA, 100 mM Tris–HCl, pH 7.4.
16. Reagents for RNAseq protocol of choice.

3 Methods

Carry out all procedures at room temperature unless otherwise specified. Use RNAse-free H_2O unless otherwise specified.

3.1 Sampling

1. Grow liquid culture in defined media containing a total of 500 μM uracil until exponential growth.
2. Assemble vacuum flask with filter support and turn on vacuum source. Test by running ultra pure water onto the filter to ensure that the liquid is being sucked out.
3. Add 4-tU solution to the growing culture to a final concentration of 500 μM. Sample the first time point by pipetting 10 mL of culture into the filter apparatus. Remove the circular filter with tweezers once the liquid has drained. Place the filter into a 2 mL eppendorf tube and immediately place in liquid nitrogen. Samples can be stored in −80 C for subsequent processing.
4. Rinse filter apparatus with ultrapure water in between samplings and continue sampling as in **step 3** for approximately 2 h. Samples should be acquired frequently at the beginning of the time course and less frequently toward the end to capture approach to equilibrium kinetics. An example time course would be sampling at 3, 5, 7, 11, 25, 35, 50, and at 160 min.

3.2 RNA Extraction

1. Add 750 µL of lysis buffer and 750 µL of acid phenol to the frozen samples and vortex until foamy and opaque. Incubate for 1 h at 65 °C and vortex every 20 min.
2. Discard the filter and put the samples on ice for 10 min.
3. Spin samples for 5 min at 5000 × *g*.
4. With a pipette, transfer the top aqueous layer to a prespun PLG tube.
5. Add 750 µL chloroform and invert to mix (*see* **Note 2**) and spin at 5000 × *g* for 5 min.
6. Pour aqueous layer into a new 2 mL eppendorf (*see* **Note 3**).
7. Add 75 µL of 3 M sodium acetate and 1.2 mL 100% ethanol, then vortex samples. Incubate samples at −80 °C for 30 min (*see* **Note 4**).
8. Spin samples at 5000 × *g* for 10 min at 4 °C and decant supernatant.
9. Wash the surface of the pellet with 400 µL of 70% ethanol and spin samples for 2 min at 5000 × *g* and discard supernatant. Repeat this step then proceed to **step 10**.
10. Air-dry samples inverted on the bench for 30 min.
11. Dissolve pellet in 100 µL water by pipetting up and down (*see* **Note 5**).

3.3 Making Synthetic Spike-In RNAs

1. Grow up *E. coli* strains DGP 104, 105, 106 in LB media overnight.
2. Follow the Qiagen miniprep protocol to extract plasmids. Quantify using Qubit DNA HS.
3. Linearize plasmids by making a reaction of 5 µL of 10× EcoRI compatible buffer, 5 µL EcoRI, 3 µg of the extracted plasmid to a total volume of 50 µL. Incubate for 2 h at 37 °C (*see* **Note 6**).
4. Clean up reactions using Qiagen PCR cleanup kit and resuspend in 30 µL of H_2O. Quantify using Qubit DNA HS. Around 100–200 ng/mL is required to proceed.
5. Add 6.25 µL of linearized plasmid DNA, 4 µL transcription optimized 5× buffer, 2 µL DTT, 0.75 µL Recombinant RNasin, 4 µL rNTP, 2 µL 4sUTP, and 1 µL of SP6 RNA Polymerase. Incubate for 1 h at 30 °C.
6. Add 1 µL RQ1 RNase-Free DNase and incubate for 15 min at 37 °C.
7. Add 19 µL acid phenol. Vortex vigorously and centrifuge at 12,000 × *g* for 2 min.
8. Transfer top aqueous layer to a new eppendorf tube and add 1.8 µL of 3 M Na Acetate and 45 µL of 100% ethanol (*see* **Note 3**). Incubate for 30 min in −80 °C (*see* **Note 4**).

9. Centrifuge samples at full speed at 4 °C for 25 min and discard supernatant.
10. Wash with 1 mL 70% ethanol then centrifuge for 5 min at full speed. Discard supernatant and repeat this step then proceed to **step 11**.
11. Resuspend pellet in 10 μL of H_2O by pipetting up and down.

3.4 Fractionation of Labeled RNA (See Note 7)

1. Dissolve RNA in 20 μL of water to a concentration of 500 ng/μL.
2. Add 147 μL H_2O, 2.5 μL 1 M HEPES, 0.5 μL 0.5 M EDTA, and 50 μL RNA sample. Add the 50 μL of biotin last and vortex briefly. Incubate samples in the dark for 2 h.
3. Add 250 μL chloroform–isoamyl alcohol (24:1) and pipet up and down until mixed (*see* **Note 8**).
4. Transfer into prespun PLG tube and centrifuge for 5 min.
5. Pour the aqueous phase of the PLG into a new 1.5 mL eppendorf (*see* **Note 9**).
6. Precipitate RNA by adding 1/10 volume of 5 M NaCl and 1.1 volume of isopropanol and incubate at room temperature for 10 min.
7. Spin for 20 min at full speed at 4 °C and discard the supernatant (*see* **Note 10**).
8. Add 400 μL of 75% ethanol and spin for 5 min at full speed at 4 °C and remove supernatant. Resuspend in H_2O to a concentration of 500 ng/μL.
9. Warm 100 μL of bead buffer per sample to 65 °C.
10. Add 200 μL beads to new eppendorf tubes and place in magnetic rack. Leave for 1.5 min until beads sediment and then discard the supernatant.
11. Remove tubes from magnetic rack and wash the beads with 200 μL bead buffer by pipetting up and down. Sediment beads in magnetic rack again for 1.5 min and discard supernatant.
12. Remove tubes from magnetic rack and resuspend in 100 μL of bead buffer. Add 100 μL of RNA sample (*see* **Note 11**) and incubate for 20 min on the bench.
13. Place samples in magnetic stand for 2 min then discard the supernatant (*see* **Note 12**).
14. Remove tubes from magnetic rack and resuspend in 100 μL of bead buffer and incubate for 5 min. Sediment beads in magnetic rack again for 2 min and discard supernatant.
15. Remove tubes from magnetic rack and resuspend in 100 μL bead buffer and incubate for 1 min. Sediment beads in magnetic rack again for 2 min and discard supernatant.

16. Remove tubes from magnetic rack and resuspend in 100 μL of the 65 °C prewarmed bead buffer and leave for 1 min. Sediment beads in magnetic rack again for 2 min and discard supernatant.
17. Remove tubes from magnetic rack and resuspend in 100 μL bead buffer and leave for 1 min. Sediment beads in magnetic rack again for 2 min and discard supernatant.
18. Remove tubes from magnetic rack and resuspend beads in 20 μL of 5% β-mercaptoethanol and incubate for 10 min at room temperature. Sediment beads in magnetic rack again for 2 min and transfer supernatant to a new eppendorf (*see* **Note 13**).
19. Repeat β-mercaptoethanol incubation at 65 °C with the beads from **step 18**, then pool that with the supernatant from **step 18**.
20. Precipitate RNA with 1/10 volume 5 M NaCl, 4 μg glycogen, and 1.1 volume Isopropanol.
21. Incubate for 10 min at room temperature and spin at maximum speed for 25 min at 4 °C.
22. Discard supernatant and wash with 75% ethanol then spin at maximum speed for 10 min.
23. Resuspend pellet in 10 μL water. Sediment beads in magnetic rack again for 2 min and transfer supernatant to a new eppendorf tube.
24. Quantify RNA concentration using Qubit RNA HS (*see* **Note 14**).
25. Use the RiboZero Kit as per instructions to remove rRNA (*see* **Note 15**).
26. Generate RNAseq libraries using a standard strand-specific RNAseq protocol.

3.5 Computational Analysis

1. Align reads to reference transcriptome using a splice-aware aligner (e.g., HiSat2). The reference genome, and annotation file (if used), must contain the sequences of the synthetic spike-in RNAs. Generate a BAM file containing mapped reads.
2. Count the number of reads mapping to each transcript using a program such as htseq-count or Rsubread and generate a matrix containing gene name and number of RNAseq counts at each time point. The annotation file (gtf or gff) used for this step must contain the relevant information for the spike-ins.
3. Test whether spike-ins decrease in relative abundance compared to native RNA counts with time (*see* Fig. 1).
4. Normalize RNAseq counts within each timepoint to spike-in abundance by determining the total proportion (*p)* of reads mapping to all spike-ins in the library i (p_i) and dividing the RNAseq counts for each RNA j in library i by p_i (RNA_{ji}/p_i).

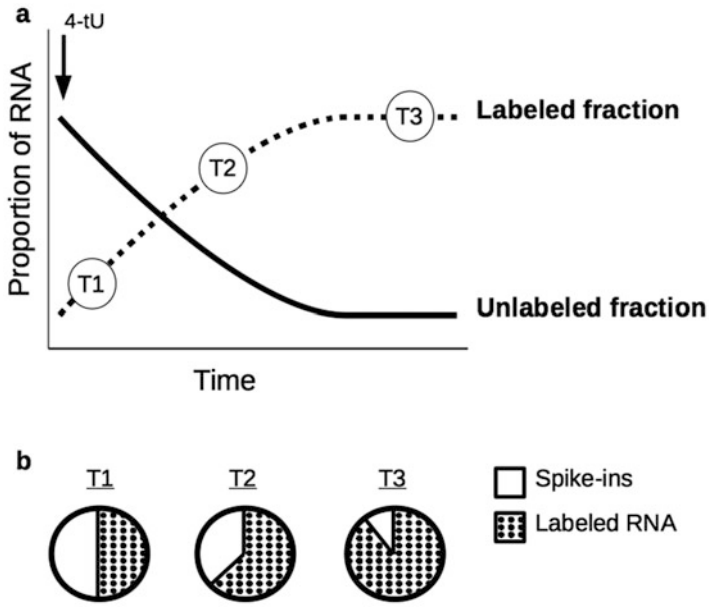

Fig. 1 Principle of RNA approach to equilibrium sequencing. (**a**) Fractionating the labeled RNA from a sample time course collected after the addition of 4-tU yields increasing amounts of RNA until equilibrium is reached. T1, T2, and T3 are time points representing the beginning, middle, and end of a typical RATE-seq time course. (**b**) Pie charts representing fraction of RNA-seq reads belonging to spike-ins compared to RNA. While the absolute quantity of spike-ins is the same for all time points, its fraction shrinks as the labeled RNA fraction increases. Normalizing RNA-seq reads to a common pool of synthetic spike-ins accounts for technical variation that is attributable to variation in RNA-seq library sizes between time points

5. Fit a nonlinear model to the normalized counts to estimate approach to equilibrium kinetics using a statistical programming language, such as R. Using regression, estimate the parameters $[\mathrm{mRNA}_{\mathrm{labeled}}]_{\mathrm{ss}}$ and α using the formula $[\mathrm{mRNA}_{\mathrm{labeled}}](t) = [\mathrm{mRNA}_{\mathrm{labeled}}]_{\mathrm{ss}} \cdot (1 - \mathrm{e}^{\alpha \cdot t})$, where $[\mathrm{mRNA}_{\mathrm{labeled}}]_{\mathrm{ss}}$ is the amount of labeled RNA at steady state, α is the combined sum of growth rate and RNA degradation rate, and t is time. Extract the degradation rate constant for each mRNA.
6. To assess model fits, plot normalized data and model fits (*see* Fig. 2).

4 Notes

1. Synthetic spike-ins were generated from *Bacillus subtilis* and *Caenorhabditis elegans* sequences cloned into a polyA vector to generate polyA-tailed RNAs using in vitro transcription. For sequence information see [4].
2. Vortexing will ruin PLG tubes.

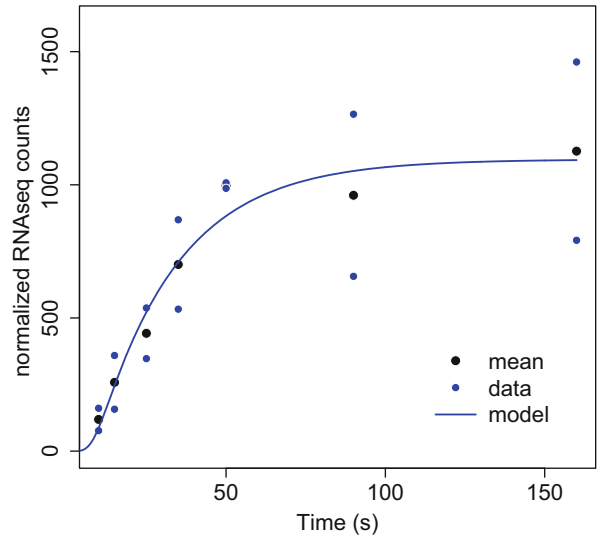

Fig. 2 Approach to equilibrium profile of normalized counts. A nonlinear model is fit to the data using the equation: $[\text{mRNA}_{\text{labeled}}](t) = [\text{mRNA}_{\text{labeled}}]_{\text{ss}} \cdot (1 - e^{\alpha \cdot t})$ to estimate the values of $[\text{mRNA}_{\text{labeled}}]_{\text{ss}}$ and α. Model fitting is ideally performed using a weighted regression that aims to account for unequal variances between time points or a maximum likelihood method that assumes negative binomial distributions of counts at each time point

3. If your sample has low amounts of RNA, use glycogen as a carrier to help precipitate the RNA.
4. Samples can also be precipitated overnight by incubating at −20 °C.
5. Begin by resuspending in a small amount of water then dilute further if the concentration is too high.
6. Run linearized and unlinearized fragments on a 1% gel to check that the reaction has proceeded as expected.
7. A dotblot should be performed as in ref. 4 to monitor the dynamics of label incorporation into bulk RNA. Once it is established that 4-tU is being incorporated into RNA as expected in your system, it is not necessary to repeat a dotblot each time RATE-seq is used.
8. Alternate RNA precipitation methods can be used here such as an ethanol precipitation or an Ampure bead cleanup.
9. You can try to pipet out the remnants of the aqueous phase unless there is white particulate inside.
10. The pellet does not stick well to the tube and could be discarded with the supernatant if the sample is not handled carefully.

11. If adding more than 50 μg, make sure the volume of bead buffer to sample is at least 1:1.
12. If interested in the unlabeled RNA, save the unbound fraction for downstream processing and sequencing.
13. The liquid should be pink/light purple color. Avoid accidentally transfering beads into the new tube.
14. An increase in RNA concentration with time should be observed.
15. This kit is expensive. Scaling down this protocol by a half has been successful.

References

1. Wiesner RJ, Zak R (1991) Quantitative approaches for studying gene expression. Am J Phys 260(4 Pt 1):L179–L188
2. Wang Y, Liu CL, Storey JD, Tibshirani RJ, Herschlag D, Brown PO (2002) Precision and functional specificity in mRNA decay. Proc Natl Acad Sci U S A 99:5860–5865. https://doi.org/10.1073/pnas.092538799
3. Grigull J, Mnaimneh S, Pootoolal J, Robinson MD, Hughes TR (2004) Genome-wide analysis of mRNA stability using transcription inhibitors and microarrays reveals posttranscriptional control of ribosome biogenesis factors. Mol Cell Biol 24:5534–5547. https://doi.org/10.1128/MCB.24.12.5534-5547.2004
4. Munchel SE, Shultzaberger RK, Takizawa N, Weis K (2014) Dynamic profiling of mRNA turnover reveals gene-specific and system-wide regulation of mRNA decay. Mol Biol Cell 22:2787–2795. https://doi.org/10.1091/mbc.E11-01-0028
5. Miller C, Schwalb B, Maier K, Schulz D, Dümcke S, Zacher B, Mayer A, Sydow J, Marcinowski L, Dölken L, Martin DE, Tresch A, Cramer P (2011) Dynamic transcriptome analysis measures rates of mRNA synthesis and decay in yeast. Mol Syst Biol 7:58. https://doi.org/10.1038/msb.2010.112
6. Neymotin B, Athanasiadou R, Gresham D (2014) Determination of in vivo RNA kinetics using RATE-seq. RNA 20:1645–1652. https://doi.org/10.1261/rna.045104.114
7. Duffy EE, Rutenberg-Schoenberg M, Stark CD, Kitchen RR, Gerstein MB, Simon MD (2015) Tracking distinct RNA populations using efficient and reversible covalent chemistry. Molecular Cell 59(5)858–866
8. Greenberg JR (1972) High stability of messenger RNA in growing cultured cells. Nature 240:102–104
9. Kim CH, Warner JR (1983) Messenger RNA for ribosomal proteins in yeast. J Mol Biol 165:79–89