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Minimal perturbation analysis of mRNA degradation rates with Tet-off and RT-qPCR

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RNA biology of fungal pathogens

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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 post-transcriptional 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.

Keywords: 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 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 (Hoek *et al*, 2019). 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 artefacts (Tutucci *et al*, 2018; Li *et al*, 2022).

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 (Cleary *et al*, 2005; Dölken *et al*, 2008), variants of pulse-chase labeling of mRNA use now chemical modification of incorporated nucleotides that can be detected by short reads sequencing (Herzog *et al*, 2017).

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-phenantrolin or thiolutin, can have various secondary effects because a stress response can be induced by such treatments (e.g. Zencir *et al*, 2020).

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 (Gossen & Bujard, 1992). 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 potential TATA box, for example (Baudrimont *et al*, 2017). 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 (Wada & Becskei, 2017).

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 (Garí *et al*, 1997) 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 minutes.
2. SD-URA medium: 2% D-glucose, 6.7g/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 minutes. 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. Stored at room temperature.
2. Phenol-chloroform-isoamyl alcohol (25:24:1), pH 5.2. Store at 4°C.

3. Ethanol-Ammonium acetate mix (6 volumes of ethanol for 1 volume of 7.5M ammonium acetate. Store at -20°C).
4. Ethanol 70% (vol/vol). Store at room temperature.
4. Thermomixer of dry bath that can hold 65°C for 1.5 ml Eppendorf tubes.
5. Optimized DNase I and its buffer containing Mg²⁺ and Ca²⁺ ions (e.g. Turbo Dnase).
6. Spectrophotometer for small volumes working at 260 nm (e.g. Nanodrop).
7. Cell culture incubator with shaking (30° C).
8. Doxycyclin 1000x stock solution at 10 mg/ml in ethanol (store at -20°C).
9. 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 pre-mix, containing SYBR Green intercalating fluorophore, thermostable DNA polymerase, dNTPs and the manufacturer buffer. Usually these pre-mixed solutions are provided as 2X stock. Store at -20°C.
4. Oligonucleotides specific for the transcript of interest^{NOTE1}. Store at 100 µM in H₂O 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. Reverse transcriptase buffer 5X stock (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl₂). 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, each deoxy-nucleotide triphosphate (dATP, dGTP, dCTP, dTTP) at 25 mM. Store at -20°C.
10. Linear polyacrylamide 5 mg/ml stock (store at -20° C).

2.4 Analysis of results and half-life estimation

1. Computer system with R, Rstudio and MASS package installed (*reshape2* is 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 diluted in 300 ml YPD medium at a 600 nm to an initial absorbance of 0.15.
2. Incubate the cells with agitation at 30°C for about 4 hours, 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 minutes. Continue incubation with agitation at 30°C.
4. Add 50 µl doxycycline stock to the flask labeled “30 min.”. Add doxycyclin 10 minutes 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 at 2000g, 4°C, for 10 minutes.
7. Transfer the cells using 1 ml of ice cold water in an Eppendorf tube. Centrifuge 30 seconds at 10 000 g. Remove supernatant and store the tubes at -80° C or process immediately.

3.2 Total RNA extract preparation with hot phenol

1. Suspend the pellet by pipetting up and down 400 µl lysis buffer. Add 400 ul phenol/choloroform/isoamyl alcohol mix. Vortex for 10 seconds.
2. Transfer the tubes to a dry block at 65°C and incubate for 30 minutes with occasional mixing.
3. Move the tubes on ice and incubate for 5 minutes.
4. Separate phases by centrifugation at 14 000 g for 5 min., 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 minutes.

7. Recover precipitated RNA by centrifugation at 14 000 g for 15 min in a refrigerated centrifuge (4° C).
8. Wash the pellet with 800 µl ethanol (70%) twice, using 5 minutes of centrifugation to recover the pellet at each wash.
9. Remove all the ethanol from the wash step 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 minutes 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 15 µl total volume, together with DNase buffer and modified DNase I (2 units). Incubate 30 min. at 37°C
2. Add 85 µl water, to increase the working volume and perform a phenol-chloroform extraction.
3. To the recovered aqueous phase, add 8 µl linear polyacrylamide solution (40 µg) and 3.5 volumes ethanol/ammonium acetate mix. Incubate on ice for 10 minutes.
4. Recover the DNase treated RNA by centrifugation for 20 min. at 14 000g at 4°C.
5. Wash once with 0.8 ml 70% ethanol. After drying the pellet, suspend in 10 µl 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 µl PCR tube, should contain 1 µl 5X RT buffer and specific oligonucleotides for the reaction, 0.5 µl of a 5 µM solution for each^{NOTE2}. A control reaction, without oligonucleotides, is optional but recommended. An additional control, without RT, can also be performed.
7. Prepare a 2X mix containing the reverse transcriptase. For 5 µl of mix, use 1 µl 5X RT buffer, 1 µl 0.1M DTT, 0.3 µl of a 25 mM dNTP mix (each nucleotide at 25 mM), 0.05 µl Actinomycin D (10 mg/ml), 2.3 µl water, 0.35 µl reverse transcriptase (*e.g.* Superscript III or IV at 200 U/µl). 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, add 5 μl of reverse transcriptase mix and mix by pipetting up and down.
10. Incubate for 30 min. at 42°C and inactivate the enzyme by an incubation 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 cDNA dilution with 20 μl of SYBR Green mix, which contains 12.5 μl of 2X commercial 2X dNTP, enzyme and SYBR Green, 1.25 μl of 10 μM solution for each of the two PCR primers and 5 μl H_2O .
13. Perform the qPCR reaction. Typical conditions are 95°C for 5 min. followed by 40 cycles of 20 sec. 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^{NOTE3}.
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 doxycyclin treatment. 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 column corresponding to a description of which replicate experiment it represents^{NOTE4}.
4. Use the *nls* function included in R to estimate the first order kinetic constant for an exponential decay model^{NOTE5}. This function uses a named vector for the initial estimates of the estimated parameter, that 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 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 value 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: $\text{half-life} = \ln(2)/k$ ^{NOTE6}.

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 (Arvidsson *et al*, 2008): <https://quantprime.mpimp-golm.mpg.de>. For spliced transcripts, oligonucleotides that cover 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 μ l of a 100 μ M solution for each reaction), the annealing reaction conditions should be adjusted, by incubating the annealing mix for 5 minutes at 65° C and switching to ice for 2 minutes 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 (Pfaffl, 2001), 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 normalization of 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 (Baudrimont *et al*, 2017). 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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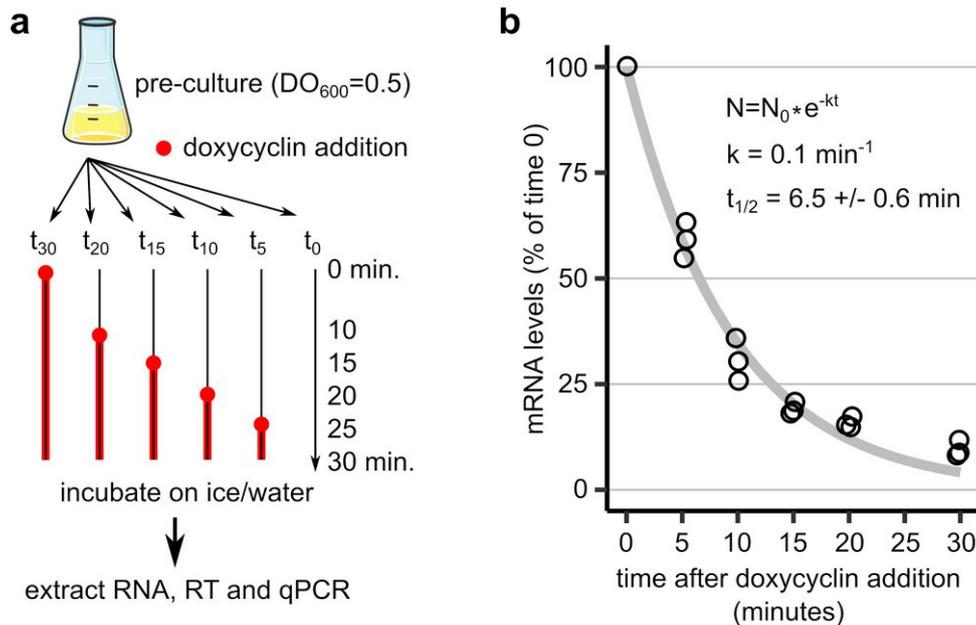


Figure 1. Typical workflow and result for the half-life estimation using RT-qPCR with a Tet-off reporter. **a)** Schematics of the culture conditions and a way to synchronize the recovered samples and ensure equal treatment for the different time points. All the time points are recovered together, but with different periods of doxycyclin treatment. **b)** Example of obtained results (Dehecq *et al*, 2018), 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. (The flask image was generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license).

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