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Metabolic Labeling and Recovery of Nascent RNA to Accurately Quantify mRNA Stability

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Abstract

Changes in the rate of mRNA decay are closely coordinated with transcriptional changes and together these events have profound effects on gene expression during development and disease. Traditional approaches to assess mRNA decay have relied on inhibition of transcription, which can alter mRNA decay rates and confound interpretation. More recently, metabolic labeling combined with chemical modification and fractionation of labeled RNAs has allowed the isolation of nascent transcripts and the subsequent calculation of mRNA decay rates. This approach has been widely adopted for measuring mRNA half-lives on a global scale, but has proven challenging to use for analysis of single genes. We present a series of normalization and quality assurance steps to be used in combination with 4-thiouridine pulse labeling of cultured eukaryotic cells. Importantly, we demonstrate how the relative amount of 4sU-labeled nascent RNA influences accurate quantification. The approach described facilitates reproducible measurement of individual mRNA half-lives using 4-thiouridine and could be adapted for use with other nucleoside analogs.

Keywords

mRNA stability; mRNA decay; 4-thiouridine labeling; digital PCR; RNA biotinylation

1. Introduction

1.1 Introduction to mRNA decay

After an mRNA is fully processed and exported to the cytoplasm, the life-span of the transcript impacts its contribution to gene expression. In fact, the stability of mRNA is thought to contribute 20–50% to overall gene regulation [1–3]. The life-span of mRNA can be adapted to accommodate environmental cues such as stress [4, 5], nutrient availability [6, 7], or in response to cellular changes during development [8–10], all of which may require rapid and precise adjustments in mRNA abundance. Control of mRNA stability provides a

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prompt and energy efficient response to stimuli that modulates the existing pool of mRNA rather than synthesizing new mRNA. Additionally, transcriptional changes occurring in response to environmental cues must be coordinated with changes in mRNA turnover to achieve appropriate mRNA abundances [1–3, 11]. mRNA decay is a highly regulated process influenced by both *cis*- and *trans*-acting factors [1, 12, 13]. The accurate and reproducible measurement of mRNA decay rates is critical to elucidating how this network of factors influences RNA stability.

1.2 Challenges and approaches to assessing mRNA decay rates

The primary challenge in this field is that the rate of decay can only be inferred by preventing synthesis of the transcript of interest and measuring the decrease in its abundance over time. There are two ways to accomplish this: inhibiting transcription, or labeling the newly transcribed RNA in such a way that it can be distinguished from the pre-existing RNA following extraction.

Global Transcription Inhibition—Classical approaches to measure RNA half-lives rely on arrest of RNA Polymerase II transcription by small molecules including actinomycin D, 5,6-Dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) and α -amanitin. Although this method has given invaluable insights into the mechanisms and factors involved in mRNA decay [1, 14–18], there are serious concerns regarding the validity of the results generated in this way. First, global inhibition of transcription is extremely toxic to any living cell and causes unwanted responses such as turning off global mRNA translation [19]. As a result, transcription inhibition cannot be used to assess more stable transcripts due to cell death. Moreover, the results obtained from this approach could reflect alternative decay pathways activated by the stress of transcription inhibition. Second, it is now clear that transcription and decay are coupled processes, such that inhibiting one is likely to directly influence the rate of the other [11, 20]. This means that the rates of decay determined following inhibition of transcription may not accurately reflect the rate when transcription is ongoing.

Regulated Promoters—An alternative to inhibiting global transcription is to use reporters driven by promoters that can be specifically inhibited without affecting RNA polymerase II. The most commonly used approach employs a tetracycline responsive promoter [21, 22] that is rapidly shut off following addition of doxycycline to the media. This method avoids issues with toxicity but has alternative drawbacks including that it cannot be used with endogenous transcripts and requires recombinant DNA manipulation and transfection. In addition, this method separates the transcript from its natural promoter which could also influence the decay rate, given the coupling between transcription and decay [11, 20].

Metabolic labeling—The first experiments using metabolic labeling to assess mRNA decay relied on incorporation of a pulse of radiolabeled nucleoside followed by chasing with unlabeled nucleotides and assessing abundance of the labeled mRNA of interest over time by hybridization to an unlabeled probe on a filter [23]. While this method did not interfere at all with the ongoing gene expression pathways, it was laborious, insensitive and required handling of large amounts of radioactivity.

The non-radioactive method described here is based on an approach first employed by Johnson *et al* in 1991 [24] which used 4-thiouridine to label newly synthesized mRNAs and then separated them from pre-existing transcripts by reversible conjugation to mercurated agarose. Two relatively recent improvements to this approach were replacement of toxic mercurated agarose with biotin/streptavidin [25–28] and the development of a more efficient means to conjugate the biotin to 4-thiouridine [29, 30]. As a result of these advances, in recent years 4sU-labeling has been applied to assess half-lives in a variety of living organisms and cell types, primarily on a global scale. The main advantage of this type of metabolic labeling is that it is less toxic than transcriptional inhibition and does not drastically interfere with ongoing transcription or other aspects of cell metabolism.

We note that other nucleoside analogs can be used for metabolic labeling and half-life determination including 5-bromouridine (5BrU) [33–36] and 5-ethynyluridine (5EU) [37]. The approach described here could be adapted to these nucleosides simply by replacing the positive control RNA with one labeled with 5EU or 5BrU and following established protocols for labeling and fractionation. However, we note that isolation of 5BrU labeled RNAs requires the use of antibodies to 5BrU which can be expensive and variable in quality, while 5EU-labeled RNAs are recovered through Click conjugation which is not reversible so cDNA must be synthesized while the RNA is attached to beads.

To date, 4sU metabolic labeling has mainly been applied to global RNA half-life analyses using RNA-seq [26, 35, 38] and has only been used for small scale experimentation sporadically despite its clear advantages over transcriptional inhibition [31, 32]. This is in part due to the difficulty of minimizing and controlling for sample-to-sample variation during the processing and fractionation. In addition, the favored approach for assessing RNA abundance, RT-qPCR, is often not sensitive enough to reliably assess small changes (10–20%) in relative abundance of nascent RNA that result from significant changes in half-life. Herein, we describe a method for half-life determination through 4sU-labeling where internal controls, and quantification by RT-dPCR facilitate accurate assessment of mRNA decay rates on a small scale. The same considerations and controls can also be applied to samples that will be analyzed through RNA-seq.

2. Materials and Methods

2.1. Overview

This method relies on the uptake of 4sU into cells and its incorporation into newly synthesized mRNAs. These labeled mRNAs, along with a spiked in artificially labeled positive control RNA, can be isolated by reversible conjugation to biotin and binding to streptavidin beads. After washing, the labeled RNA is recovered via application of the reducing agent, dithiothreitol (DTT). The RNA of interest is quantified by reverse transcription-digital PCR (RT-dPCR) and the relative amounts in the nascent and total RNA fractions are used to derive a half-life estimate. We detail the steps of this approach including experimental design, cell culture considerations, isolation and quality control of RNA, as well as biotinylation and fractionation of RNA for analysis by dPCR (Fig. 1). Furthermore, we discuss critical quality control standards, analysis and interpretation of resulting data, as well as limitations of the approach and means to overcome them. A

detailed protocol is provided along with an example from our studies examining PCBP2 as a regulator of decay of the GPR56/ADGRG1 mRNA. Finally, we propose a set of guidelines to ensure resulting 4sU-associated data are accurate and reproducible.

2.2. Experimental Design

2.2.1 Manipulations performed prior to half-life analysis—Typically, experiments in this field are aimed at establishing the effects of manipulating culture conditions or gene expression on rates of mRNA decay. Examples of conditions that are expected to influence mRNA stability include: altering levels of an RNA-binding protein (RBP) or ribonuclease (by depletion, knockout or over-expression) [11], differentiation [8, 10], and exposure to small molecules, cytokines, growth factors [39] or stress [5]. The appropriate conditions must be determined prior to embarking on the metabolic labeling described below. In the example provided for this protocol, we performed siRNA-mediated knockdown of the RNA-binding protein PCBP2 in induced pluripotent stem cells (iPSCs) in order to assess the effect on decay of the GPR56 mRNA which is known to be directly regulated by PCBP2 [18]. Optimal conditions for generating reliable knockdown of PCBP2 were empirically determined and verified by western blotting prior to performing the half-life analysis (Supplementary Material).

2.2.2 Assumptions—There are many methods to quantify mRNA abundance (qRT-PCR, northern blot, RNase protection etc.), but no approach to directly assess decay (or synthesis) of mRNAs in living cells exists. Metabolic labeling provides a way to separate the individual contributions of transcription and decay to overall mRNA abundance by allowing us to isolate newly transcribed (4sU-labeled) RNA from the (unlabeled) population which was transcribed prior to the addition of 4sU. Assuming that there is no change in the abundance, or rates of synthesis and decay over the course of the experiment, it is possible to derive the mRNA half-life from the proportion of total RNA that is 4sU labeled at time (t_L) after labeling began using the equation shown in Figure 1.

It is important to note that under conditions where cells are actively inducing or repressing expression of the mRNA of interest, for example in response to stress or other stimulus, this approach cannot accurately determine the half-life (Figure 2). Due to this limitation, it is vital to avoid changing the culture conditions during the experiment. Addition of fresh serum/media, temperature fluctuations and reaching confluence should all be avoided immediately before and during the labeling period. In some experiments changes in gene expression are unavoidable, such as when attempting to discern the effect of a specific stimulus on mRNA decay. In these cases, it may be informative to use a short labeling period (to minimize the changes in overall mRNA abundance) and/or to perform the analysis at multiple time points during the response.

In addition, the equation used to derive the half-life requires that the population of transcripts is homogenous and decays according to first order exponential kinetics. If the transcript of interest does not fit this assumption, it may be possible to get a more accurate picture of the decay kinetics by quantifying the pre-existing population at multiple time points during treatment [40].

2.2.3. 4sU Concentration and Labeling Time—4sU has been shown to influence cell viability when applied for long periods or in high concentrations [27, 41] and this in turn can alter mRNA decay rates. In addition, the efficiency of uptake and incorporation of 4sU into RNA can vary with cell type and culture conditions [25–27]. Poor uptake or incorporation of 4sU can result in overestimation of the mRNA half-life. Once a cell line and culture conditions have been selected, it is therefore wise to verify that 4sU does not drastically interfere with viability during the course of the experiment and that 4sU is incorporated efficiently (for example, by evaluating the half-life of an unstable or short RNA or by directly quantifying the amount of 4sU incorporated into RNA via mass spectrometry [25, 27]). Table 1 lists human cell lines and 4sU concentrations that have been used successfully in other studies. To date, we have used concentrations of 4sU ranging between 100–500 μ M in primary and transformed mammalian cell lines (HeLa, HEK293, human iPSCs, human foreskin fibroblasts and C2C12 mouse myoblasts) with little evidence of toxicity, supporting that this approach is widely applicable.

The labeling time should be adjusted depending on the expected half-life, such that 15%–90% of the mRNA of interest is labeled by the end of the incubation period. Experiments where labeling falls outside of this range should be repeated with a shorter (if labeling is >90%) or longer (if labeling is <15%) incubation with 4sU, as the half-life generated will not be reliable (Figure 3A). A good rule of thumb is to label for 50–100% of the expected half-life, but we generally favor shorter incubation periods to minimize toxicity and changes in culture conditions during the experiment. We recommend 30–60 min for a short-lived mRNA (<2hr half-life), 2–6 hr for a transcript with a 4–8 hr half-life and 6–8hr for very stable mRNAs (>12 hr). If the half-life of an RNA is unknown, a 4 hour labeling period is a good starting place as it works reasonably well for half-lives ranging from 1–17 hr (Figure 3B).

2.3 Protocol

2.3.1. 4sU labeling—The experiment should be performed using sufficient cells to generate 25–50 μ g of total RNA for each treatment. This is generally equivalent to a 6 or 10 cm dish for most adherent cell lines. At the start of the labeling period, the growth media from the cells is transferred to a sterile 15 ml conical tube, 4sU (Sigma #T4509) is added to a final concentration of 400 μ M (diluted from a 100 mM stock), and the treated media is put back onto the cells. The published half-life of the GPR56 transcript is 3–4 hours [18]; therefore, we labeled siRNA-transfected iPSCs with 4sU for 2 hours and 5 minutes. The additional 5 minutes is the estimated time for the cells to take up the 4sU and begin to incorporate it into newly transcribed RNA [26, 27].

2.3.2 RNA isolation and quality control—At the end of the labeling period, the media is removed and total RNA is immediately extracted using TRIzol[®] reagent (ThermoFisher Scientific #15596018) according to the manufacturer's recommendations. As an alternative to TRIzol[®], column-based RNA purification systems can be used, although total RNA yield can be reduced with this approach. Either method of RNA extraction is suitable for this protocol as long as 25–50 μ g total RNA is recovered. After RNA is isolated, DNase I treatment (ThermoFisher Scientific #DN0525) is performed for 30 min at 37°C to remove

genomic DNA and the RNA is recovered by phenol extraction and ethanol precipitation. At least 25 µg, and preferably 50 µg of high quality total RNA is required for effective recovery following fractionation. We recommend either simple gel electrophoresis to visually assess 18S and 28S rRNA integrity (no smearing of bands should be observed in high quality RNA) or the use of a Bioanalyzer/Tapestation and resulting RNA Integrity Number (RIN) [48] to verify adequate RNA quality in starting samples.

2.3.3. Biotinylation of total RNA and spike-in controls—The next step is to link biotin specifically to the thiol group of the labeled transcripts to allow their retention on streptavidin beads. Before biotinylation, a synthetic 4sU-labeled positive control RNA is spiked into each total RNA sample. This positive control transcript can be used to assess biotinylation efficiency and recovery from the streptavidin beads and normalize for sample-to-sample variability. If desired, unlabeled synthetic RNA or RNA from an unrelated organism can be used to assess non-specific binding to the streptavidin beads. In our experience, the background binding of non-biotinylated (i.e. unlabeled) RNA to µMacs streptavidin beads (Miltenyi) is in the 2–4% range. This was estimated by processing a sample of total RNA isolated from cells that had not been treated with 4sU and assessing the percentage of three different transcripts recovered in the eluate. Other brands of streptavidin beads may give different background and should be evaluated empirically.

Based on our estimates, if overall binding of the transcript of interest is less than ~15–20%, background binding can contribute to a significant error in half-life calculations. If desired, an unlabeled control can be used to assess and subtract background binding in each experiment, but given the increased uncertainty when nascent RNA is <15% of the total (Figure 3), it is still wisest to perform the experiment with a long enough labeling period that background is negligible.

2.3.3.1. 4sU Control RNA: For each 50 µg total RNA sample, 50 fmol of a 4sU-labeled synthetic RNA is included in the biotinylation reaction. Our experiments use a custom synthesized RNA (Dharmacon) with the following sequence:

5' -
AUUUAGGUGACACUAUAGGAUCCUCUAGAGUCGACCUUCUCCCUAUAGUGAGU
CGUAUUAGCA[4-S-U]CAG-3'

The two underlined regions are the binding sites for the dPCR primers. The sequence is derived from the polylinker of pGEM®4 (Promega) and contains sequences from SP6 and T7 promoters. The thiolated residue lies outside of the region amplified during RT-dPCR in order to minimize its effects on the efficiency of reverse transcription. Note that any individual synthetic RNA sequence or even a complex RNA sample from an unrelated organism [11] can serve the same purpose, as long as it can be distinguished from endogenous transcripts present in your sample and can be detected by RT-dPCR or RT-qPCR.

The 4sU control RNA is relatively expensive and the thiol modification is photoreactive, thus this reagent, and indeed any thiolated RNA, should be stored in small aliquots in the dark.

2.3.3.2 Biotinylation reaction: Biotinylation is accomplished by assembling a 150 μ l reaction containing 25–50 μ g of 4sU labeled total RNA, 50 fmol 4sU Ctrl RNA, 15 μ l 10 x biotinylation buffer (100 mM HEPES [pH 7.5], 10 mM EDTA) and 10 μ l MTSEA-biotin-XX (1mg/mL dissolved in dimethylformamide; Biotium #90066). The reaction is incubated for 30 minutes at room temperature in the dark with gentle agitation. We have found that 30 minutes incubation is sufficient for reproducible half-life estimates [30] and gives results comparable to the 2 hour incubation suggested elsewhere [35, 38]. After biotinylation, excess biotin must be removed. The reaction volume is raised to 200 μ l with RNase-free water and an equal volume of chloroform/isoamylalcohol (CHCl_3 :IAA (24:1)) is added. After vortexing briefly, the reaction is transferred to a pre-spun Phase-Lock Gel heavy tube (5 PRIME #2302810) and inverted several times to mix (vortexing is not advised as it disturbs the Phase-Lock Gel). Samples are centrifuged at 16,000g at 4°C for 5 minutes. The aqueous phase contains the RNA and is removed to a fresh microcentrifuge tube. Biotinylated RNA is recovered by precipitation with 0.1 volumes 5M NaCl and 2.5 volumes 100% ethanol, centrifuged at 16,000g at 4°C for 10 min, washed with 70% ethanol and resuspended in 105 μ l RNase-free water. No carrier (e.g. glycogen) or incubation is necessary at this step due to the large amount of RNA in the sample. RNA recovery and concentration should be evaluated by Nanodrop, and, if there are any concerns, can also be verified by Bioanalyzer/Tapestation or gel electrophoresis (see section 2.3.5).

2.3.4. Fractionation of total, nascent and pre-existing RNA—Prior to fractionation, all samples should be adjusted to the same concentration with RNase-free water. This ensures that an equal amount of RNA is being fractionated for each sample. A 50 μ l aliquot of each biotinylated RNA sample is reserved in a tube labeled “total RNA”. A second 50 μ l aliquot of RNA is mixed with 100 μ l of the streptavidin magnetic bead solution (μ Macs streptavidin kit; Miltenyi #130-074-101) and incubated at room temperature in the dark with gentle agitation for 15 minutes. After the incubation, an empty column (provided with the beads) is placed on the magnetic stand (Miltenyi # 130-042-602) and equilibrated with 100 μ l of nucleic acid equilibration buffer (provided with the beads) immediately before application of the bead/RNA mixture. Equilibrating the column too soon before binding biotinylated RNA can reduce RNA recovery. The RNA/bead solution is applied to the column and the flow-through is collected in a tube labeled “pre-existing”. Note that the RNAs in this fraction should not contain 4sU nor be biotinylated. The column is washed once with 100 μ l of column wash buffer (100mM Tris-HCl [pH 7.4], 10mM EDTA, 1M NaCl, 0.1% Tween-20) and the eluate is added to “pre-existing” tube to ensure quantitative recovery of ‘pre-existing’ RNA. An additional wash is performed with 500 μ l of column wash buffer and discarded. Elution is achieved by removing the column from the magnetic stand and applying 100 μ l of freshly diluted 100mM DTT (from 1M stock) to reduce the disulfide bond between the biotin and the thiolated uridine and release the RNA. The DTT solution flows through by gravity and the eluate is collected into a tube labeled “nascent”. After 5 minutes, a further 100 μ l of 100mM DTT is added and the eluate collected into the same tube during

another 5 minute period. In parallel, 150µl of 100mM DTT is added to the total RNA tube and incubated at room temperature for 5 minutes.

The RNA contained in each fraction is recovered by ethanol precipitation after adjusting the NaCl concentration to ~ 0.5M and adding 1 µl glycogen (20mg/ml, Thermofisher Scientific #R0561) as a carrier to ensure quantitative RNA precipitation (Table 2). The glycogen is particularly important for the eluate which has a much lower quantity of RNA. All three fractions are incubated at –80°C for at least 20 min prior to centrifugation at 4°C at 16,000g for 10 minutes. Pellets should be visible at this time with the total RNA being white/opaque while the pre-existing RNA and nascent RNA pellets are slightly brown in color due to the streptavidin beads. This is no cause for concern as it has no impact on RNA integrity or downstream cDNA synthesis. The RNA pellets are washed with 70% ethanol and air dried. Finally, each fraction is resuspended in a volume of RNase-free water equivalent to the number of µg of starting material. In our example, we added 50µl of water to each fraction because we started with 50µg total RNA from each sample.

2.3.5. Quality Control of Fractionated RNA—It is possible to verify recovery of RNA at this step by spectrophotometry, or more accurately by fluorometry. Integrity of the RNA can be assessed by Bioanalyzer/Tapestation (Agilent) or gel electrophoresis, although the nascent RNA fraction cannot always be visualized by gel electrophoresis due to its comparatively low concentration. In addition, the nascent RNA is refractory to accurate quantification by spectrophotometry because 4sU influences the absorbance spectrum [27]. The pre-existing fraction is not required for half-life calculations, but can be useful to retain for troubleshooting analysis (see section 3.4). Typical results of Tapestation and fluorometric analyses via the Qubit RNA Broad Range Assay Kit (ThermoFisher Scientific #Q10210) for two sets of fractionated samples are shown in Figure 4. Note that the concentration of the nascent RNA fraction is generally less than that for the pre-existing and total RNA fractions but it remains intact and gives an acceptable RIN. The abundance of rRNA in the eluate is generally lower than in the total RNA fraction due to the fact that rRNA has a relatively long half life.

2.3.5. Quantification of mRNA abundance using RT-dPCR—In order to quantify RNA abundance, the RNA must first undergo reverse transcription. We utilize 1 µl of each fraction in a standard reaction with a 3:1 mixture of random hexamers (Integrated DNA Technologies #51-01-18-01) and oligo(dT)₁₈ (Integrated DNA Technologies #51-01-15-07) as primers. Improm II reverse transcriptase (Promega #A3800) performs well in our hands, but other enzymes are acceptable.

Upon the completion of cDNA synthesis, the abundance of specific RNAs from each fraction can be determined using gene specific primers in either quantitative (real-time) PCR (qPCR) or digital PCR (dPCR). Here we use Bio-Rad digital droplet PCR combined with EvaGreen chemistry (Bio-Rad #1864034) to determine the RNA abundance in each fraction. The use of dPCR allows for the detection of relatively small changes in abundance (<10%) that can be hard to quantify by qPCR but can reflect significant changes in half-life. However, if large changes (much greater than 2-fold) in half-life are anticipated, qPCR can give reliable results.

Digital PCR is more sensitive than qPCR [49–51] but has a smaller linear range, requiring that there be between 1 and 100,000 copies of the template in each 20 µl reaction for accurate quantification. High abundance transcripts (such as GAPDH or 16S rRNA) will require significant dilution of the cDNA (1:250–1:1000 fold) prior to dPCR while very rare transcripts may not require dilution at all. PCR reactions are assembled as follows: 10 µl QX200™ ddPCR™ EvaGreen Supermix (Bio-Rad # 1864034), 1 µl Forward Primer (2.5mM), 1 µl Reverse Primer (2.5mM), 1–5 µl cDNA (diluted as necessary). RNase-free water is added to bring the volume to 20 µl. Specific primers are required to detect the mRNA of interest, and the positive control spike. In this study we used the following primers for dPCR: 4sU Ctrl F: 5'-ATTAGGTGACACTATAGGATCCTCTAG-3', 4sU Ctrl R: 5'-GCTAATACGACTCACTATAGGGAGAAG-3', GPR56 F: 5'-GCTTCACCTTCTCCTTCCACAGTC-3' GPR56 R: 5'-ACATGTCCACCGAGGCATTGTG-3'. Primers designed for real-time PCR are generally adequate for dPCR, but can also be designed using dPCR-optimized criteria [49–51]. A no template control and no reverse transcriptase control are included for each primer set, but technical replicates are not required for dPCR.

After the reactions are set up, 8 reactions are partitioned at time using the Bio-Rad Droplet Generator (Bio-Rad #1864002) as follows: Each 20µl sample is loaded into a sample well on the 8 well cassette (Bio-Rad #1864008). Next, 70µl of EvaGreen droplet generation oil (Bio-Rad #1864006) is added to each oil well. The cassette is then covered with a gasket (Bio-Rad #1863009) and inserted into the droplet generator which applies a vacuum to partition the reaction into droplets. The droplets are transferred to a 96-well plate (Eppendorf # 951020346), sealed with a foil seal (Bio-Rad cat# 1814040) and subjected to thermocycling (95°C for 5 min, 40 cycles of (95°C for 30 sec followed by 60°C for 1 min), 90°C for 5 min, hold at 4°C) in a BioRad C1000 Touch thermal cycler (Bio-Rad #1851197). The fluorescence in each droplet is read using the QX200™ Droplet Reader (Bio-Rad #1864003) to count positive and negative droplets. The QuantaSoft™ software (provided with QX200™ droplet reader) applies a Poisson distribution to the data to infer the number of copies of template cDNA per µl of sample, which accurately reflects the abundance of the mRNA of interest.

Controls

We note that a reference gene is not required for half-life analysis because the ratios of nascent to total RNA will be consistent within each sample set. However, a reference gene is essential to accurately quantify overall differences in the abundance of the mRNA of interest between the total RNA samples if so desired. In addition, it can be useful to assess the half-life of a control transcript that is not expected to vary between samples. Ideally, an endogenous control transcript should have a half-life in the same range as that of your gene of interest to ensure that it is recovered appropriately during the same labeling period (Figure 3). A reliable endogenous control can also substitute for the positive control synthetic RNA as a means to normalize recovery between different samples.

3. Data analysis

In this section we describe how to utilize the mRNA abundance measurements derived by dPCR to estimate mRNA half-life.

3.1 Adjustment of copy number values

In general, it is not necessary to adjust the copy number values to reflect the cDNA dilution factor unless different dilutions of cDNA are used for the nascent and total RNA fractions. If different dilutions were used, then simply multiply the copy number by the dilution factor for each fraction.

3.2. Normalization of nascent RNA recovery

Efficiency of biotinylation, binding to and elution from the streptavidin beads, and efficiency of RNA precipitation can all vary between samples. Immediately before the biotinylation of total RNA a short 4sU labeled control RNA was added to serve as an internal standard to assess and control for variability during sample processing. Table 3 shows the abundance of the 4sU control RNA in each fraction from samples treated with siRNAs targeting GFP (as a negative control) or targeting PCBP2. Samples 1 and 2 underwent processing at the same time and Sample 3 was processed separately. The values generated in the total and nascent fractions are utilized to normalize the recovery of nascent RNA. The Nascent/Total Ratio (R) for the positive control RNA is assumed to be constant between samples. Note that a maximum of ~75% of the 4sU control RNA, which is thiolated at just one uridine, is recovered in the eluate. This is lower than the theoretical maximum of 100%, but within expectations given that RNA structure and sequence influence biotinylation efficiency. Based on the behavior of the positive control RNA, and on analyses reported by others [29, 38] >99% of any transcript with 4 or more thiolated uridines should be successfully biotinylated and recovered in the eluate using this protocol. The vast majority of transcripts should easily meet this requirement but short RNAs, especially those with a low uridine content, may require higher concentrations of 4sU.

Next, a Normalization Factor (F) is determined for each sample by dividing the R of the positive control in each sample by the R for the sample that has the highest recovery of the control RNA in the eluate. In the example, Sample 3 had the highest recovery so Samples 1 and 2 were normalized to Sample 3.

If the recovery of the positive control in the nascent fraction is drastically lower than expected (less than half of the maximum value) this can be an indication of problems with RNA degradation or failure at one step in the procedure. In general, these samples should be discarded.

3.3. Transcript half-life analysis

In order to calculate the half-life of a particular RNA we assume first order kinetics. Making this assumption, the half-life of an RNA can be calculated using the following equation [26] (also shown in Figure 1):

$$t_{1/2} = -t_L * \ln(2) / \ln(1-R)$$

t_L = labeling time (minus 5 minutes for 4sU incorporation to begin)

R = abundance in nascent RNA fraction/abundance in total RNA fraction, normalized to R calculated for the positive control RNA.

The results of this calculation for three samples are shown in the last column of Table 2.

3.4. Trouble-shooting

The spiked in control 4sU RNA is vital to minimize the influence of day-to-day and sample-to-sample variation. A synthetic control is favored over an endogenous control RNA because it fractionates reproducibly and efficiently regardless of the labeling period and cell treatment. As well as allowing for normalization, the control RNA provides a valuable means to easily identify and diagnose problems in the biotinylation and fractionation procedure, especially when used in conjunction with approaches that monitor overall RNA quality and quantity. For instance, if the 4sU control RNA is not recovered in the nascent fraction, possible explanations include loss or degradation of the RNA, failure of the biotinylation step, or failure to elute the biotinylated RNA from the column. RNA loss or degradation can be identified by assessing RNA quantity/quality via Tapestation or Bioanalyzer (Figure 4). Assessing the control RNA in the pre-existing fraction may also be helpful to determine if the biotinylation reaction failed. If this was the case, all the RNA, including the spiked-in control, will be found in that fraction. If there is evidence for failure of the biotinylation step, then the 4sU-labeled spike cannot be relied upon for normalization because it is more sensitive than the endogenous transcripts to failure at this step (due to the fact that it contains only one thiolated uridine). On the other hand, if the control RNA behaves as expected but the mRNA of interest is not recovered in the nascent fraction, then it is likely that 4sU incorporation into nascent RNA failed at the cell culture step, or the starting RNA was significantly degraded prior to addition of the spike.

4. Results

Table 3 provides the values and calculations for the GPR56 transcript in three samples. These data were combined with data from additional replicates to generate the chart shown in Figure 5. This experiment demonstrates that in iPSC cells the GPR56 mRNA has a half-life of ~1.7 hr and that PCBP2 depletion results in a two-fold increase in the half-life to ~3.1 hr. This is consistent with previous data generated using actinomycin D shut off in cardiomyocytes, which also showed stabilization of GPR56 following PCBP2 knockdown [18]. Details of how PCBP2 knockdown was accomplished and verified are provided in Supplementary Data.

5. Conclusions and proposed guidelines

5.1. Conclusions

The use of metabolic labeling of nascent RNA to determine endogenous RNA half-lives can replace standard methods that rely on transcription inhibition followed by repeated analysis over a time course. Metabolic labeling is less toxic, it requires just a single time point and it avoids the need to inhibit transcription. This last benefit is significant as recent studies have proposed a feedback mechanism between mRNA decay and transcription to maintain a steady-state level of RNA [11, 20]. In order to generate reliable and reproducible half-lives it is important to (i) use an appropriate 4sU labeling period (Figure 3), (ii) normalize for variability in fractionation using a spiked in positive control RNA, and ideally (iii) quantify the RNA using dPCR as it is inherently more reliable for small differences.

5.3. Proposed guidelines

1. Evaluate toxicity of 4sU in the cell line of interest.
2. Minimize variation in cell culture conditions during the experiment. This approach requires that the system be at steady state with minimal change in mRNA abundance, transcription and decay. (Figure 2)
3. Determine the 4sU labeling time required to recover 15–90% of the mRNA of interest in the nascent fraction. Results are reliable only when the nascent RNA recovery falls in the center of this range (Figure 3).
4. Evaluate fractionation efficiency by comparing the abundance of the 4sU control RNA in the total and nascent fractions. For the synthetic RNA used here, 37.5–75% of the RNA should be recovered in the nascent fraction. If an RNA with different sequence is used, an acceptable range should be determined empirically.
5. Normalize the nascent RNA recovery to that of the positive control RNA and determine half-lives.
6. If desired, assess half-life of an endogenous control transcript that is not affected by your treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

4sU 4-thiouridine

DRB	5,6-Dichloro-1- β -D-ribofuranosylbenzimidazole
iPSCs	induced pluripotent stem cells

References

1. Cheadle C, Fan J, Cho-Chung YS, Werner T, Ray J, Do L, Gorospe M, Becker KG. Control of gene expression during T cell activation: alternate regulation of mRNA transcription and mRNA stability. *BMC Genomics*. 2005; 6:75. [PubMed: 15907206]
2. Garcia-Martinez J, Aranda A, Perez-Ortin JE. Genomic run-on evaluates transcription rates for all yeast genes and identifies gene regulatory mechanisms. *Molecular Cell*. 2004; 15(2):303–313. [PubMed: 15260981]
3. Huang L, Lou CH, Chan W, Shum EY, Shao A, Stone E, Karam R, Song HW, Wilkinson MF. RNA homeostasis governed by cell type-specific and branched feedback loops acting on NMD. *Molecular Cell*. 2011; 43(6):950–961. [PubMed: 21925383]
4. Miller MA, Russo J, Fischer AD, Lopez Leban FA, Olivas WM. Carbon source-dependent alteration of Puf3p activity mediates rapid changes in the stabilities of mRNAs involved in mitochondrial function. *Nucleic Acids Research*. 2014; 42(6):3954–3970. [PubMed: 24371272]
5. Russo J, Olivas WM. Conditional regulation of Puf1p, Puf4p, and Puf5p activity alters YHB1 mRNA stability for a rapid response to toxic nitric oxide stress in yeast. *Molecular Biology of the Cell*. 2015; 26(6):1015–1029. [PubMed: 25631823]
6. Brauer MJ, Huttenhower C, Airoidi EM, Rosenstein R, Matese JC, Gresham D, Boer VM, Troyanskaya OG, Botstein D. Coordination of growth rate, cell cycle, stress response, and metabolic activity in yeast. *Molecular Biology Of The Cell*. 2008; 19(1):352–367. [PubMed: 17959824]
7. Slavov N, Botstein D. Decoupling nutrient signaling from growth rate causes aerobic glycolysis and deregulation of cell size and gene expression. *Molecular Biology of the Cell*. 2013; 24(2):157–168. [PubMed: 23135997]
8. Asaoka-Taguchi M, Yamada M, Nakamura A, Hanyu K, Kobayashi S. Maternal Pumilio acts together with Nanos in germline development in *Drosophila* embryos. *Nature Cell Biology*. 1999; 1(7):431–437. [PubMed: 10559987]
9. Wreden C, Verrotti AC, Schisa JA, Lieberfarb ME, Strickland S. Nanos and pumilio establish embryonic polarity in *Drosophila* by promoting posterior deadenylation of hunchback mRNA. *Development*. 1997; 124(15):3015–3023. [PubMed: 9247343]
10. Neff AT, Lee JY, Wilusz J, Tian B, Wilusz CJ. Global analysis reveals multiple pathways for unique regulation of mRNA decay in induced pluripotent stem cells. *Genome Research*. 2012; 22(8):1457–1467. [PubMed: 22534399]
11. Sun M, Schwalb B, Pirkel N, Maier KC, Schenk A, Failmezger H, Tresch A, Cramer P. Global analysis of eukaryotic mRNA degradation reveals Xrn1-dependent buffering of transcript levels. *Molecular Cell*. 2013; 52(1):52–62. [PubMed: 24119399]
12. Miles WO, Tschoep K, Herr A, Ji JY, Dyson NJ. Pumilio facilitates miRNA regulation of the E2F3 oncogene. *Genes & Development*. 2012; 26(4):356–368. [PubMed: 22345517]
13. Nahas GR, Murthy RG, Patel SA, Ganta T, Greco SJ, Rameshwar P. The RNA-binding protein Musashi 1 stabilizes the onco tachykinin 1 mRNA in breast cancer cells to promote cell growth. *FASEB Journal*. 2015
14. Lee JE, Lee JY, Trembly J, Wilusz J, Tian B, Wilusz CJ. The PARN deadenylase targets a discrete set of mRNAs for decay and regulates cell motility in mouse myoblasts. *PLoS Genetics*. 2012; 8(8):e1002901. [PubMed: 22956911]
15. Masuda A, Andersen HS, Doktor TK, Okamoto T, Ito M, Andresen BS, Ohno K. CUGBP1 and MBNL1 preferentially bind to 3' UTRs and facilitate mRNA decay. *Scientific Reports*. 2012; 2:209. [PubMed: 22355723]
16. Perez-Ortin JE, Alepuz P, Chavez S, Choder M. Eukaryotic mRNA decay: methodologies, pathways, and links to other stages of gene expression. *Journal of Molecular Biology*. 2013; 425(20):3750–3775. [PubMed: 23467123]

17. Molin C, Jauhiainen A, Warringer J, Nerman O, Sunnerhagen P. mRNA stability changes precede changes in steady-state mRNA amounts during hyperosmotic stress. *RNA*. 2009; 15(4):600–614. [PubMed: 19223440]
18. Zhang Y, Si Y, Ma N, Mei J. The RNA-binding protein PCBP2 inhibits Ang II-induced hypertrophy of cardiomyocytes through promoting GPR56 mRNA degradation. *Biochemical & Biophysical Research Communications*. 2015; 464(3):679–684. [PubMed: 26116532]
19. Friedel CC, Dolken L. Metabolic tagging and purification of nascent RNA: implications for transcriptomics. *Molecular BioSystems*. 2009; 5(11):1271–1278. [PubMed: 19823741]
20. Haimovich G, Medina DA, Causse SZ, Garber M, Millan-Zambrano G, Barkai O, Chavez S, Perez-Ortin JE, Darzacq X, Choder M. Gene expression is circular: factors for mRNA degradation also foster mRNA synthesis. *Cell*. 2013; 153(5):1000–1011. [PubMed: 23706738]
21. Shyu AB, Belasco JG, Greenberg ME. Two distinct destabilizing elements in the c-fos message trigger deadenylation as a first step in rapid mRNA decay. *Genes & Development*. 1991; 5(2):221–231. [PubMed: 1899842]
22. Shyu AB, Greenberg ME, Belasco JG. The c-fos transcript is targeted for rapid decay by two distinct mRNA degradation pathways. *Genes & Development*. 1989; 3(1):60–72. [PubMed: 2496006]
23. Ross J. mRNA stability in mammalian cells. *Microbiological Reviews*. 1995; 59(3):423–450. [PubMed: 7565413]
24. Johnson TR, Rudin SD, Blossey BK, Ilan J, Ilan J. Newly synthesized RNA: simultaneous measurement in intact cells of transcription rates and RNA stability of insulin-like growth factor I, actin, and albumin in growth hormone-stimulated hepatocytes. *Proceedings of the National Academy of Sciences of the United States of America*. 1991; 88(12):5287–5291. [PubMed: 1711226]
25. Dolken L. High resolution gene expression profiling of RNA synthesis, processing, and decay by metabolic labeling of newly transcribed RNA using 4-thiouridine. *Methods in molecular biology*. 2013; 1064:91–100. [PubMed: 23996251]
26. Radle B, Rutkowski AJ, Ruzsics Z, Friedel CC, Koszinowski UH, Dolken L. Metabolic labeling of newly transcribed RNA for high resolution gene expression profiling of RNA synthesis, processing and decay in cell culture. *Journal of Visualized Experiments : JoVE*. 2013; (78)
27. Dolken L, Ruzsics Z, Radle B, Friedel CC, Zimmer R, Mages J, Hoffmann R, Dickinson P, Forster T, Ghazal P, et al. High-resolution gene expression profiling for simultaneous kinetic parameter analysis of RNA synthesis and decay. *RNA*. 2008; 14(9):1959–1972. [PubMed: 18658122]
28. Duan J, Shi J, Ge X, Dolken L, Moy W, He D, Shi S, Sanders AR, Ross J, Gejman PV. Genome-wide survey of interindividual differences of RNA stability in human lymphoblastoid cell lines. *Scientific Reports*. 2013; 3:1318. [PubMed: 23422947]
29. Duffy EE, Rutenberg-Schoenberg M, Stark CD, Kitchen RR, Gerstein MB, Simon MD. Tracking Distinct RNA Populations Using Efficient and Reversible Covalent Chemistry. *Molecular cell*. 2015; 59(5):858–866. [PubMed: 26340425]
30. Duffy EE, Simon MD. Enriching s4 U-RNA Using Methane Thiosulfonate (MTS) Chemistry. *Current Protocols In Chemical Biology*. 2016; 8(4):234–250. [PubMed: 27925666]
31. Borowski LS, Szczesny RJ. Measurement of mitochondrial RNA stability by metabolic labeling of transcripts with 4-thiouridine. *Methods In Molecular Biology*. 2014; 1125:277–286. [PubMed: 24590796]
32. Burow DA, Umeh-Garcia MC, True MB, Bakhaj CD, Ardell DH, Cleary MD. Dynamic regulation of mRNA decay during neural development. *Neural Development*. 2015; 10:11. [PubMed: 25896902]
33. Maekawa S, Imamachi N, Irie T, Tani H, Matsumoto K, Mizutani R, Imamura K, Kakeda M, Yada T, Sugano S, et al. Analysis of RNA decay factor mediated RNA stability contributions on RNA abundance. *BMC Genomics*. 2015; 16:154. [PubMed: 25879614]
34. Paulsen MT, Veloso A, Prasad J, Bedi K, Ljungman EA, Magnuson B, Wilson TE, Ljungman M. Use of Bru-Seq and BruChase-Seq for genome-wide assessment of the synthesis and stability of RNA. *Methods*. 2014; 67(1):45–54. [PubMed: 23973811]

35. Tani H, Akimitsu N. Genome-wide technology for determining RNA stability in mammalian cells: historical perspective and recent advantages based on modified nucleotide labeling. *RNA Biology*. 2012; 9(10):1233–1238. [PubMed: 23034600]
36. Tani H, Mizutani R, Salam KA, Tano K, Ijiri K, Wakamatsu A, Isogai T, Suzuki Y, Akimitsu N. Genome-wide determination of RNA stability reveals hundreds of short-lived noncoding transcripts in mammals. *Genome Research*. 2012; 22(5):947–956. [PubMed: 22369889]
37. Abe K, Ishigami T, Shyu AB, Ohno S, Umemura S, Yamashita A. Analysis of interferon-beta mRNA stability control after poly(I:C) stimulation using RNA metabolic labeling by ethynyluridine. *Biochemical & Biophysical Research Communications*. 2012; 428(1):44–49. [PubMed: 23063848]
38. Schwarzl T, Higgins DG, Kolch W, Duffy DJ. Measuring Transcription Rate Changes via Time-Course 4-Thiouridine Pulse-Labeling Improves Transcriptional Target Identification. *Journal of Molecular Biology*. 2015; 427(21):3368–3374. [PubMed: 26362006]
39. Thiele BJ, Doller A, Kahne T, Pregla R, Hetzer R, Regitz-Zagrosek V. RNA-binding proteins heterogeneous nuclear ribonucleoprotein A1, E1, and K are involved in post-transcriptional control of collagen I and III synthesis. *Circulation Research*. 2004; 95(11):1058–1066. [PubMed: 15514164]
40. Azarkh Y, Dolken L, Nagel M, Gilden D, Cohrs RJ. Synthesis and decay of varicella zoster virus transcripts. *Journal of Neurovirology*. 2011; 17(3):281–287. [PubMed: 21484478]
41. Burger K, Muhl B, Kellner M, Rohrmoser M, Gruber-Eber A, Windhager L, Friedel CC, Dolken L, Eick D. 4-thiouridine inhibits rRNA synthesis and causes a nucleolar stress response. *RNA Biology*. 2013; 10(10):1623–1630. [PubMed: 24025460]
42. Windhager L, Bonfert T, Burger K, Ruzsics Z, Krebs S, Kaufmann S, Malterer G, L'Hernault A, Schilhabel M, Schreiber S, et al. Ultrashort and progressive 4sU-tagging reveals key characteristics of RNA processing at nucleotide resolution. *Genome Research*. 2012; 22(10):2031–2042. [PubMed: 22539649]
43. Stubbs SH, Conrad NK. Depletion of REF/Aly alters gene expression and reduces RNA polymerase II occupancy. *Nucleic Acids Research*. 2015; 43(1):504–519. [PubMed: 25477387]
44. Blackinton JG, Keene JD. Functional coordination and HuR-mediated regulation of mRNA stability during T cell activation. *Nucleic Acids Research*. 2016; 44(1):426–436. [PubMed: 26490963]
45. Payne TL, Blackinton J, Frisbee A, Pickeral J, Sawant S, Vandergrift NA, Freil SA, Ferrari G, Keene JD, Tomaras GD. Transcriptional and posttranscriptional regulation of cytokine gene expression in HIV-1 antigen-specific CD8+ T cells that mediate virus inhibition. *Journal of Virology*. 2014; 88(17):9514–9528. [PubMed: 24899193]
46. Donato E, Croci O, Sabo A, Muller H, Morelli MJ, Pelizzola M, Campaner S. Compensatory RNA polymerase 2 loading determines the efficacy and transcriptional selectivity of JQ1 in Myc-driven tumors. *Leukemia*. 2016
47. Bresson SM, Hunter OV, Hunter AC, Conrad NK. Canonical Poly(A) Polymerase Activity Promotes the Decay of a Wide Variety of Mammalian Nuclear RNAs. *PLoS Genetics*. 2015; 11(10):e1005610. [PubMed: 26484760]
48. Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, Lightfoot S, Menzel W, Granzow M, Ragg T. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Molecular Biology*. 2006; 7:3. [PubMed: 16448564]
49. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, Bright IJ, Lucero MY, Hiddessen AL, Legler TC, et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Analytical Chemistry*. 2011; 83(22):8604–8610. [PubMed: 22035192]
50. Hindson CM, Chevillet JR, Briggs HA, Gallichotte EN, Ruf IK, Hindson BJ, Vessella RL, Tewari M. Absolute quantification by droplet digital PCR versus analog real-time PCR. *Nature Methods*. 2013; 10(10):1003–1005. [PubMed: 23995387]
51. Pinheiro LB, Coleman VA, Hindson CM, Herrmann J, Hindson BJ, Bhat S, Emslie KR. Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. *Analytical Chemistry*. 2012; 84(2):1003–1011. [PubMed: 22122760]

Highlights

- Accurate assessment of RNA stability is key to characterizing gene expression
- 4-thiouridine labeling has major advantages over methods that inhibit transcription
- Quality control and normalization are essential to generate reproducible half-lives
- The relative amount of 4sU-labeled nascent RNA influences accurate quantification

Figure 1. Typical work-flow for the assessment of individual mRNA half-lives using 4sU metabolic labeling

Sub-confluent cell cultures are incubated with 4sU for a pre-determined time. Following incubation, total RNA is extracted and DNase treated. A positive control (4sU labeled) RNA is spiked into the total RNA sample for downstream normalization and overall quality control. The RNA is conjugated to biotin followed by clean up and precipitation. Fractionation of total RNA to separate nascent and pre-existing transcripts is performed using magnetic streptavidin beads and nascent RNA is recovered by elution with DTT. The nascent and total RNA fractions are analyzed by reverse transcription and digital PCR.

Abundance of the mRNA of interest and the positive control mRNA are determined in the total and nascent fraction and used in the equation shown to calculate half-life.

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Figure 2. The experimental system must be at steady state in order to accurately estimate mRNA half-life

A key assumption of this approach is that the experimental system is at steady state. In other words, the mRNA abundance, transcription rate, and rate of decay do not vary during the labeling period. The bar charts show the amounts of pre-existing and newly synthesized mRNA during a 2 hour labeling period for *hypothetical* transcripts with different expression profiles. The times above each bar indicate the half-life that would be calculated based on the ratio of nascent to total RNA. **Panel A** shows a theoretical transcript with a 60 minute half-life, while **B** depicts a transcript with a much longer half-life of around 13 hr. The total amount of RNA does not change during the course of the experiment in either case and the same half-life would be calculated whether the RNA was harvested at 60 min or 120 min. However, the half-life calculation would be significantly more reliable for A than B because in B the nascent:total ratio is 0.9 and therefore outside of the acceptable range (see Figure 3). **Panels C and D** depict the same short-lived theoretical transcript as in A but the half-life (C) or transcription rate (D) change between 60 and 120 min. This leads to changes in the overall abundance of the transcript and a different assessment of the mRNA half-life at 120 versus 60 min.

Figure 3. 4sU labeling periods should be adjusted depending on half-life of the RNA of interest
Panel A depicts the dependence of the calculated half-life on nascent/total ratios determined for a 2 hour labeling period. When the N/T ratio falls within the red shaded areas (below 0.15 or above 0.9), background binding, pipetting inaccuracies, partial loss or decay of RNA samples, and other errors have a disproportionate impact on the half-life calculation. When the ratio falls in the center of the curve (green shaded areas) normal experimental variation will have less impact on the half-life. The chart shown in **Panel B** depicts the range of half-lives that can be reliably determined with labeling periods ranging from 0.5 to 8 hours. It is recommended that the 4sU labeling period be adjusted so that the expected half-life of the mRNA of interest falls close to the center of the range shown.

Figure 4. Tapestation assessment of the quality of the RNA fractions used in half-life calculations Equal volumes of total (T), nascent (N) and pre-existing (P) RNA from iPSCs treated with siRNAs targeting GFP or PCBP2 were interrogated by Tapestation. RNA integrity number equivalents (RIN_e) are shown. A RIN value ≥ 8 is acceptable for downstream abundance measurements [39]. Concentration of each fraction was determined using a Qubit with the Qubit RNA Broad Range Assay Kit.

Figure 5. Knockdown of PCBP2 results in the stabilization of the GPR56 transcript

iPSCs were transfected with siRNAs targeting GFP or PCBP2 as described in Supplementary Data. 4-thiouridine metabolic labeling was used to determine the half-life of GPR56 mRNA. Error bars are SEM derived from 4 independent replicates.

Table 1

Examples of studies using 4sU labeling to assess mRNA stability in human cell lines

Cell Line	4sU Concentration & Labeling Time	Citation
BL41 (lymphoma)	500 μ M for 30 min 100 μ M for 1 hr	Dölken 2008 [27]
	100 μ M for 1 hr	Friedel 2009 [19]
DG75 (B lymphocyte)	500 μ M for 30 min 100 μ M for 1 hr	Dölken 2008 [27]
	500 μ M for 5–60 min	Windhager 2012 [42]
HeLa (cervical carcinoma)	500 μ M for 30 min 100 μ M for 1 hr	Dölken 2008 [27]
	250 μ M for 1 hr	Borowski 2014 [31]
HEK293T (embryonic kidney)	250 μ M for 1 hr	Borowski 2014 [31]
293A (embryonic kidney)	2.5 μ M for 2 hr	Stubbs 2014 [43]
iPS (stem cell)	400 μ M for 2 hr	This study
Jurkat (T lymphocyte)	500 μ M for 30 min 100 μ M for 1 hr	Dölken 2008 [27]
	200 μ M for 1 hr	Blackinton 2015 [44]
LCL (lymphoblast)	200 μ M for 2 hr	Duan 2013 [28]
MEWO (melanoma)	500 μ M for 1 hr	Azarkh 2011 [40]
Primary T-cells	200 μ M for 1–6 hr	Payne 2014 [45]
RAJI (lymphoblast)	300 μ M for 30 min	Donato 2016 [46]
RCC (renal carcinoma)	2 μ M for 2 hr	Bresson 2015 [47]
SH-SY5Y (neuroblastoma)	500 μ M for 0–4 hr	Schwarzl 2015 [38]
143B (osteosarcoma)	250 μ M for 1 hr	Borowski 2014 [31]

Table 2

Volumes of reagent added to fractions for precipitation

	Total	Pre-Existing (Flow-thru)	Nascent (Eluate)
Initial Volume	50	200	200
100mM DTT	150	-	-
20 mg/ml glycogen	1	1	1
5M NaCl	25	5	20
100% Ethanol	500	500	500

Table 3

Example Calculations for GPR56 Half-life in Three Samples

Biological treatment	Fraction	Ctrl Copy Number	GPR56 Copy Number	Ctrl RNA $R_{ctrl} (= N_{ctrl}/T_{ctrl})$	Normalization Factor F ($= R_{high}/R_{sample}$)	GPR56 $R_{GPR} (= N_{GPR}/T_{GPR})$	Normalized GPR56 R_{final} ($= R_{GPR} \cdot F$)	Normalized GPR56 Half-life (hr)
(1) siGFP-A	Total (T)	363	106	0.64	1.156	0.377	0.436	2.42
	Nascent (N)	233	40					
(2) siPCBP2	Total (T)	324	146	0.67	1.104	0.288	0.317	3.63
	Nascent (N)	218	42					
(3) siGFP-B	Total (T)	113	64	0.74	1.000	0.594	0.594	1.54
	Nascent (N)	85.9	38					

Copy number = # of copies of the cDNA template per ul of reaction volume as estimated by dPCR

Ctrl RNA = 4sU-labeled spike RNA. GPR56 = GPR56 mRNA

R= ratio of nascent (N) copy number to total (T) copy number

R_{high} = N/T for the 4sU-labeled spike in the sample that showed best recovery (in this case, the siGFP-B sample)

Half-life is determined using the R_{final} in the equation shown in Figure 1.