Published in final edited form as:

Curr Protoc Mol Biol. 2019 January ; 125(1): e71. doi:10.1002/cpmb.71.

Targeted Profiling of RNA Translation

Ben B. Li, Changli Qian, Thomas M. Roberts, and Jean J. Zhao

Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts

Abstract

This unit describes a reverse transcription-quantitative PCR (RT-qPCR)-based method for gene-targeted measurement of RNA translation levels. In brief, the method comprises washing and lysis of cells with a buffer containing cycloheximide to enrich ribosomal accumulation at translation initiation sites (TIS), followed by enzymatic treatment to generate ribosomal footprints, reverse transcription targeted towards TIS of specific transcripts of interest to generate complementary DNA (cDNA), and qPCR to measure the abundance of these footprints. In this manner, this method enables time- and cost-effective assessment of changes in translation level across focused panels of genes and across numerous samples.

Keywords

RNA translation; protein synthesis; RT-qPCR

INTRODUCTION

The transformation of genetic information is mediated by two distinct and essential cellular processes - transcription and translation. RNA translation, as the second of these two steps, is carried out by the ribosome complex, together with numerous translation factors that enable and regulate its catalytic activity. This process is controlled by various key intracellular signaling pathways, including mTOR signaling (Morita *et al.*, 2015; Saxton *et al.*, 2017) and the integrated stress response (Pakos-Zebrucka *et al.*, 2016), and is known to be dysregulated in various diseases, including many cancers. In this unit, we describe a method that enables rapid and cost-effective measurement of relative changes in translation across focused groups of genes. In broad terms, the assay comprises enrichment of footprints at translation initiation sites (TIS), followed by their isolation, reverse transcription, and relative quantitation by qPCR, and incorporates optimization at various steps to increase detection specificity and data quality. Our method permits interrogation of RNA translation changes across numerous samples with relative ease, potentially enabling greater amounts of in-depth analyses into the translation process and its regulation.

Correspondence to ben_li@dfci.harvard.edu (B.B.L.).

Competing Interests

B.B.L. and J.J.Z. are co-inventors on patent application PCT/US2017/039001. All other authors declare no competing interests.

BASIC PROTOCOL

NOTE: The protocol, as described, is for adherent cells cultured to near-confluence in one well of 6-well plates and can be scaled as appropriate. The actual cell density at harvest will vary by cell line but will likely be $0.5 - 1 \times 10^6$ cells per well for 6-well plates.

NOTE: It is important that molecular biology grade reagents (RNase-, DNase-, and nucleic acid-free) are used, in particular after extraction of ribosomal footprints, to prevent degradation and/or contamination of desired template RNA and cDNA. Standard molecular biology techniques should likewise be applied to prevent introduction of contaminating nucleases and nucleic acid material into the workflow.

NOTE: It is preferable to make master mixes of reagents and solutions to ensure uniformity between samples. Remember to make slight more than volume required (e.g., 10% excess) to account for dispensing limitations and errors.

Materials

Buffer WB (see recipe)

Buffer LB (see recipe)

Solution Q3 (see recipe)

Solution QT (see recipe)

Primers (see recipe)

Nuclease-free water (Ambion)

100% ethanol (Sigma Aldrich)

100% isopropanol (Sigma Aldrich)

100% chloroform (Sigma Aldrich)

1X PBS (Life Technologies)

1 M Tris-HCl solution, pH 7.4 (Ambion)

5 M NaCl solution (Ambion)

1 M MgCl₂ solution (Ambion)

3 M NaOAc (Ambion)

1 M DTT solution (Sigma Aldrich)

100 mM DTT solution (Invitrogen)

1 N NaOH solution (Sigma Aldrich)

100% Triton X-100 (Sigma Aldrich)

100 mg/ml cycloheximide (Sigma Aldrich)

TRIzol reagent (Ambion)

15 mg/ml GlycoBlue (Ambion)

Qubit RNA HS assay kit and assay tubes (Invitrogen)

100 U/ul RNase I (Ambion)

2 U/ul TurboDNase (Ambion)

20 U/ul SUPERase-In (Ambion)

200 U/ul Superscript III (Invitrogen)

5X Superscript III First-Strand buffer (Invitrogen)

40 mM dNTP mixture (10 mM of each dNTP) (New England Biolabs)

2X SYBR-Select Master Mix (Applied Biosystems)

1.5 ml low-retention microcentrifuge tubes

Disposable cell scrapers

Blue ice

Dry ice

Liquid nitrogen

Standard thermocycler

Standard qPCR analyzer

Refrigerated microcentrifuge

Qubit fluorometer (Invitrogen)

Rotary mixer

Solution E (see recipe)

Solution Q1 (see recipe)

Solution Q2 (see recipe)

NOTE: We validated this protocol using human mammary epithelial cell lines, such as MDA-MB-468.

NOTE: In our experiments, we use a Bio-Rad C1000 Thermo Cycler and an Applied Biosystems 7300 Real-Time PCR System. Many alternatives systems are available and can be used.

Harvest and prepare cell lysate

1. Aspirate media, add 4 ml of ice-cold buffer WB, keep on cells for 60 s on ice, and aspirate buffer WB.
2. Add 200 μ l of ice-cold buffer LB, disperse evenly by tilting the plate, and transfer plate to liquid nitrogen bath to snap freeze.

The addition of cycloheximide in buffers WB and LB enhances ribosomal accumulation at the translation initiation site, resulting in increased qPCR signal.

The plate must be cooled to around 0°C prior to transfer to liquid nitrogen to avoid the plastic shattering upon contact.

3. Thaw plate on ice.

Lysis buffer on the cell culture surface requires approximately 20 min to 30 min to completely thaw.

4. Scrape cells into thawed buffer, transfer lysate to pre-cooled 1.5 ml microcentrifuge tube, and snap freeze in liquid nitrogen.

Repeatedly freeze-thaw cycles promote efficient cell lysis. Alternatively, other means of physical lysis can be utilized, such as trituration through a narrow needle.

For storage, transfer to vapor phase of liquid nitrogen.

5. Thaw samples on ice.

Lysate requires approximately 1 h to 2 h to completely thaw.

6. Centrifuge at 20,000g for 5 min at 4°C, retrieve supernatant, and transfer to pre-cooled 1.5 ml microcentrifuge tube.

This step removes cell debris, leaving a clear supernatant that contains RNA, some genomic DNA and intracellular proteins.

Low-retention microcentrifuge tubes from this point onwards are preferable to minimize loss of nucleic acid material.

For storage, flash freeze in LN2, store in vapor phase of liquid nitrogen.

7. Quantitate RNA concentration using the Invitrogen Qubit RNA HS kit, per manufacturer's protocol, testing 2 μ l of sample for each test.

The Qubit RNA HS kit uses a proprietary dye that specifically detects RNA, and is used to ensure approximately equal samples are used for subsequent enzymatic treatment steps.

When starting with MDA-MB-468 cells seeded at 0.5×10^6 cells per well of 6-well plates, quantitation at this point typically yields 5-20 ng/ μ l.

8. Dilute sample with buffer LB to achieve approximately 500 ng of RNA in 100 μ l final volume.

The amount of sample added can be varied according to experimental need. The important point is that samples under comparison should have approximately equal amounts of RNA, to ensure degree of enzymatic treatment is comparable between samples.

Enzymatic treatment of cell lysate

9. Add 15 μ l of solution E to sample, mix by gently flicking tube, and centrifuge briefly.

Solution E contains high concentrations of RNase, to generate ribosomal footprints, and DNase, to remove contaminating genomic DNA. Mix well as enzymes are suspended in glycerol stocks.

10. Incubate for 1 h at 4°C with gentle mixing.

Enzymatic digest at 4°C is preferable to prevent any aberrant ribosomal movement that might occur at higher temperatures. Gentle mixing on a rotary mixer is sufficient.

Isolate RNA

11. Add 460 μ l of TRIzol, vortex well to mix, centrifuge briefly, and incubate for 5 min at room temperature.

TRIzol addition is necessary to halt enzymatic digestion, and should be carefully timed.

12. Add 92 μ l of chloroform, shake vigorously for 30 s, and incubate for 3 min on ice.

13. Centrifuge at 12,000g for 15 min at 4°C.

Proceed to retrieve supernatant immediately after centrifugation is complete.

14. Retrieve 250 μ l of supernatant and transfer to pre-cooled 1.5 ml microcentrifuge tube.

Take care not to contact the interface or the lower phase, as this will increase contaminant content, such as genomic DNA and phenol.

Remaining solution from TRIzol extraction are not needed, and can be discarded.

15. Add 2 μ l of 15 mg/ml GlycoBlue, vortex briefly, add 375 μ l of 100% isopropanol, and vortex well to mix.

Glycogen generally increases yield of nucleic acid precipitation, and GlycoBlue is preferable as it colors the pellet to prevent accidental discard. Isopropanol is slightly immiscible with aqueous solutions, so needs to be vortexed well to mix completely.

16. Incubate for 30 min on dry ice, or overnight at -80°C.

17. Centrifuge at 20,000g for 30 min at 4°C, and cleanly remove supernatant.

18. Wash with 1 ml of ice-cold 70% ethanol, centrifuge at 20,000g for 10 min, cleanly remove supernatant, and dry for 10 min at room temperature.

It is preferable to centrifuge and remove supernatant several times to ensure all ethanol is removed, and this also shortens time necessary for drying. Adequate drying can be achieved by leaving the microcentrifuge tube open in a clean, well-ventilated area at room temperature. Given that resuspension is in a small volume, ensure that the pellet is completely dry before proceeding to prevent excessive ethanol contamination.

19. Resuspend pellet in 10 µl of water, and transfer to 0.2 ml PCR tubes.

Ensure pellet is completely dissolved before proceeding.

For storage, transfer to -80°C freezer.

Reverse transcription

20. Program a thermocycler with protocols RTD, RTR and RTH as listed below.

Program RT

Step 1: 80°C for 60 s

Step 2: Temperature ramp of 80°C to 30°C at 0.3°C/s

Step 3: 30°C for 10 s

Step 4: 4°C until use

Program RTR

Step 1: Temperature ramp of 40°C to 50°C in 2°C step-wise manner, 600 s per step

Step 2: 4°C until use

Program RTH

Step 1: 98°C for 1200 s

Step 2: 4°C until use

We use a Bio-Rad C1000 Thermo Cycler; other suitable systems are available.

21. Add 2 µl of solution Q1 to sample, mix by gently flicking tube, and centrifuge briefly.

Solution Q1 contains the reverse transcription primer mix.

22. Transfer samples to a thermocycler, and run program RTD.

Program RTD comprises a high temperature denaturation step, followed by a gradual temperature ramp downwards to promote complementary binding between the reverse transcription primers and the ribosomal footprint templates.

23. Add 8 µl of solution Q2 to sample, mix by gently flicking tube, and centrifuge briefly.

Solution Q2 contains the reverse transcriptase master mix.

24. Return samples to the thermocycler and run program RTR.

Program RTR is a graduated temperature ramp from 40°C to 50°C to promote efficient reverse transcription.

25. Add 2.2 µl of 1 N NaOH, vortex well to mix, and centrifuge briefly.

26. Return samples to the thermocycler and run program RTH.

Program RTH is a high temperature incubation to promote base-mediated hydrolysis of RNA template, so that only cDNA (and reverse transcription primers) should remain after isopropanol precipitation below.

27. Add sample to 176 µl of solution Q3, vortex briefly, add 2 µl of 15 mg/ml GlycoBlue, vortex briefly, add 300 µl of 100% isopropanol, and vortex well to mix.

GlycoBlue and NaOAc (pH adjustment) promotes efficient RNA precipitation. Continue with nucleic acid precipitation and resuspension as Steps 16 - 19, also described as follows.

28. Incubate for 30 min on dry ice, or overnight at -80°C.

29. Centrifuge at 20,000g for 30 min, and cleanly remove supernatant.

30. Wash with 1 ml of ice-cold 70% ethanol, centrifuge at 20,000g for 10 min, cleanly remove supernatant, and dry for 10 min at room temperature.

It is preferable to centrifuge and remove supernatant several times to ensure all ethanol is removed, and this also shortens time necessary for drying. Given that resuspension is in a small volume, ensure that the pellet is completely dry before proceeding to prevent excessive ethanol contamination.

31. Resuspend pellet in 20 µl of water, then add 480 µl of water, and vortex well to mix.

Ensure pellet is completely dissolved before proceeding.

For storage, transfer to -80°C freezer.

Quantitative PCR

32. Add 5 µl of sample to 15 µl of solution QT in optical qPCR plate, centrifuge briefly, vortex gently, centrifuge briefly.

Solution QT contains the footprint-specific qPCR forward primer, with a common qPCR reverse primer, and the qPCR master mix.

33. Transfer samples to an RT-qPCR instrument and run program QP.

Program QP is a PCR-based amplification of specific footprints, with a fluorescence detection step at the end of each cycle.

Program Q

Step 1: 95°C for 120 s

Step 2: 40 cycles of

Step 2.1: 95°C for 15 s

Step 2.2: 50°C for 15 s

Step 2.3: 55°C for 15 s

Step 2.4: 60°C for 15 s

Step 2.5: 65°C for 45 s

Step 2.6: Measure fluorescence

Step 3: Standard dissociation curve

Standard fluorescence measurement settings and dissociation curves, broadly suitable for other RT-qPCR analyses, should be used. These settings are often pre-programmed into the instrument.

We use an Applied Biosystems 7300 Real-Time PCR System; other suitable systems are available.

34. Analyze data per standard qPCR applications for gene expression analysis.

See Current Protocols in Molecular Biology, Unit 15.8 High-throughput real-time quantitative reverse transcription PCR, Bookout et al., for an analysis protocol of RT-qPCR data. [*Copy Editor: Please add CPMB literature citation and link.]

REAGENTS AND SOLUTIONS

Buffer WB

PBS supplemented with cycloheximide to 100 µg/ml final concentration. Make fresh, cool in ice-water, protect from light.

Buffer LB

Add to water to obtain the following final concentrations:

20 mM Tris-HCl, pH 7.4

150 mM NaCl

5 mM MgCl₂

1 mM DTT

1 mg/ml cycloheximide

1% v/v Triton X-100

Mix well after adding reagents, taking care to avoid excessive bubbles, and centrifuge briefly prior to use. Mix in advance if preferable, store at -80°C, protect from light.

It is preferable to make a 20% v/v Triton X-100 solution for subsequent dilution to 1% v/v. Tris-HCl, pH 7.4 (1 M) can be made by combining Tris-HCl, pH 7 (1 M) and pH 8 (1 M) (both from Ambion) in a 4:1 ratio.

Solution E—1000 U of RNase I, and 10 U of TurboDNase, in 15 µl volume.

Obtain by mixing 10 µl of RNase I (100 U/ul) (Ambion) and 5 µl of TurboDNase (2 U/µl) (Ambion). Mix gently by pipetting and flicking, do not vortex. Centrifuge briefly prior to use. Make fresh

Solution Q1—Reverse transcription primer mixture at 1 µM each in water. Mix by vortexing well. Mix in advance if preferable, store at -20°C.

Solution Q2—For each reaction, mix in the following order to obtain 8 µl of solution Q2:

4 µl of 5X SuperScript III First-Strand buffer (Invitrogen)

1 µl of 40 mM dNTP mixture (10 mM of each dNTP)

1 µl of 0.1 M DTT (Invitrogen)

1 µl of 20 U/ul SUPERase-In (Ambion)

1 µl of 200 U/ul Superscript III reverse transcriptase (Invitrogen)

Mix gently by pipetting and flicking, do not vortex. Centrifuge briefly prior to use. Make fresh.

Solution Q3—For each reaction, mix in the following order to obtain 176 µl of solution Q3:

156 µl of water

20 µl of 3 M NaOAc

Mix by vortexing well. Centrifuge prior to use. Mix in advance if preferable, store at room temperature

Solution QT—For each reaction, mix in the following order to obtain 15 µl of solution QT:

10 µl of 2X SYBR-Select Master Mix (Applied Biosystems)

4 µl of qPCR primer mix

1 µl of water

Mix well by gentle vortexing. Centrifuge briefly prior to use.

Primer mix comprises 1 µM each of footprint-specific qPCR forward primer and common reverse primer, diluted in water. Make fresh

Primers

Reverse transcription primer and qPCR forward primers are designed against the translation initiation site of specific genes as using the general scheme shown in Figure 1. The qPCR reverse primer is designed against the 5' region of the reverse transcription primer and is common amongst all targets tested. As examples, the primer sequence against three common genes are listed in Table 1, together with the sequence of the translation initiation site of the genes.

COMMENTARY

Background Information

RNA translation is an essential process in the transformation of genetic information into functional protein products. Ribosome profiling and polysome profiling, and their variations, represent some of the most frequently used methods for directly assessing fundamental characteristics of this process.

Polysome profiling, which has been used for many years, aims to fractionate intact ribosomal-transcript complexes across a sucrose gradient according to their mass, and to measure transcript abundance across the different fractions, based on the reasoning that “heavier” complexes have greater ribosomal occupation on each transcript within these fractions, and therefore are more highly translated. The relative transcript abundance across the fractions can then be measured by qPCR, microarray, or RNA sequencing-based methods.

Ribosome profiling, a more recent technique, aims to generate RNase-resistant ribosomal footprints, in a sample containing intact ribosomal-transcript complexes, and to extract, sequence, map, and count these footprints across transcripts (Ingolia *et al.*, 2011; Ingolia *et al.*, 2012). This technique is based on the reasoning that transcripts with more ribosomal protection have generally greater ribosomal occupancy, and are therefore likely to be more highly translated. This approach is designed as a sequencing-based, transcriptome-wide method and has been shown to provide nucleotide-resolution information on ribosomal occupation. By design, the technique gives information across the entire (or near entire) transcriptome without focus on any specific transcripts. Furthermore, this method has been modified in various ways to give other information on translation, such as in QTI-seq for transcriptome-wide mapping and measurement of translation initiation (Lee *et al.*, 2012; Gao *et al.*, 2015).

While both techniques have proven to be immensely useful in giving numerous, novel insights into the role and regulation of RNA translation in normal and disease physiology, their application towards larger scale experiments have, however, been somewhat limited. This is in large part due to technical challenges posed by these techniques, in particular, due to their demands on time and resources. Here, we built upon the ribosomal profiling approach to develop an RT-qPCR-based method for more rapid and cost-effective measurement of RNA translation levels across focused groups of genes. In general, our method aims to measure the relative abundance of specific footprints across transcripts of interest, as a surrogate for general ribosomal occupation across those transcripts and their

level of translation. In particular, we optimized RT and qPCR probe designs to improve the magnitude and specificity of measurement signals for short (approximately 30 nt) ribosomal footprints. Specifically, probes are designed to detect ribosomal footprints located at translation initiation sites to improve signal intensity by making use of the ability of certain translation inhibitors, such as cycloheximide, to cause ribosomal accumulation at these sites (Ingolia *et al.*, 2011; Ingolia *et al.*, 2012; Gerashchenko and Gladyshev, 2014); to use footprint-specific sequences at both the RT and qPCR stages in order to increase detection specificity for footprints of interest; and, to use RT primers which are artificially elongated to overcome challenges of detecting small templates in order to improve qPCR signal quality.

Critical Parameters and Troubleshooting

Reagents purity and consumables quality—Following nuclease digestion, some of the remaining footprints may be low in abundance. Therefore, it is imperative that nuclease-free reagents, tubes, and other consumables that directly contact samples, together with low-binding tubes, are utilized to minimize loss of sample.

Formation of primer dimers in qPCR—Given that there may be some overlap between the RT and qPCR forward primers, it is possible that primer dimers may be formed in the qPCR stage. Therefore, it is essential that melting curves are generated at the end of the qPCR reaction to assess primer dimer formation. In the event that substantial amounts of primer dimers do form, it may be necessary to re-design the footprint-specific primers to minimize overlap.

Low signal/High Ct values with high variability in qPCR—Lower qPCR signals (Ct greater than 30 cycles), possibly associated with higher variability between qPCR technical replicates, may be indicative of low footprint abundance. It may be possible to increase sample input into the RT and qPCR reactions to increase signal strength. Alternatively, this could be indicative of contaminating chemicals in the RT or qPCR reactions that inhibit the enzymes involved. In this case, during TRIzol extraction, ensure the aqueous supernatant is separated cleanly from the lower layers, and following nucleic acid precipitation steps, ensure that pellets are washed thoroughly and completely dry prior to resuspension.

Changes in total transcript levels—Changes measured by this method are indicative of gross changes in translation, which could also be brought about by changes in transcript levels. To take this into account, it is advisable to dedicate separate “total mRNA” samples that are likewise processed with the exception of omitting steps 9 and 10 (nuclease treatment) in order to assess changes in transcript levels. As there is no nuclease treatment in this case, a smaller input sample amount is usually sufficient, e.g. 100 ng.

Anticipated Results

qPCR analysis should yield fluorescence values at the conclusion of each PCR cycle for each well. These raw values should be normalized to internal ROX fluorescence and baseline-subtracted (most qPCR analysis instrument should be capable of carrying out these analysis steps), and C_t values determined for each sample as the interpolated cycle number

at which fluorescence value crosses a given threshold within the exponential phase of amplification. Change in abundance of footprints of interest, relative to internal control footprints and reference samples, can then be analyzed as for standard qPCR analysis of mRNA levels. A number of analysis methods are available, with the most commonly used being the C_t approach (See Unit 15.8 High-throughput real-time quantitative reverse transcription PCR). Across multiple biological replicates, in our hands, it is usually possible to reliably detect changes as little as 25% between different conditions for cell culture-based experiments. We have demonstrated this technique in its application towards the study of translational regulation of genes encoding ribosomal proteins (Li *et al.*, *in press*).

Time Considerations

This method is most effective when applied as a routine assay over a focused group of genes and across moderate sample numbers. Analysis of 8-12 samples over 4-8 transcripts can be expected to require approximately 2-3 days for one biological replicate.

Acknowledgements

This work was supported by NIH R35 CA210057 (J.J.Z.), R01 CA187918 (T.M.R. and J.J.Z.), P50 CA168504 (T.M.R. and J.J.Z.), P50 CA165962 (T.M.R. and J.J.Z.) and the Breast Cancer Research Foundation BCRF-17-176 (J.J.Z.).

Demonstrates the application of this method towards the study of translational regulation of genes encoding ribosomal proteins.

Literature Cited

- Bookout AL, Cummins CL, Mangelsdorf DJ 2005, High-throughput real-time quantitative reverse transcription PCR. *Curr. Protoc. Mol. Biol.* 15.8.1-15.8.21
- Gao X, Wan J, Liu B, Ma M, Shen B, and Qian SB 2015 Quantitative profiling of initiating ribosomes in vivo. *Nat. Methods.* 12(2):147–153. [PubMed: 25486063]
- Gerashchenko MV, and Gladyshev VN 2014 Translation inhibitors cause abnormalities in ribosome profiling experiments. *Nucleic Acids Res.* 42(17):e134. [PubMed: 25056308]
- Ingolia NT, Lareau LF, and Weissman JS 2011 Ribosome profiling of mouse embryonic stem cells reveals the complexity and dynamics of mammalian proteomes. *Cell* 147(4):789–802. [PubMed: 22056041]
- Ingolia NT, Brar GA, Rouskin S, McGeachy AM, and Weissman JS 2012 The ribosome profiling strategy for monitoring translation in vivo by deep sequencing of ribosome-protected mRNA fragments. *Nat. Protoc.* 7(8):1534–1550. [PubMed: 22836135]
- Lee S, Liu B, Lee S, Huang SX, Shen B, and Qian SB 2012 Global mapping of translation initiation sites in mammalian cells at single-nucleotide resolution. *Proc. Natl. Acad. Sci. U S A* 109(37):E2424–2432. [PubMed: 22927429]
- Li BB, Qian C, Gameiro PA, Liu CC, Jiang T, Roberts TM, Struhl K, and Zhao JJ Targeted profiling of RNA translation reveals mTOR-4EBP1/2-independent translational regulation of mRNAs encoding ribosomal proteins. *In press.*
- Morita M, Gravel SP, Hulea L, Larsson O, Pollak M, St-Pierre J and Topisirovic I 2015 mTOR coordinates protein synthesis, mitochondrial activity and proliferation. *Cell Cycle* 14(4): 473–480. [PubMed: 25590164]
- Pakos-Zebrucka K, Koryga I, Mnich K, Ljubic M, Samali A and Gorman AM (2016). “The integrated stress response.” *EMBO Rep* 17(10): 1374–1395. [PubMed: 27629041]
- Saxton RA and Sabatini DM 2017 mTOR Signaling in Growth, Metabolism, and Disease. *Cell* 169(2): 361–371.

Key References

- Li BB, Qian C, Gameiro PA, Liu CC, Jiang T, Roberts TM, Struhl K, and Zhao JJ Targeted profiling of RNA translation reveals mTOR-4EBP1/2-independent translational regulation of mRNAs encoding ribosomal proteins. In press.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1. General scheme for design of reverse transcription (RT) and qPCR primers. (A) RT and qPCR forward primers are designed against the sequence of the translation initiation site (TIS) for genes of interest. (B) Example of common region in the RT primer, and the sequence of the qPCR common reverse primer based on this common region.

Table 1.

Examples of primer sequences.

In the translation initiation site sequences, regions targeted by RT primer alone are shown as yellow, by qPCR forward primer alone shown as blue, and by both primers (i.e., overlap) as red. Red regions are kept to a minimum to avoid primer dimer formation in qPCR stage. T_m are calculated using IDT OligoAnalyzer 3.1.1, with parameters: for RT, target type is RNA, 0.1 μM oligo, 75 mM monovalent cation, 3 mM divalent cation, 2 mM dNTPs total; for qPCR, target type is DNA, 0.2 μM oligo, 50 mM monovalent cation, 1.5 mM divalent cation, 0.8 mM dNTPs.

Gene	Translation initiation site sequence		Primer type	Primer sequence		Tm
PPIA	CGTGTA	TATTAGCC	RT	AGCGGATAACAATTTCACACAGGAAACAGCTATGAC	CGGTGGGGTTGA	46.6
		ATG	qPCR forward	CGTGTA	CTATTAGCCATGGTCA	59.3
TUBA1B	TAATCCCTAGC	CACT	RT	AGCGGATAACAATTTCACACAGGAAACAGCTATGAC	GAGATGCAC	43.7
		ATG	qPCR forward	TAATCCCTAGC	CACTATGCCGT	60.4
ACTB	CGCCGCCAGCT	CACC	RT	AGCGGATAACAATTTCACACAGGAAACAGCTATGAC	GGCGATATCATCATC	45.1
		ATG	qPCR forward	CGCCAGCTCACC	ATGGA	61.3
			qPCR common reverse			61.1
			AGCGGATAACAATTTCACACAGG			