Published in final edited form as:

Methods Mol Biol. 2014 ; 1125: 353–371. doi:10.1007/978-1-62703-971-0_28.

Quantitative analysis of deadenylation-independent mRNA decay by a modified MBACE assay

Julie A. Dougherty^{1,2,3}, Roshan Mascarenhas^{1,2,4}, and Daniel R. Schoenberg^{1,2,3,*}

¹Center for RNA Biology, The Ohio State University, Columbus, OH 43210

²Department of Molecular and Cellular Biochemistry, The Ohio State University, Columbus, OH 43210

³Biomedical Sciences Graduate Program, The Ohio State University, Columbus, OH 43210

Summary

Endonuclease cleavage is the rate-limiting step in the decay of nonsense-containing human β -globin mRNA in erythroid cells. The 5'--truncated intermediates thus generated are polyadenylated and more stable than the parent mRNA. Northern blotting is commonly used to measure the decay rate of full-length mRNA, and S1 nuclease protection is used to assay the fate of decay intermediates. We have adapted the more sensitive and facile MBACE assay (1) to quantitatively monitor the decay process by detecting full-length β -globin and its decay intermediates.

Keywords

β -globin mRNA; MBACE assay; molecular beacons; mRNA decay; nonsense codon; endonuclease

1. Introduction

A premature termination codon (PTC) can arise within an mRNA due to mutation, errors in splicing, or via alternative splicing. Translation of PTC--containing mRNAs can cause deleterious effects in cells by producing C-terminally truncated proteins. A surveillance mechanism termed nonsense-mediated mRNA decay (NMD) recognizes and targets these PTC--containing mRNAs for accelerated degradation via two mechanisms. NMD target mRNAs can be internally cleaved by an endonuclease and then rapidly degraded by exonucleases, with Xrn1 catalyzing 5'--3' degradation of the downstream fragment and the exosome--associated exonucleases catalyzing 3'--5' degradation of the upstream fragment.

*Address all correspondence to: Daniel R. Schoenberg, Ph.D., Department of Molecular and Cellular Biochemistry, The Ohio State University, Columbus, OH 43210-1218, schoenberg.3@osu.edu.

⁴Current address: Department of Pharmacology, The Ohio State University, Columbus, OH 43210

¹³The conditions for these particular constructs and experiments and may not be the optimal conditions for other constructs. These conditions could be used as a starting point for optimization. Parameters to optimize include: primer concentration, beacon concentration, template concentration (sometimes less is better), annealing temperature, and qPCR reagent. Also, due to the sensitivity of the MBACE assay, use well-maintained and calibrated pipettes, as small deviations can become large differences.

¹⁴An alternative to using molecular beacons would be to use SYBR green chemistry -- if it can be proven to be highly specific. Designing the forward primer to hybridize to the RNA linker--mRNA junction sequence would serve to differentiate the products. Standard curves and specificity testing would be necessary to guarantee specific detection. qPCR products should be electrophoresed on an agarose gel and the 5' end sequences of the resulting product(s) verified by 5' RACE as above to additionally ensure specificity. Bioline's SensiFAST SYBR reagents have shown superior sensitivity.

Alternatively, the target mRNAs may undergo deadenylation and decapping followed by exonucleolytic decay from the 5' and 3' ends (reviewed in (2)). In erythroid cells SMG6 catalyzes endonuclease cleavage of PTC--containing human β --globin mRNA (manuscript submitted) but unlike other forms of NMD this uniquely gives rise to *stable* 5'--truncated products (3, 4).

The conventional approach to studying the decay of nonsense--containing mRNA is to monitor the disappearance of the full--length mRNA; however, this does not fully illustrate the decay process. Stable decay products of PTC--containing β --globin mRNA were originally detected by S1 nuclease protection and primer extension assays (4). Each of these approaches is time--consuming, insensitive, difficult to quantify, and impractical for use with large numbers of samples. To monitor changes in both full--length β --globin mRNA and its decay products we adapted a highly sensitive assay based on *molecular beacon rapid amplification of cDNA ends* (MBRACE) (1). MBRACE involves ligation of an RNA linker onto the 5' end of monophosphorylated mRNA and detection of the resulting product using a probe specific for the linker--mRNA junction. Molecular beacons are single-stranded, dual-labeled fluorescent probes held in a hairpin structure by complementary stem sequences at the ends of the probe with target-specific sequence in the loop region. While in the hairpin configuration the proximity of the quencher blocks fluorescent emission by the reporter. Hybridization of the molecular beacon to the target sequence opens the hairpin structure, separating the reporter dye and quencher and enabling fluorescent emission. Since only one beacon binds per target molecule the fluorescent signal is proportional to the amount of target DNA. Molecular beacons have several advantages over other quantitative PCR--based assays. They do not interfere with primer binding and extension, they fluoresce only when hybridized to the target sequence, and, unlike Taqman probes, they are not dependent on the exonuclease activity of the polymerase (5).

2. Materials

2.1 General Supplies

1. Table top centrifuge
2. Refrigerated microcentrifuge
3. 1.7 mL microcentrifuge tubes
4. Pipette tips with filter
5. Spectrophotometer to quantify RNA
6. PCR tubes
7. Thermocycler
8. Agarose gel electrophoresis apparatus
9. Illumina Eco Real--Time PCR system or a comparable instrument

2.2 Enzymes

1. Calf intestinal alkaline phosphatase, CIAP
2. Tobacco acid pyrophosphatase, TAP (Epicentre, Wisconsin)
3. T4 RNA ligase I
4. Reverse transcriptase, SuperScript III (Life Technologies, New York)
5. Taq DNA polymerase (Sigma--Aldrich, Massachusetts)

6. T4 DNA ligase (Promega, Wisconsin)

2.3 Reagents

1. Phosphate buffered saline, PBS
2. TRIzol (Life Technologies, New York)
3. Acid phenol: chloroform (5:1, pH 4.5)
4. Chloroform
5. 80% Ethanol, freshly diluted or stored at -20°C
6. 70% Ethanol, cold
7. Isopropanol
8. Carrier for nucleic acid precipitation, such as BSA or glycogen
9. RNase-free water
10. RNase inhibitor, such as RNase OUT (Life Technologies, New York)
11. 10 mM ATP
12. 2 $\times$ PerfeCTa qPCR FastMix –ROX, UNG (QuantaBiosciences, Maryland)
13. 10 mM dNTP mix
14. 25 mM MgCl_2
15. 0.1 M Dithiothreitol, DTT
16. 50 μM oligo(dT)₂₀
17. 100 μM RNA linker, 40–44 base pairs in length
18. Forward primer specific to 5' end of RNA linker
19. Gene--specific reverse outer primer
20. Gene--specific reverse nested primer
21. Cloning vector, such as pGEM--T (Promega, Wisconsin)
22. Luria broth, LB
23. LB plus ampicillin at 50 $\mu\text{g/mL}$
24. LB agar plates with ampicillin at 50 $\mu\text{g/mL}$
25. Gel extraction kit
26. QIAquick PCR purification kit (Qiagen, California)
27. Gene--specific reverse qPCR primers
28. Molecular beacons specific for junction between RNA linker and mRNA sequence
29. Gene-specific qPCR forward and reverse primers for internal control mRNA
30. *In vitro* transcription kit – such as MAXIscripT T7/SP6 kit (Ambion/Life Technologies, New York)

2.4 Buffers

1. Erythroid fractionation lysis buffer (EFLB): 100 mM NaCl, 10 mM Tris HCl pH 8.0, 2 mM EDTA, 1% NP-40, 1% β -mercaptoethanol, 1 mM DTT, RNase OUT (*see Note 1*)
2. CIAP reaction buffer: 50 mM Tris HCl pH 8.5, 0.1 mM ZnCl₂, 5% glycerol (v/v) (10× stock provided with enzyme)
3. Stop solution: 5 M Ammonium acetate, 100 mM EDTA
4. TAP buffer: 50 mM sodium acetate pH 6.0, 0.1 mM EDTA, 0.1% β -mercaptoethanol, 0.01% Triton-X-100 (10× provided with enzyme)
5. T4 RNA ligase I buffer: 50 mM Tris HCl pH 7.5, 10 mM MgCl₂, 1 mM DTT, 1 mM ATP (10× provided with enzyme, with or without ATP)
6. SuperScript III RT buffer: 20 mM Tris HCl pH 8.4, 50 mM KCl (10× provided with kit)
7. 1× TBE: 89 mM Tris, 89 mM boric acid, 2 mM EDTA pH 8.0
8. Rapid ligation buffer: proprietary content including polyethylene glycol (PEG) and ATP (2× provided with pGEM-T vector)
9. 2× Rapid ligation buffer, T4 DNA ligase: 60 mM Tris-HCl pH 7.8, 20 mM MgCl₂, 20 mM DTT, 2 mM ATP, 10% polyethylene glycol (Promega, Wisconsin)
10. Taq DNA polymerase buffer: 10 mM Tris HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.1 mM DTT, stabilizers, 5% glycerol (10× provided with enzyme)

3. Methods

3.1. Erythroid cell harvest, fractionation and RNA extraction (*see Note 2*)

1. Scrape $1-5 \times 10^6$ cells into a 15 mL culture tube and centrifuge at 1000×g for 5 min then remove media.
2. Wash pellet with 1 mL ice cold PBS and transfer to 1.7 mL microcentrifuge tube. Centrifuge at 1000×g for 5 min and remove all PBS.
3. Resuspend cell pellet in 3–5 volumes of EFLB by pipetting up and down 4–5 times, tap the tube and incubate on ice 5 min. Tap tube and incubate on ice 5 min more, repeat until lysate becomes clear (*see Note 3*).
4. Centrifuge at 5000×g for 10 min at 4°C to pellet nuclei and debris. Transfer cytoplasmic supernatant to a new tube *pre-chilled on ice* (*see Note 4*).
5. Add 1 mL TRIzol, gently invert to mix, and incubate at room temperature for 5 min.

¹Prepare an incomplete stock of EFLB composed of all components except DTT and the RNase inhibitor; this can be stored at –20°C. Prior to use, thaw it on ice and complete buffer by adding DTT and RNase inhibitor then keep it on ice. Discard any remaining unused completed buffer.

²Erythroid cells are difficult to lyse so this method will not be appropriate for all cell types. Use an established fractionation protocol for the cell type being used.

³The lysate should become clear but not viscous as this denotes lysis of the nuclear membrane. Three cycles of tapping/incubation have been adequate for erythroid cells. RNA can be assessed for proper cyto-nuclear fractionation by denaturing gel electrophoresis and observation of the mature 18S and 28S rRNAs highly concentrated in the cytoplasm and 2 larger pre-rRNA bands only seen in the nucleus.

⁴To lyse the nucleus, resuspend the pellet in 3–5 volumes of EFLB and process as with cytoplasm -- tapping and incubating on ice. Centrifuge at 5000×g to pellet cell debris. Transfer nuclear supernatant to a new tube *pre-chilled on ice*.

6. Add 200 μL chloroform and shake vigorously by hand for 15 sec, incubate at room temperature 10–15 min. Centrifuge at $>12,500\times g$ for 15 min at 4°C to separate phases. Transfer upper aqueous phase to new tube.
7. Add 500 μL isopropanol and 20 μg glycogen, invert to mix and incubate on ice for 10 min. Centrifuge at $>7500\times g$ for 10 min at 4°C to pellet RNA. Discard supernatant.
8. Wash pellet with 1 mL 80% ethanol. Centrifuge at $>7500\times g$ for 10 min at 4°C to pellet RNA. Remove ethanol and air-dry for 5–10 min. Resuspend the pellet in an appropriate amount of RNase--free water to keep the RNA concentrated. Incubate at 60°C for 10 min to fully dissolve pellet. Centrifuge briefly to collect and keep on ice (sample may be stored at -80°C at this point). Quantify RNA using a spectrophotometer, such as the NanoDrop1000.

3.2. 5' RLM--RACE to identify the sequence at 5' ends

1. Treat cytoplasmic RNA with CIAP to remove 5' monophosphates from RNA.

1. In a 20 μL total reaction volume, combine: 4 μg RNA, 2 μL 10 $\times$ CIAP buffer, 2 μL (2 U) CIAP, and RNase--free water to 20 μL . Mix gently, centrifuge briefly to collect.
2. Incubate at 37°C for 1 hr.
3. Terminate reaction by adding 115 μL RNase--free water and 15 μL Stop Solution. (*see* Note 5)
4. Extract RNA by adding 150 μL acid phenol: chloroform. Vortex and centrifuge 5 min at $12,500\times g$ at room temperature. Transfer upper, aqueous phase to a new microcentrifuge tube.
5. Add 150 μL chloroform. Vortex thoroughly. Centrifuge 5 min at $12,500\times g$ at room temperature. Transfer upper, aqueous phase to a new microcentrifuge tube.
6. Add 150 μL isopropanol and 20 μg glycogen. Vortex and incubate on ice 10 min. Centrifuge 20 min at $12,500\times g$ at room temperature.
7. Rinse pellet with 500 μL of cold 70% ethanol. Centrifuge 5 min at $12,500\times g$ at room temperature. Remove ethanol and air dry for 10–15 min.
8. Resuspend pellet in 10 μL of RNase--free water. Keep on ice. (sample may be stored at -80°C at this point)

2. Treat CIAP--reacted RNA with TAP to remove 5' cap and leave a 5' monophosphate end. (*see* Note 6)

1. In a 16 μL total reaction volume, combine: 5 μL CIAP reaction product (2 μg RNA), 1.6 μL 10 $\times$ TAP buffer, 0.3 μL of TAP (3 U), 0.5 μL RNase OUT, and RNase--free water to 16 μL .
2. Incubate at 37°C for 2 hr, place on ice (sample may be stored at -80°C at this point).

⁵CIAP *must* be inactivated/removed or it will continue to dephosphorylate RNA after subsequent TAP--mediated cleavage of the 5' cap, which would leave all the RNA without a 5' monophosphate.

⁶As of this writing, TAP is exclusively sold by Epicentre and is quite expensive. To economize costs, scale up the reactions and use an aliquot of the TAP reaction for subsequent work. Save the remainder of the TAP reaction at -80°C .

3. **Ligate RNA linker to 5' ends of RNA** (*see Note 7*)

T4 RNA ligase I ligates the 3' hydroxyl of the RNA linker to the 5' monophosphate of RNA molecules.

1. Combine 8 μL of TAP reaction product (1 μg RNA) and 2 μL 100 μM RNA linker. Heat at 65°C for 5 min and snap chill on ice to relax secondary structures.
2. For a total reaction volume of 16 μL , combine per reaction: 1.6 μL of 10 $\times$ T4 RNA ligase I buffer, 1.6 μL of 10 mM ATP (if not in 10 $\times$), 1 μL of T4 RNA ligase I (10 U), 0.5 μL of RNase OUT, and RNase--free water to 16 μL .
3. Incubate at 37°C for 2 hr, heat--inactivate enzyme by incubating at 65°C for 15 min, place on ice (sample may be stored at -80°C at this point).

4. **First strand cDNA synthesis**

Perform cDNA synthesis using a gene--specific reverse primer so only β --globin mRNAs are amplified in subsequent PCR reactions.

1. In a PCR tube, combine: 8 μL ligation reaction product (500 ng RNA), 1 μL 10 mM dNTPs, and 1 μL 5 μM reverse primer. Heat contents at 65°C for 5 min, snap chill on ice.
2. In a separate tube, prepare a 2 $\times$ reaction mix as follows: 2 μL 10 $\times$ RT buffer, 4 μL 25mM MgCl_2 , 2 μL 0.1 M DTT, 1 μL RNase OUT, and 1 μL SuperScript III RT.
3. Add 10 μL 2 $\times$ reaction mix to sample tube and incubate at 50°C for 50 min. Inactivate enzyme by heating reaction at 70°C for 15 min. Place tubes on ice.
4. Add 1 μL RNase H to tube, gently mix and spin to collect. Incubate at 37°C for 20 min. RNase H will remove the RNA from the RNA:cDNA hybrids. Place tubes on ice (sample may be stored at -20°C at this point)

5. **Nested PCR to amplify and visualize full--length and truncated products** (*see Fig. 1*).

1. Perform primary PCR using a forward primer complementary to the 5' end of the RNA linker and a reverse primer specific to the gene of interest. This will amplify all target gene products with a ligated adapter, full--length and truncated. Prepare the primary PCR in 50 μL total reaction volume under the following conditions: 10 mM Tris--HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl_2 , 200 μM dNTPs, 400 nM forward primer, 400 nM reverse outer primer, 15 ng cDNA, and 2.5 U *Taq* DNA polymerase (SigmaAldrich). Thermal cycle conditions: 94°C 3 min $\rightarrow$ [94°C 30 sec $\rightarrow$ 60°C 30 sec $\rightarrow$ 72°C 1 min] $\times$ 35cycles $\rightarrow$ 72°C 5 min.
2. The secondary PCR reaction utilizes the same forward primer as the primary and a reverse primer that binds upstream of that used in the

⁷Order an RNA linker without a 5' monophosphate, the lack of a 5' monophosphate will prevent concatamerization of the linker molecules. Excess adapter needs to be used in the reaction to ensure as many molecules as possible are ligated. Aliquot the RNA linker for storage at -80°C to avoid freeze--thaw cycles. The RNA linker sequence was adapted from GeneRacer[®] protocol by Invitrogen. To complete the 5' RACE experiments a 25mer RNA linker was used, which was later changed for the MBACE qPCR to a 44mer RNA linker to provide room for hybridization of the forward primer and the beacon.

primary reaction. Dilute the primary PCR 1:10, 1:100 and 1:1000 in water for use as template for the secondary reaction. Diluting the primary reaction results in increased detection of the nested product after gel electrophoresis (6).

3. Perform secondary PCR under the following reaction conditions: 1 μ L diluted primary PCR reaction, 10 mM Tris--HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 200 μ M dNTPs, 400 nM forward primer, 400 nM reverse nested primer, and 2.5 U *Taq* DNA polymerase (Sigma) in 50 μ L total reaction. Thermal cycle conditions: 94°C 3 min→[94°C 30 sec→60°C 30 sec→72°C 45 sec] ×35 cycles→72°C 5 min.

6. Identification of 5' sequence

1. Electrophorese 8 μ L of the secondary PCR reaction on a 3% low melting point (LMP) agarose gel using 1× TBE buffer. Visualize and document bands with a Bio--Rad GelDoc or similar imager. Size markers allow for identification of products based on size (*see* Fig. 1). The 3% LMP agarose gel will resolve <500 bp bands very well.
2. Excise bands from the gel and purify using a gel extraction kit. Ligate purified bands into pGEM--T cloning vector in a 1.7 mL microcentrifuge tube. For a 10 μ L reaction add the following: 20 ng purified DNA, 1 μ L pGEM--T vector, 5 μ L 2× rapid ligation buffer, 1 μ L T4 DNA ligase (3 U), and RNase--free water to 10 μ L. Incubate the reaction at 4°C overnight. Thaw competent *E. coli* cells, such as DH5 α , on ice and transform by adding 2 μ L of the ligation reaction to 50 μ L of cells and keeping on ice for 5 min. Heat shock the cells at 42°C for 45 sec and quickly return tubes to ice for another 5 min. Add LB to 1 mL and incubate at 37°C with shaking for 1 hr. Plate 100 μ L of the transformed culture on LB agar plates with 50 μ g/mL ampicillin, or appropriate antibiotic, and grow overnight in 37°C incubator. Choose single colonies, verify ligation of target band with PCR, and sequence multiple positive clones using vector--specific forward and reverse primers (*see* Note 8).
3. Identify the RNA linker's 3' end sequence and following it will be the 5' sequence of the mRNA of interest. Align sequencing results with mRNA of interest using NCBI's specialized alignment engine (*bl2seq*) or other alignment software. Multiple identical sequencing results ensures confidence in the 5' end of the mRNA of interest.

3.3. Molecular beacons

Molecular beacons are single--stranded, dual--labeled fluorescent probes held in a hairpin structure by complementary stem sequences at the ends of the probe. The loop region is complementary to the target sequence; hybridization of the molecular beacon to the target sequence opens the hairpin--loop structure and separates the reporter dye and quencher resulting in fluorescent emission. Since only one beacon binds per target molecule the measured fluorescence is quantitatively proportional to the amount of target DNA.

1. Molecular Beacon and primer design

⁸Use of vector--specific forward and reverse primers will allow for sequencing the entire insert. Using forward and reverse sequencing primers also lends greater assurance of junction sequence as it is determined by two separate reactions. Sequencing several independent colonies provides verification of junction sequence.

Most importantly, the beacon needs to be designed to efficiently hybridize to and detect the proper target while reducing nonspecific fluorescence. Beacon design may vary as to the purpose of the assay -- standard target detection or allele discrimination (refer to (5) for detailed design tips). A qPCR master mix specific for probes is to be used in these reactions, do not use a SYBR green master mix. These experiments utilized QuantaBiosciences PerfeCTa qPCR FastMix --ROX since one of the beacons is detected in the ROX channel.

1. Design molecular beacon target sequences to span the junction between the RNA linker and the target product sequence.
2. Forward and reverse primers should have a T_m of 50–65°C, with 50–60% GC content. The forward primer needs to be at least 6 nucleotides upstream of the molecular beacon binding site to provide room for the polymerase. NOTE: this means the molecular beacon cannot be designed to the 5' most end of a target (*see* Note 9). Design the reverse primer so the resulting amplification product is 75–250 nucleotides in length.
3. Optimize molecular beacon conditions empirically by testing varying primer and beacon concentrations. For these experiments, conditions were found to be 250 nM beacon and 500 nM each primer. Illumina technical support is very helpful and a tutorial is available on their website: http://www.ecoqpcr.com/Files/training/Eco--Real_Time_PCR_Assays/Published/index.htm

2. Determining the optimal beacon annealing temperature

The annealing temperature of the reaction should be chosen so that the beacon specifically detects the correct target sequence. The optimal annealing temperature can be determined using a thermal denaturation profile of (1) beacon with correct target, (2) beacon with incorrect target, and (3) beacon alone. Choose the annealing temperature that provides maximal detection of the target sequence and minimal nonspecific detection.

1. Measure molecular beacon fluorescence in (1) the absence of target, (2) in the presence of perfectly complementary target, and (3) in the presence of mismatched target. The target oligonucleotides are created by *in vitro* transcription and subsequent gene--specific cDNA synthesis (*see* Note 10)
2. Prepare 3 reactions containing 200 nM molecular beacon in 1× final qPCR buffer. Add to one reaction a 5--fold molar excess of perfectly complementary cDNA, to the next add a 5--fold molar excess of mismatched cDNA, and to the last add only buffer (*see* Note 11).

⁹Avoid selecting a molecular beacon target sequence in an area of strong secondary structure.

¹⁰Specific target cDNAs are needed to perform the denaturation profiles, standard curves, and verify specificity of the beacons since the linker--specific sequence is shared. Synthetic 90mer DNA oligonucleotides of reverse complementarity to T7 promoter sequence --RNA linker--5' end mRNA sequence were ordered through Sigma Aldrich. 1 µg of 90mer DNA was combined with 2 µL of 50 µM T7 promoter--specific primer in a 0.65 mL tube. This tube was placed in a 1.7 mL tube filled with RNase--free water and the set was heated to 95°C on a heat block for 10 min. The set of tubes was then removed and placed at room temperature to cool slowly and allow the primer to properly hybridize to the 90mer and form a double--stranded T7 promoter. T7--directed *in vitro* transcription was completed using the Ambion MAXIScript T7/SP6 kit in a 30 µL reaction volume per manufacturer's instructions. The reaction was then treated with Turbo DNase to degrade the DNA template. Resulting RNA was stored at --80°C. The full--length mRNA product served as proper target for the full--length beacon and improper target for the decay product beacon. Alternatively the decay product served as proper target for the decay beacon and improper target for the full--length beacon.

¹¹For detection with the beacons, the original RNA linker sequence needed to be altered to increase the annealing temperature for the beacon. Additionally, the RNA linker length was increased to 44 base pairs to accommodate binding of the forward primer and beacon.

3. Using the Illumina Eco Real--Time PCR machine (Eco) choose the “other” detection chemistry and the high--resolution melt option. Select to detect the fluorescence for each preparation while the temperature decreases from 80°C to 30°C.
4. Normalize the data to the buffer control to get the denaturation profiles. The annealing temperature chosen provides the largest window of discrimination between matched and mismatched target detection.
5. This can be completed on other instruments by running the same samples and measuring fluorescence of the sample while decreasing temperature from 80°C to 30°C in 1°C increments for 1 minute each and comparing detection curves.

3. Standard curves

Standard curves allow for absolute quantification of data and ensure reliable detection by the beacon. Standard curves also determine the efficiency of the qPCR, which is needed to perform relative quantification calculations.

1. Using the cDNA from the thermal denaturation profiles, create a 10--fold dilution series of standards. The dilution series needs to cover a broad range to ensure a proper curve is generated.
2. In the Eco software, choose the “other” detection chemistry and the standardization option. Prepare a master mix of 2× qPCR buffer, primers, and beacon at the optimized conditions and aliquot into wells. Add template, running each standard in triplicate. Be sure to include a no template control for background detection.
3. The Eco software will generate a standard curve graph and provide values for the standard curve line. For 10--fold dilutions the ideal slope is -3.32 , which indicates an efficiency of 100%. Acceptable curves will have slopes that range from -3.10 to -3.58 , indicating an efficiency from 90% to 110%, with an R^2 value > 0.98 (7). If the standard curve is not acceptable, then try adjusting the reaction conditions. Always use a freshly diluted series of standards and run multiple standard curves on different days.

4. Beacon Specificity

Determining the degree of nonspecific detection by the beacon is essential to assure confidence in positive readings.

1. To determine the specificity of the molecular beacons, a template containing mutations only at the 3' binding side of the beacon is needed along with the correct template. For these experiments, full--length beacon was reacted with decay product target and the decay product beacon was reacted with full--length target, as the beacons differ only in their 3' sequence.
2. Run triplicate qPCR reactions of the 10--fold dilution series using a similar molar dilution series. Compare Ct values to those for samples of similar molarity, if the Ct value for nonspecific template is 7 cycles higher than the Ct for correct template, then nonspecific detection is less than 1%, according to the $2^{-\Delta\Delta C_t}$ method of calculation (8). In our experiments, nonspecific detection was less than 1%.

3.4. Processing Experimental Samples for MBRACE (see Fig. 2)

1. TAP treatment to remove 5' cap.

A reaction with 2 µg RNA will be used as an example.

1. Thaw RNA and 10× TAP buffer on ice.
2. Incubate RNA at 65°C for 5 min and snap chill on ice to relax secondary structures.
3. Prepare 10 µL total volume reactions as follows: 2 µg RNA, 1 µL 10× TAP buffer, 0.3 µL TAP (3 U), 0.5 µL RNase OUT (20 U), and RNase--free water to 10 µL
4. Incubate at 37°C for 1 hr.
5. Return tubes to ice (samples may be stored at –80°C at this point).

2. RNA linker ligation

1. Thaw RNA, 100 µM RNA linker, 10× T4 RNA Ligase I buffer, and 10 mM ATP on ice.
2. Combine 5 µL TAP reaction (1µg RNA) with 2 µL 100uM RNA linker and incubate at 65°C for 5 min then snap chill on ice.
3. Prepare reaction master mix, using the following per reaction: 1.6 µL 10× buffer, 1.6 µL 10 mM ATP (if not in 10×), 1 µL T4 RNA ligase I (10 U), 0.5 µL RNase OUT and RNase--free water to 16 µL total reaction volume.
4. Add reaction mix to tubes and incubate at 37°C for 1 hr.
5. Heat--inactivate enzyme at 65°C for 15 min. Return tubes to ice (samples may be stored at –80°C at this point).

3. First strand cDNA synthesis

cDNA synthesis will now be performed using an oligo(dT)₂₀ primer, which will bind to RNA with an intact poly(A) tail. This allows for detection of an internal control mRNA species for normalization of data later.

1. Thaw ligation reaction, oligo(dT)₂₀ primer, dNTP mix, 10× RT buffer, MgCl₂, and DTT on ice.
2. For a 20 µL total volume reaction, prepare a master mix of: 1µL 50 µM oligo(dT)₂₀ primer and 1uL 10 mM dNTP mix per reaction. Aliquot 2 µL master mix into 0.2 mL PCR tubes and add 8 µL ligation reaction (500 ng RNA). Denature secondary structure by heating at 65°C for 5 min and snap chilling on ice.
3. Prepare a 2× reaction master mix on ice, containing the following per reaction: 2 µL 10× RT buffer, 4 µL 25 mM MgCl₂, 2 µL 0.1 M DTT, 1 µL SuperScript III reverse transcriptase and 1 µL RNase OUT. Add 10 µL 2× reaction master mix to each tube.
4. Incubate at 50°C 50 min. Heat--inactivate the enzyme at 70°C for 15 min, place tubes on ice.
5. Add 1µL RNase H to each reaction, mix gently and centrifuge briefly to collect. Incubate at 37°C for 20 min.
6. Place tubes on ice (samples may be stored at –20°C at this point).

4. Column purification of cDNA (*see* Note 12)

Qiagen QIAquick PCR purification kit columns were chosen as they demonstrated the greatest recovery of cDNA (9).

1. Mix cDNA reaction with 5 volumes (100 μ L) of buffer PB. Load onto column and, using a tabletop centrifuge, spin at 12,500 $\times g$ for 1 min. Discard the flow through and return columns to collection tubes.
2. Add 750 μ L buffer PE to each column. Spin at 12,500 $\times g$ for 1 min, discard flow through and return columns to tubes.
3. Remove residual ethanol by spinning at 12,500 $\times g$ for 2 min. Discard collection tubes and place columns in new 1.7 mL elution tubes.
4. Add 52 μ L of elution buffer (2 μ L will be lost to the column) directly to column and incubate at room temperature for 5 min.
5. Elute by centrifuging at 12,500 $\times g$ for 2 min. Current concentration of cDNA is approximately 10 ng/ μ L. Keep tubes on ice (samples may be stored at -20°C at this point).

5. MBACE qPCR

1. Thaw purified cDNA, forward and reverse primers, beacons, and qPCR reagent on ice; keep qPCR reagent and beacons under foil to limit light exposure.
2. For a 20 μ L total reaction volume, prepare a master mix as follows: 10 μ L 2 $\times$ PerfeCTa qPCR FastMix, 1 μ L 10 μ M forward primer, 1 μ L 10 μ M reverse primer, 1 μ L 5 μ M beacon, and 5 μ L RNase--free water per reaction.
3. Aliquot 18 μ L master mix per well, including wells for a no template control and a positive control, such as a standard from curves generated earlier.
4. Add 2 μ L cDNA (~20 ng) per well and apply cover to plate. Run experimental samples in triplicate. Agitate plate to mix and centrifuge to collect.
5. Using Eco software, choose "other" detection chemistry. Set the thermal profile: 95 $^{\circ}\text{C}$ 3 min $\rightarrow$ [95 $^{\circ}\text{C}$ 10 sec $\rightarrow$ 61 $^{\circ}\text{C}$ 30 sec* $\rightarrow$ 72 $^{\circ}\text{C}$ 15 sec] $\times$ 40 cycles (* denotes fluorescence detection). Remember to set the fluorescent detection for the annealing step as this is when the beacons will fluoresce.
6. Once the run is completed, set the threshold and base line to "Auto". Export the data for analysis.
7. Set a separate qPCR reaction for an internal control mRNA, such as β -actin or GAPDH, using SYBR green chemistry. Run each sample in triplicate for the control mRNA. Again, set the Eco software to automatically determine baseline and threshold. Export the data for analysis.

6. Analyzing qPCR Data

¹²cDNA column purification was necessary in our experiments to yield proper detection amplification curves with the molecular beacons.

1. Absolute quantification

1. Absolute quantification calculates the input amount of the target by relating the qRT--PCR signal to a standard curve of known quantity. The quantity of the gene of interest is *interpolated* along the linear regression line generated by the standard curve. The range of standards must be broad enough to generate a curve containing amounts relevant to the experiment -- quantity may NOT be extrapolated from the standard curve, as the relationship may no longer be linear outside of the standard curve range.
2. The equation for the linear regression line will allow for absolute quantification of unknowns. The equation will be given in the $y = mx + b$ format -- where y is the C_t , x is the quantity, m is the slope, and b is the y -intercept. The unknown C_t values substitute in for y and the equation is solved for x to determine the quantity.
3. Absolute quantification assumes the qPCR reactions for each target have *equal amplification efficiencies*. If efficiencies are not equal then relative quantification should be used. Additionally, absolute quantification may not be effective when small changes in mRNA are observed, thus relative quantification may prove more fruitful.

2. Relative quantification

1. Relative quantification reports the fold change between the target transcript of one sample versus another. Changes in fold expression across treatments or groups may prove more useful in some studies. An internal reference transcript, a housekeeping gene, is to be used to account for experimental variation. A proper internal reference must not be affected by the treatment; in our study we used β -actin. If a housekeeping gene is not the desired normalization control then an exogenous RNA, such as luciferase, may be spiked into the cell lysate prior to RNA extraction to act as a calibrator.
2. If the amplification efficiencies of the target and reference gene are *similar* then the Livak method is used for calculations (10). In this method of calculation the relative expression ratio, R , is given as

$$R = 2^{-\Delta\Delta C_t}$$

To determine the value of $\Delta\Delta C_t$, ΔC_t values are first calculated for both the target and reference mRNA using

$$\Delta C_t = C_{t_{sample}} - C_{t_{control}}$$

where, control refers to the housekeeping gene. This step is also called normalization of the data as the reference control

may differ due to experimental variations. Calculate the standard deviation for ΔCt using the equation

$$s = \sqrt{s_1^2 + s_2^2}$$

where s_1 and s_2 represent the standard deviations of the Ct values. The reference sample needs to be chosen, to which the other samples will be compared -- untreated control or wildtype is a good choice for the reference sample. Next, $\Delta\Delta Ct$ is calculated by the equation

$$\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{ref}$$

The resulting values are substituted into the relative expression ratio equation. Note that the reference sample will always have a fold expression equal to 1. The standard deviation for $\Delta\Delta Ct$ is the same as the standard deviation of the ΔCt_{sample} value. In our experiments, β -actin acted as the control and the wildtype cell line acted as the reference (see Fig. 3).

3. If the amplification efficiencies *differ*, then the Pfaffl efficiency--corrected calculation model based on multiple samples is used (11). This model states the relative expression ratio, R , is calculated by the equation

$$R = \frac{(E_{ref})^{Ct_{sample}}}{(E_{tar})^{Ct_{sample}}} \div \frac{(E_{ref})^{Ct_{control}}}{(E_{tar})^{Ct_{control}}}$$

Control refers to the housekeeping gene, or a calibrator. E refers to the efficiency of amplification of a standard curve for those particular qPCR reaction conditions; the slope of the linear regression line determines the amplification efficiency as:

$$E = 10^{(-1/slope)}$$

E_{ref} refers to the qPCR efficiency of the reference sample. Likewise, E_{ref} refers to the qPCR efficiency of the target sample. $Ct_{control}$ refers to the Ct value of the internal normalization control mRNA, the housekeeping gene or calibrator.

Acknowledgments

We wish to thank members of the Schoenberg lab for their helpful comments in developing this methodology. This work was supported by the National Institute of General Medical Science of the National Institutes of Health under award number GM079707.

References

1. Lasham A, et al. A rapid and sensitive method to detect siRNA-mediated mRNA cleavage in vivo using 5' RACE and a molecular beacon probe. *Nucleic Acids Res.* 2010; 38:e19. [PubMed: 19942683]
2. Schoenberg DR, Maquat LE. Regulation of cytoplasmic mRNA decay. *Nat Rev Genet.* 2012; 13:246–259. [PubMed: 22392217]
3. Stevens A, et al. Beta-Globin mRNA decay in erythroid cells: UG site-preferred endonucleolytic cleavage that is augmented by a premature termination codon. *Proc Natl Acad Sci USA.* 2002; 99:12741–12746. [PubMed: 12242335]
4. Lim S-K, et al. Nonsense codons in human beta-globin mRNA result in the production of mRNA degradation products. *Molecular and Cellular Biology.* 1992; 12:1149–1161. [PubMed: 1545796]
5. Vet, JA.; Marras, SA. Design and optimization of molecular beacon real-time polymerase chain reaction assays. In: Herdewijn, P., editor. *Methods in Molecular Biology: Oligonucleotide Synthesis.* New Jersey: Humana Press; 2005. p. 273-290.
6. Youseff BH, Dougherty JA, Rappleye CA. Reverse genetics through random mutagenesis in *Histoplasma capsulatum*. *BMC Microbiol.* 2009; 9:236. [PubMed: 19919692]
7. Bio-Rad Laboratories. Hallmarks of an optimized qPCR assay. 2012
8. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 2001; 29:e45. [PubMed: 11328886]
9. Molas ML, Kiss JZ. The effect of column purification on cDNA indirect labelling for microarrays. *Plant Methods.* 2007; 3:9. 2007. [PubMed: 17597522]
10. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} Method. *Methods.* 2001; 25:402–408. [PubMed: 11846609]
11. Pfaffl, SL. Relative Quantification. In: Dorak, MT., editor. *Real-time PCR (Advanced Methods).* New York: Taylor & Francis Group; 2006. p. 63-82.

Fig. 1. 5'-RLM-RACE to identify 5' ends of full-length β-globin and its decay products

Cytoplasmic RNA was treated with CIAP to remove the 5' monophosphates from uncapped RNAs. This is then treated with TAP to convert capped RNAs to RNAs with 5' monophosphate ends for ligation to the 3'-hydroxyl of an RNA linker. Gene-specific cDNA synthesis produces cDNAs of all β-globin mRNA species. The first strand PCR amplifies the 515 bp full-length RNA linker-β-globin cDNA and any decay products with a ligated 5' linker. The second round of PCR amplifies the 312 bp full-length RNA linker-β-globin cDNA and 5'-truncated decay products.

Fig. 2. MBRACE schematic

Cytoplasmic RNA is treated with TAP to convert capped 5' ends to 5' monophosphates. Next, the RNA linker is added to the RNA via ligation of its 3' hydroxyl to the 5' monophosphates of the RNA. Oligo(dt)--specific cDNA synthesis creates templates only from RNAs with an intact poly(A) tail. The MBRACE assays uses beacons to detect the junction between the RNA linker and (1) full--length β --globin and (2) a decay product by fluorescing upon hybridization. SYBR green qPCR may be used as an alternative detection method by designing the forward primer to be specific to the linker--mRNA junction sequence.

Sample	full-length β -globin Average Ct	β -actin Average Ct	Δ Ct full-length - β -actin	$\Delta\Delta$ Ct Δ Ct(sample) - Δ Ct(WT)	Fold expression relative to WT $2^{-\Delta\Delta$ Ct}
WT β -globin	23.79 $\pm$ 0.21	18.60 $\pm$ 0.22	5.19 $\pm$ 0.30	0.00 $\pm$ 0.30	1.00 (0.81-1.23)
PTC β -globin	26.32 $\pm$ 0.21	19.04 $\pm$ 0.21	7.27 $\pm$ 0.30	2.08 $\pm$ 0.30	0.24 (0.19-0.29)

Sample	β -globin decay product Average Ct	β -actin Average Ct	Δ Ct decay product - β -actin	$\Delta\Delta$ Ct Δ Ct(sample) - Δ Ct(WT)	Fold expression relative to WT $2^{-\Delta\Delta$ Ct}
WT β -globin	26.09 $\pm$ 0.13	18.60 $\pm$ 0.22	7.49 $\pm$ 0.26	0.00 $\pm$ 0.26	1.00 (0.84-1.20)
PTC β -globin	22.26 $\pm$ 0.07	19.04 $\pm$ 0.21	3.21 $\pm$ 0.22	-4.28 $\pm$ 0.22	19.38 (16.68-22.63)

Fig. 3. Effect of PTC on β -globin expression

Data analysis of MBACE for WT and PTC--containing β -globin in MEL cells. Values and standard deviations are shown for Ct, Δ Ct, and $\Delta\Delta$ Ct. Value and range shown for $R = 2^{-\Delta\Delta$ Ct}. The presence of a PTC significantly ($p < 0.01$) reduces full-length β -globin and significantly ($p < 0.01$) stabilizes the decay product.