Published in final edited form as:

Methods Mol Biol. 2013 ; 980: 157–173. doi:10.1007/978-1-62703-287-2_8.

Quantitative Real-Time PCR Expression Analysis of Peripheral Blood Mononuclear Cells in Pancreatic Cancer Patients

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Abstract

The ability of peripheral blood mononuclear cells (PBMCs) to act as a surrogate window into the presence and physiologic effects of pancreatic cancer is becoming increasingly apparent. In this chapter, we describe the techniques for isolation, lysis, RNA extraction, cDNA synthesis, and Q-RT PCR analysis of PBMCs as well as reasonable alternatives and the advantages and disadvantages of each. We further discuss the noteworthy considerations necessary for successful isolation and conversion of the high-quality PBMC RNA required to acquire interpretable and reproducible results for PBMC genetic expression analysis.

Keywords

Peripheral blood mononuclear cells (PBMCs); Pancreatic cancer; Blood; Cancer immunity; Genetic expression

1. Introduction

Peripheral blood mononuclear cells (PBMCs), consisting of circulating monocytes, T-cells, and B-cells, have been proving to be of increasing importance in cancer, both physiologically and as a potential diagnostic mechanism (1, 2). As we gain further understanding into the recognition of cancer by the immune system in early disease as well as the immuno-altering effects of cancer immune evasion, it is becoming more and more clear that the genetic profiling of this easily accessible cell population may be able to act as a surrogate window into the cancer disease state.

There is no cancer in which the utilization of this potential is more needed than in pancreatic cancer (PC), a disease in which the overall 5-year survival rate is a dismal 5%, owing mostly to the lack of specific symptoms, resulting in consistently late diagnoses (3). Importantly, preliminary analysis of the specificity of PBMC genetic profiling indicates little overlap in expression alterations across multiple disease states, and even multiple cancers. Consequently, emphasis placed on the study of PBMCs to improve the understanding of their genetic profiles, and how to interpret them in the context of PC, is increasing. Excitement for this field of research is further enhanced as it becomes clear that PBMC expression profiling as a surrogate marker for diagnosis is not reliant on significant tumor burden, as is the case with other potential diagnostic markers derived directly from the tumor or tumor microenvironment. It may be recognizable either as soon as immune cells recognize the cancer or as soon as immune evasion begins.

The process of PBMC isolation and expression analysis is not difficult, though it must be conducted in a timely fashion. Overall, it consists of five steps prior to expression analysis: (1) obtain whole blood, (2) isolate PBMCs, (3) PBMC lysis, (4) RNA extraction, and (5) RNA to cDNA conversion. Steps (1)–(3) can be performed in approximately 90 min and must be completed on the day the blood sample is drawn (started within 2 h of venopuncture

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¹Throughout the process, from sample collection to cell lysis (Steps 3.1–3.4.1), it is imperative to move quickly and methodically, as RNA can denature easily. This will greatly affect your results. In total, these procedures, from dilution of whole blood with saline to PBMC lysis, should take no more than 90 min. These restraints make it necessary to use the time occupied by sample centrifugation to prepare tubes and reagents for subsequent steps.

²Choice of anticoagulant in blood collection tubes is important for optimal PBMC expression analysis. While multiple variations of vacutainer tubes containing various anticoagulants are available and clinically used, it is strongly recommended that tubes containing heparin *not* be used. Heparin for vacutainer tubes is often isolated from bacterial cultures and may risk contamination with LPS. LPS contamination can result in a strong transcriptional and translational reaction from PBMCs, which will significantly confound any results of a PBMC study.

³From a typical patient, 7 mL of blood will yield a total PBMC number of $8\text{--}10 \times 10^6$. Consequently, if multiple analysis types requiring high RNA yield or PBMC subpopulation sorting are desired, it is recommended that the total volume of blood obtained from each patient be scaled up to 14–21 mL to ensure ample cell number for the desired study.

⁴An alternative strategy for PBMC isolation, omitting the use of a Ficoll density gradient, is also available. This strategy consists of centrifuging whole blood using a swing-bucket rotor for 10 min at 1,800 RCF is equivalent to G-force to pellet the cells. Plasma is then removed and the pellet is resuspended in 50 mL of Red Cell Lysis Buffer and incubated in the dark for 30 min. Cells are then re-pelleted (400 RCF for 15 min), the supernatant aspirated, and pellets are assessed for residual RBC contamination as described in Subheading 3.3, step 1, previously. If necessary, RBC lysis is repeated. Otherwise, the pellet is then resuspended in 1 mL PBS, transferred to a 1.7 mL microcentrifuge tube, and centrifuged to wash. The sample is then lysed and RNA is purified as described previously. Though this strategy is simpler and less time intensive than the use of a Ficoll gradient, it carries significant risk of granulocyte contamination.

⁵Making a choice between utilization of the Ambion *mirVana* and the Qiagen Qiashtredder/RNeasy kit(s) for RNA extraction may prove to be an important one depending on your study design and the RNA yield expected. In our experience, *mirVana* provides more efficient RNA isolation and should be utilized for studies in which few cells ($<6 \times 10^6$) per patient/sample are expected. In contrast, the protocols from the Qiashtredder and RNeasy kits are easier to follow and the kits are less expensive by comparison. Consequently, if ample PBMCs are expected for your endpoints based on study design and the volume of blood collected, the Qiagen kit combination works well. Kit choice, however, should be made prior to initiation of the study and the same kit should be used for all study samples.

⁶If the OD_{260/280} ratio is too low, it indicates protein contamination. If too high, it indicates contamination by a UV absorber in the OD₂₆₀ range, such as an organic solvent like phenol or chloroform. Both chloroform and phenol are used in the Ambion kit. In the event of either a high or low OD_{260/280} ratio, the RNA extraction procedure should be repeated prior to cDNA synthesis, omitting the homogenization step if using the Qiagen kit.

⁷The choice between Oligo dT or random hexamers for cDNA conversion can be based upon the methods of expression analysis that will subsequently be conducted in your study. Oligo dT functions by binding to the 3' end of the RNA and acting as a primer for the reverse transcriptase. The reverse transcriptases commonly used for cDNA conversion often only amplify efficiently for the first kb after the primer. Consequently, cDNA conversion using Oligo dT will only amplify the first 1,000 bp on the 3' end of the RNA, which is of particular concern for larger genes. This method of cDNA synthesis is sufficient if PBMC expression analysis is to be accomplished by quantitative real-time PCR or microarray using primers/oligonucleotide sequences based upon the 3' end of analyzed genes. The use of random hexamers rectifies this restriction in analysis because they can bind throughout the mRNA sequence and thus allow for the entire mRNA, even for larger genes, to be represented in the converted cDNA pool. As such, random hexamers allow for greater freedom in primer design and microarray platform choice to future analyses.

⁸In the event that division of the PBMC population pool into individual subpopulations (T-cells, B-cell, and monocytes) prior to lysis is desired, it is strongly recommended that this be done through negative exclusion procedures such as those available from Miltenyi Biotech. These methods act to bind to all cells except for the ones desired and hold them in a column or other extraction platform, allowing the desired purified cells to flow through unaltered. Positive exclusion, in contrast, separates subpopulations by binding the specific surface receptors of the desired cells and holding them in the extraction platform, thus allowing the undesired cells to flow through. Through the use of a solvent, the desired cells can then be eluted. This method risks activating the cells of interest, resulting in alteration of their genetic expression and thereby confounding any analysis that can be subsequently obtained.

⁹Deciding between SYBR Green and TaqMan for Q-RT PCR expression analysis is often made as a balancing act between specificity and economy. SYBR Green acts by binding into the minor groove of double-stranded DNA and fluorescing. Consequently, SYBR Green has the significant potential for fluorescing in the context of primer dimers and off-target primer binding, as well as in the event of contaminating double-stranded DNA in the sample cDNA. TaqMan-based reactions paired with hydrolysis probes avoid these issues as fluorescence can only occur in the event that both primers bind to DNA with the probe binding between them. This makes it theoretically impossible for primer dimers to affect the results and significantly reduces the likelihood that off-target binding will produce fluorescence. Hence, TaqMan-based results can be more easily interpreted without worry or inclusion of additional steps to verify their validity. However, TaqMan-based assays are inherently more cumbersome to perform as the probes are highly light sensitive and cost more than ten times as much as the same analysis performed by SYBR Green. TaqMan-based Q-RT PCR also lends itself toward the potential for multiplexing of assays, though this often requires significant optimization.

¹⁰If resources are of particular concern, PBMC expression can also be analyzed semiquantitatively using standard lab reverse-transcriptase PCR protocol. Though nonquantitative and suboptimal, these methods will often provide ample data to allow for determination of significant alterations in the level of transcription. Additionally, these methods can be used to judge the likelihood of success of a quantitative PCR assay before significant resources are invested into it, allowing for the most economical utilization of resources. In the event that these methods are used, we recommend that PBMC isolation, RNA extraction, and cDNA synthesis continue to be performed as described in this chapter.

often beneficial to wait until multiple samples have been obtained and processed prior to RNA isolation and cDNA conversion for the sake of efficiency as the time required for multiple samples is often the same as that for one.

In this chapter, we discuss the reagents necessary and the proper protocols to follow to ensure the quality RNA and cDNA necessary for interpretable and reproducible PBMC gene expression analyses. Reagents can be substituted with equivalents from other companies, though the concentrations and volumes listed are optimized for the reagents suggested in this protocol.

2. Materials

2.1. Blood Collection

1. Citrate vacutainer tubes (7 mL).

2.2. PBMC Isolation

1. 50 mL disposable polypropylene centrifuge tubes.
2. Ficoll, density 1.077 (i.e., Accuprep, Accurate Chemical and Scientific Corporation, Cat: AN5511).
3. Swing-bucket centrifuge at room temperature.
4. Saline Solution (0.9% wt/vol): Into a 1 L bottle, place 9 g of NaCl and fill up to 1 L using distilled water. Stir until dissolved using a stir bar and stir plate and store at room temperature.
5. 15 mL disposable polypropylene or polystyrene centrifuge tubes.
6. 1.7 mL microcentrifuge tubes.

2.3. Red Blood Cell Lysis

1. Red Cell Lysis Buffer: In a 50 mL disposable polypropylene centrifuge tube, add 4.5 mL of 10× stock Red Cell Lysis Buffer and fill up to 45 mL using distilled water. Store at 4°C.
2. 10× stock Red Cell Lysis Buffer (i.e., BD Pharm Lyse, Cat: 555899).

2.4. Washing Cells

1. 1× Sterile Filtered Phosphate-Buffered Saline (DPBS), diluted from 10× stock (see Subheading 2.4, item 2).
2. 10× PBS: In a 1 L bottle, add 80 g NaCl, 2 g KCl, 14.4 g Na₂HPO₄, and 2.4 g KH₂PO₄. Fill the bottle up to 1 L with distilled water and mix via stir bar and stir plate until dissolved. Using diluted HCl and NaOH, adjust the pH to 7.4 and store at room temperature.

2.5. PBMC Lysis and mRNA Isolation

1. To ensure high-quality and timely mRNA extraction, we recommend utilization of an RNA extraction kit, such as the Ambion *mirVana* Isolation Kit (Cat: AM1561) or the combination of the Qiagen QIAshredder cell lysis kit (Cat: 79656) and the Qiagen RNeasy RNA extraction kit (Cat: 74106).
2. If using the Qiagen QIAshredder and RNeasy combination, be sure to add β-mercaptoethanol to buffer RLT prior to cell lysis.

3. 1.7 mL microcentrifuge tubes.

2.6. Storage of Extracted mRNA

1. -70°C freezer.

2.7. The cDNA Synthesis

1. Spectrophotometer.
2. PCR tubes.
3. Thermocycler (preferably with heated lid).
4. PCR Grade Nucleotide Mix (dATP, dCTP, dGTP, and dTTP, 10 nM each, Roche, Cat: 12111424).
5. Oligo d(T)₁₆ (50 μM , Applied Biosystems, Cat: N15501) or Random Hexamers (50 μM , Applied Biosystems, Cat: N18917).
6. 0.1 M DTT (Invitrogen, Cat: Y00147).
7. 5 $\times$ First Strand PCR Buffer (Invitrogen, Cat: Y002321).
8. Reverse Transcriptase (SuperScript II, 200 U/mL, Invitrogen, Cat: 100004925).

2.8. Storage of cDNA

1. -20°C freezer.

2.9. Quantitative Real-Time PCR

1. Gene-specific primers.
2. Gene-specific hydrolysis probes (if using the TaqMan-based approach).
3. PCR master mix (LightCycler 480 SYBR Green I Master Mix, Roche, Cat: 12706520 or TaqMan Universal PCR Master Mix, Applied Biosciences, Cat: 4304437).
4. Nuclease-free water.
5. Real-time PCR thermocycler.

3. Methods

Carry out all steps at room temperature unless otherwise noted. Prior to PBMC cell lysis, all steps in which the sample is open to the environment should be carried out inside a designated biologic hood to prevent potential pathogen exposure.

3.1. Sample Collection

1. Blood collection: Blood should be collected in 7 mL vacutainer tube(s) containing citrate to prevent coagulation and immediately stored at 4°C until processed as described below. To ensure accurate and reproducible results from PBMC expression analysis, whole blood should be processed within 2 h of collection.

3.2. PBMC Isolation

1. Dilution of whole blood: Prior to isolation of the PBMCs via Ficoll density gradient, blood should be transferred to a 50 mL polypropylene centrifuge tube and diluted 1:1 with room-temperature saline (0.9% wt/vol).

2. Layering diluted sample over Ficoll: In a separate 50 mL polypropylene tube, add a volume of room-temperature Ficoll equal to that of the undiluted blood from Subheading 3.1 via pipette. Be sure to add the Ficoll directly to the bottom of the tube so that none gets on the sides. Next, you will layer the diluted blood on top of the Ficoll via pipette by gently pressing the tip of the pipette perpendicular to the side of the tube and slowly releasing the blood so as to allow it to dribble down the side of the tube without disturbing the Ficoll's surface tension. Once finished, the tube should appear to have two distinct layers with the blood above the clear Ficoll.
3. Centrifugation: To separate the red blood cells (RBCs), PBMCs, and plasma, gently place the tube from Subheading 3.2, step 2, into a room-temperature centrifuge with a swing-bucket rotor, being sure not to disturb the layers. Centrifuge the sample at 800 RCF for 20 min. This method results in aggregation of the RBCs with only slight aggregation of lymphocytes, leading the RBCs to be denser than the Ficoll, while the PBMCs maintain a density less than that of the Ficoll. Consequently, after centrifugation a layering of the sample as shown in Fig. 1 should occur, with the PBMCs forming a distinct layer between the plasma and the Ficoll.
4. Collection of PBMCs: With a pipette, depress the plunger prior to insertion of the pipette tip into the sample so as not to disturb the layers. Slowly lower the pipette tip to approximately 0.5 mm above the PBMC layer and suck up the PBMCs in a slow and fluid motion. Remove the pipette and dispense the PBMCs into a new 15 mL polypropylene or polystyrene centrifuge tube. Repeat this process until the entire PBMC layer has been transferred to the 15 mL tube. The 50 mL tube containing the remaining layers can be disposed of in a biohazard container.
5. Pelleting the PBMCs: To pellet the PBMCs, dilute them 2:1 with 0.9% (wt/vol) room-temperature saline and centrifuge in the swing-bucket rotor used in Subheading 3.2, step 3, for 10 min at 250 RCF.
6. Cell washing and transfer to a 1.7 mL microcentrifuge tube: Once complete, remove the supernatant and resuspend the pellet in 1 mL of PBS. Transfer the total volume to a clean 1.7 mL microcentrifuge tube and centrifuge at 500 RCF for 10 min. After centrifugation, remove the supernatant and proceed to Subheading 3.3.

3.3. Red Blood Cell Lysis

1. Analyzing for necessity of RBC lysis: Despite completion of the Accuprep PBMC isolation, RBCs can still contaminate the resulting PBMC pellet. The most common reasons for this are using cold Accuprep, resulting in an overlap of the RBC and lymphocyte layers, or disturbing the RBC layer during removal of the PBMCs. Should RBC contamination occur, the contaminating RBCs must be lysed prior to further PBMC processing lest you risk their presence altering the results of your expression assays. RBC contamination can be easily assessed by examining the color of the pellet, with a red pellet signaling the need to undergo an RBC lysis step. If the pellet appears to be white, grey, or tan in color it is safe to proceed to Subheading 3.4.
2. RBC lysis: In the event that RBC lysis is necessary, resuspend the PBMC pellet in 1 mL of Red Cell Lysis Buffer and place in the dark at room temperature for 20 min. After lysis is complete, centrifuge the cells at 500 RCF for 5 min, remove the supernatant, resuspend the pellet in 1 mL PBS, and repeat the centrifugation to wash the cells prior to proceeding to Subheading 3.4.

3.4. PBMC Lysis and mRNA Isolation

1. **PBMC lysis:** Cell lysis should take place immediately after PBMC isolation and PBS washing are complete. Once lysis has been performed, RNA can either be extracted immediately or lysates can be stored at -70°C for RNA extraction at a later date. To ensure rapid and complete lysis with maintenance of RNA integrity, we recommend the use of the cell lysis buffer included in either the Ambion *mirVana* or the Qiagen RNeasy Isolation Kits. The choice of kit will depend on user preference, with each having particular pros and cons described below in the Subheading 4. If using the Qiagen kit, be sure to add β -mercaptoethanol (β -ME) to buffer RLT (10 μL β -ME for every 1 mL RLT) prior to cell lysis to inhibit RNase activity. Of note, it can be expected that 7 mL of human blood will yield eight to ten million PBMCs when isolated as described above, necessitating the use of 600 μL of lysis buffer per sample for both the Qiagen and Ambion kits. To ensure efficiency of cell lysis when using the Qiagen kit, we recommend homogenizing the lysates by passing them through the columns included in the QIAshredder (Qiagen) kit. Alternatively, samples lysed with the Qiagen RLT buffer can be homogenized by passing them through a 10-gauge needle attached to an RNase-free syringe at least five times. Regardless of the kit choice, we have found that the instructions included with each kit yield complete cell lysis and should be followed.
2. **RNA extraction:** RNA extraction should be carried out using the same kit utilized for cell lysis (*mirVana* or RNeasy). As with cell lysis, we have found that the instructions included with each kit yield high-quality RNA and should be followed directly. Final elution of RNA should be done using 35 μL nuclease-free water if using the Qiagen RNeasy kit or 100 μL nuclease-free water (heated to 95°C) if using the Ambion *mirVana* kit.
3. **Storage of extracted RNA:** Extracted RNA can be stored at -70°C for several years. Multiple freeze–thaw cycles should be avoided to prevent significant RNA breakdown.

3.5. cDNA Synthesis

1. **Spectrophotometric quantification of extracted RNA concentration:** Prior to cDNA synthesis, RNA extracted from Subheading 3.5 must be quantified and the RNA purity ($\text{OD}_{260}/\text{OD}_{280}$) must be noted. So as not to use an exorbitant amount of purified RNA for quantification, creating a 1:100 dilution with your sample in nuclease-free water prior to quantification and purity analysis is recommended. Diluted RNA should be spectrophotometrically measured for its absorbance at 260 and 280 nm. An $\text{OD}_{260}/\text{OD}_{280}$ ratio between 1.7 and 2.0 indicates that RNA is of reasonable purity. Any ratio above or below this indicates the presence of varying contaminants and RNA should be further purified prior to cDNA synthesis (see Subheading 4). If RNA is deemed to be of reasonable purity, its concentration can be calculated using the formula

$$\text{RNA concentration (ug/mL)} = \frac{\text{OD}_{260} \times \text{dilution factor} \times 40 \text{ ug/mL}}{1,000}.$$

2. **Conversion of mRNA to cDNA:** cDNA conversion consists of two steps, comprising denaturation followed by amplification. Between these two steps, additional reagents must be added to the sample PCR tubes. When outside of the thermocycler, all portions of the cDNA conversion procedure should be conducted

on wet ice. If the utilized thermocycler is so equipped, lid heating/cooling should also be activated to ensure even temperature regulation throughout the sample.

- i. Denaturation step: Pipette 2 μg of extracted RNA into a PCR tube and add nuclease-free water to make a total volume of 10 μL . To this mixture, add 2 μL of the dNTP mix and 1 μL of Oligo dT. Mix the sample well by pipetting up and down eight to ten times. In place of Oligo dT, an equal volume of random hexamers can also be used (see Subheading 4). Place the sample into the thermocycler and denature the sample at 70°C for 10 min. After this denaturation, allow the thermocycler to cool to 4°C and hold at this temperature until the samples are removed for the addition of reagents necessary for the amplification step.
- ii. Amplification step: Remove samples from the 4°C thermocycler and place on ice. Add 5 μL of 5 $\times$ PCR buffer, 2 μL of DTT, and 1 μL of Reverse Transcriptase (SuperScript II or equivalent). Mix the sample thoroughly by pipetting up and down eight to ten times. Replace the samples in the thermocycler and allow cDNA amplification to occur at 42°C for 60 min followed by inactivation of the DNA polymerase enzymes at 72°C for 15 min. After inactivation is complete, allow the thermocycler to cool to 4°C and hold at this temperature until the samples can be removed.

Note: If RNA concentration is insufficient to allow for 2 μg of RNA to be used for cDNA synthesis (i.e., if concentration is less than 200 $\mu\text{g}/\text{mL}$), this procedure can be followed using 1 μg RNA with the appropriate dilution following the amplification step.

3. Dilution of cDNA for analysis: After completion of the amplification step in Subheading 3.6, remove the samples from the thermocycler, place them on ice, and dilute them to a proper working concentration for future expression analysis. If 2 μg of RNA were used for cDNA synthesis, dilute the sample with 50 μL nuclease-free water. If 1 μg of RNA were used, dilute the sample with 15 μL of nuclease-free water. Though it is not recommended to attempt cDNA conversion with less than 1 μg RNA, if such conversion is attempted do not dilute the sample after the amplification step. Once diluted, cDNA is ready for use in quantitative real-time PCR and/or microarray expression analyses.
4. Storage of cDNA for future use: After proper dilution, cDNA can be stored at -20°C and used for analysis for up to 1 year.

3.6. Quantitative Real-Time PCR

1. Primer and probe design.
 - i. Primer design: If possible, primers should be designed for the 3' untranslated region (UTR), particularly if Oligo dT was utilized in the cDNA synthesis step. Ideally, amplicon size should be between 70 and 150 bp. Probes should be designed for an optimal GC content of 50%, with an acceptable range between 35 and 65%. Primer lengths should be between 18 and 30 bp and have a melting temperature between 60 and 64°C. Once designed, all primers should be analyzed through a BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to ensure specificity with your gene of interest. Primers should be diluted to a stock concentration of 200 mM with nuclease-free water and stored at -20°C. Stock primer solutions should be further diluted with nuclease-free water to working concentrations prior to preparation of Q-RT PCR reactions. For TaqMan-

based reactions, a working concentration of 20 mM is recommended, while a concentration of 2 mM is recommended for SYBR Green-based reactions.

- ii. Hydrolysis probe design: If using SYBR Green, no probes are necessary for expression analysis. For TaqMan-based assays, probes should be designed to have a length between 20 and 28 bp with a GC content of 35–65% (optimal: 50%) and a melting temperature of 66–70°C (optimal: 68°C). Upon design, ensure that the chosen fluorophore and quencher have overlapping respective emissions and absorption spectra, such as Cy3 (emission wavelength: 570 nm) and Black Hole Quencher-II (maximum absorption wavelength: 580 nm). As with primers, all probes should be analyzed through a BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to ensure sequence specificity with the gene of interest. The binding sites of the probes must always be between their respective primer-binding sites and should be verified during BLAST analysis. Probes should be diluted to a stock concentration of 50 mM with nuclease-free water and stored at –20°C away from light. Stock probe solutions should be further diluted with nuclease-free water to a working concentration of 5 mM prior to use in Q-RT PCR reactions. All probe dilutions and use in reaction mixtures should be done in the dark as hydrolysis probe fluorophores are light sensitive.

2. Preparing reaction for Q-RT PCR analysis.

- i. SYBR Green method: The PCR reaction mix using SYBR Green dye for analysis consists of a total of five components: (1) cDNA template, (2) SYBR Green I Master Mix, (3) forward primer, (4) reverse primer, and (5) nuclease-free water. As detection by SYBR Green is by nature nonspecific, assay-by-assay optimization of final primer concentrations may be necessary to ensure reproducible results. However, utilization of final primer concentrations of 50 nM is often able to provide high-quality and consistent results. While total reaction volumes can vary, we have found that a reaction volume of 10 µL is often sufficient for obtaining quality data. Based on a working primer concentration of 20 mM, the following volumes of each component should be used per reaction (Table 1):
- ii. TaqMan method: The PCR reaction mix using TaqMan-based chemistry consists of six components: (1) cDNA template, (2) TaqMan Universal PCR Master Mix, (3) forward primer, (4) reverse primer, (5) hydrolysis probe, and (6) nuclease-free water. Final concentrations of the forward and reverse primers should be 900 nM, while the probe should be at a 250 nM final concentration in the reaction solution. While total reaction volumes can vary, a reaction volume of 20 µL provides consistently reproducible results without a waste of reagents or sample by our hands. Thus, based on a working primer concentration of 20 µM and a working probe concentration of 5 µM, the following volumes of each component should be used per reaction (Table 2):

To allow for establishment and elimination of outliers as well as to allow for interpretation of reproducibility, reactions should always be done in replicates of 2–4.

3. PCR reaction conditions.

- i. SYBR Green method: Reaction conditions for PCR analysis using SYBR Green-based chemistry require a total of three steps: (1) preincubation (melting), (2) amplification, and (3) cooling. As melt curve analysis can be useful in determining the production of primer dimers or off-target primer binding when using SYBR Green, it is recommended that a melt curve be placed in the PCR reaction between the amplification and cooling steps. We have found that the following conditions work well for SYBR Green analysis (Table 3):
- ii. TaqMan method: TaqMan-based reaction conditions also require a total of three steps: (1) preincubation (melting), (2) amplification, and (3) cooling. Melt curve analysis cannot be performed when hydrolysis probes are used and thus cannot be included in the reaction. The following conditions work well for TaqMan-based analysis (Table 4):
- iii. Concentration analysis: Concentration analysis should be performed using the maximum second derivative for each amplification curve as the respective C_p . Concentrations can be calculated using either a serially diluted standard curve or using the 2^{-ddC_p} method. Appropriate reference genes should be utilized in all analyses and may vary depending on study design. Generally, β -actin works well for this purpose.

4. Analysis for result quality.

To the fullest extent that is possible, Q-RT PCR results should be analyzed and reported as per the MIQE criteria (4). In general, however, quality of results can be assessed through the utilization of three tools:

- i. Cycle number: Amplification should occur between cycles 10 and 35. Samples for which amplification occurs at a cycle number lower than 10 have cDNA which is too concentrated and should be rerun with appropriate further cDNA dilution. Any sample that is found to amplify later than 35 cycles into the amplification phase of the PCR reaction had too little transcript for reproducible interpretation and should be rerun using an increased volume of cDNA with a corresponding decrease in nuclease-free water volume so as to maintain the total reaction volume.
- ii. Melt curve analysis: Melt curve analysis is a highly useful tool for assessing the potential of off-target binding or primer-dimer formation as confounders in Q-RT PCR results using SYBR Green-based chemistry. As both off-target transcripts and primer dimers will have melting temperatures that are nonidentical to that of your target transcript, the formation of either of these will result in multiple peaks of fluorescence being visualized by the melting curve. As such, the presence of a single peak indicates that amplification occurred for your target sequence alone and signifies that the data is safe for interpretation (Fig. 2).
- iii. Reproducibility: Data reproducibility is highly important to assess as it can significantly alter both interpretation and future impact of Q-RT PCR results. Two methods are most acceptable for analysis of reproducibility of Q-RT PCR data: standard deviation (SD) and coefficient of variation (Cv) (4). Both intra-run and inter-run variability should be assessed for reproducibility, thus necessitating that the sample be run in replicates within a single run and each run should be repeated two or more times. In either case, the cycle number and uncalculated concentration should be used in this analysis unless you are using SD for intra-run variation, in

which case, a calculated concentration may be used. It is our opinion that that Cv often yields the most interpretable results for variability assessment and consequently is the method we recommend. Cv can be calculated by

$$Cv (\%) = \frac{\text{Standard deviation} \times 100}{\text{mean}}.$$

Data producing intra-run Cvs of <10% and inter-run Cvs of <15% can be considered to have high reproducibility.

Acknowledgments

The authors on this work are supported, in part, by grants from the National Institutes of Health (EDRN UO1 CA111294, RO1 CA131944, and SPORE P50 CA127297).

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Fig. 1.

Layering of blood as visualized following ficoll density gradient centrifugation. Upon centrifugation of diluted whole blood layered over ficoll, as described, separation of various components occurs based on density. In total, four layers should be seen, with the aggregated erythrocytes forming a dark bottom layer, followed by the ficoll, then a thin, white layer of PBMCs, and finally the top layer of diluted plasma. Often, the PBMC layer is difficult to visualize but can be assumed to be present at the interface of the plasma and ficoll layers. In this case, PBMCs should be isolated by placing the tip of the pipette approximately 1 mm above the plasma/ficoll interface. Care should be taken not to disrupt the erythrocyte layer upon removal of the PBMCs or further RBC lysis will be necessary.

Fig. 2. Melt curve analyses of Q-RT PCR results. Specificity of Q-RT PCR primers used with SYBR-Green-based chemistry can be easily accessed via melt curve analysis. As primer dimers and off-target primer associations will have different melting temperatures than the target amplified sequence, lack of primer specificity can be visualized as multiple peaks on melt curve. **(a)** A melt curve analysis showing a single peak, indicating primer specificity and valid interpretability of results. **(b)** Melt curve analysis showing two peaks, signifying off-target or primer-dimer associations of the utilized primers, preventing valid expression level quantification derived from the Q-RT PCR analysis. In the case of this, new primers should be designed for the Q-RT PCR analysis with greater care taken for sequence specificity using BLAST software.

Table 1

Reaction contents for SYBR Green-based Q-RT PCR reactions

Reagent	Stock concentration	Volume (μ L)	Final concentration (nM)
SYBR Green I Master Mix	2 $\times$	5.0	
Forward primer	2 μ M	0.25	50
Reverse primer	2 μ M	0.25	50
cDNA	N/A	1.0	
Nuclease-free water	N/A	3.5	
Total		10	

Table 2

Reaction contents for TaqMan-based Q-RT PCR reactions

Reagent	Stock concentration	Volume (μ L)	Final concentration (nM)
TaqMan Universal PCR Master Mix	2×	10.0	
Forward primer	20 μ M	0.9	900
Reverse primer	20 μ M	0.9	900
Hydrolysis probe	5 μ M	1.0	250
cDNA	N/A	4.0	
Nuclease-free water	N/A	3.2	
Total		20	

Table 3

Reaction conditions for SYBR Green-based Q-RT PCR analysis

Name	Number of cycles	Step	Target temperature (°C)	Hold time	Ramp rate (°C/s)	Notes
Preincubation	1	1	95	5 min	4.4	
Amplification	45	1	95	10 s	4.4	
		2	60	10 s	2.2	
		3	72	10 s	4.4	Fluorescence acquisition
Melt curve	1	1	95	5 s	4.4	
		2	65	1 min	2.2	
Cool	1	3	97	N/A	0.11	Continuous fluorescence acquisition (5°C)
		1	40	30 s	2.2	

Table 4

Reaction conditions for TaqMan-based Q-RT PCR analysis

Name	Number of cycles	Step	Target temperature (°C)	Hold time	Ramp rate (°C/s)	Notes
Preincubation	1	1	95	10 min	4.4	
Amplification	45	1	95	10 s	4.4	
		2	60	30 s	2.2	Fluorescence acquisition
		3	72	1 s	4.4	
Cool	1	1	40	30 s	2.2	