High-Throughput Real-Time Quantitative Reverse Transcription PCR

This unit describes the use of real-time quantitative PCR (QPCR) for high-throughput analysis of RNA expression. The topics covered include: the standard curve method (see Basic Protocol 1); production and quantification of RNA standards (see Support Protocol 1); an efficiency-corrected ΔCt (cycle time, also called cycle threshold or crossing point) method (see Basic Protocol 2); the comparative cycle time, or $\Delta\Delta$Ct method (see Alternate Protocol); and design and validation of QPCR primers and probes for both SYBR Green– and TaqMan-based assays (see Support Protocol 2). While the unit describes the use of the Applied Biosystems 7900HT (high-throughput, 384-well) instrument, the protocols may be utilized for any real-time PCR instrument. The high-throughput design allows analysis of the levels of transcripts from a number of genes of interest (GOIs) at one time by using the appropriate primer set for each gene. (Within this unit, the term GOI will refer to the actual gene of interest as well as its RNA product or cDNA copy.)

Absolute quantification means that the absolute copy number of the GOI is measured. Relative quantification means that a quantitative difference in copy number between two samples, experimental and control, is measured by normalizing both samples to an endogenous reference.

Because of the simplicity of the mathematical application, the relative standard curve method (Basic Protocol 1) is the most basic and straightforward QPCR assay described in the unit. In this method, standard curves are constructed for all of the GOIs from which RNA expression is being measured, and linear regression analysis is applied to interpolate unknown sample values. The standard curve assay may be performed even if the PCR amplification efficiencies of the primer sets (as determined by the template dilution assay in Support Protocol 2) are not equal, since correction for unequal efficiencies is intrinsic to the linear regression formula. One drawback of the standard curve method is that standard curves must be run for each of the primer sets on an assay plate. This results in less space on the plate for the unknown samples, and requires the use of additional reagents. This use of resources is particularly excessive when the PCR amplification efficiencies of the primer sets have been determined to be essentially 100% and relative fold-change is the preferred outcome of the measurements. In such cases, the $\Delta\Delta$Ct method (see Alternate Protocol) should be employed instead. Another limitation is that unless the levels of all of the GOIs in the cDNAs used to construct the standard curves are known, the relative concentration of one GOI cannot be compared to that of another GOI. If comparison between the levels of different GOIs (without the knowledge of the relative level of the transcripts in the standards) is desired, the efficiency-corrected ΔCt method (see Basic Protocol 2) should be applied. Alternatively, absolute standards can be generated (Support Protocol 1).

The efficiency-corrected ΔCt method builds upon the relative standard curve method by incorporating PCR efficiency (E) into the quantity calculations. The standard curve slopes are used to calculate PCR efficiency according to the relationship $E = 10^{(-1/\text{slope})}$. The efficiency has a maximum value of 2 for perfect doubling of the PCR template (see Basic Protocol 2 for an in-depth explanation of E). Note that in this method the standard curve is used only to determine slope and not to interpolate the RNA values of the unknown samples. The efficiency correction is then applied to determine the relative

amount of RNA using the measured Ct values for the test samples, and is particularly important when comparing the levels of different RNAs whose standard-curve slopes deviate from each other by greater than ±0.1. This application is useful for comparing the expression profiles of many different RNAs, e.g., those belonging to gene families or related biological pathways. The sample-space and reagent-use limitations mentioned for the standard curve method also apply here.

The $\Delta\Delta$Ct method is the method of choice when the desired output is "fold-change," because standards are not necessary, thus saving both reagents and space on the re-action plate. However, this method requires that the amplification efficiencies of the primer/probe sets be 100%. If the amplification efficiencies are suboptimal, but the primers generate a single product as determined by melting curve analysis (Support Protocol 2), Basic Protocol 1 should be used to determine fold-changes.

Chapter 4 describes the isolation of RNA from several sources and *UNIT 15.5* details the traditional procedure for reverse transcription of RNA into complementary DNA (cDNA). For QPCR, it is recommended that total RNA be resuspended in diethylpyrocarbonate (DEPC)–treated water or an equivalent nuclease-free buffer that does not contain EDTA. The RNA should be treated with DNase and then reverse transcribed using 0.08 μg/μl (final concentration) random hexamer or nonamer primers. Purified messenger RNA—i.e., poly(A)$^+$ RNA (*UNIT 4.5*) or RNA that has been reverse transcribed using oligo(dT) or gene-specific reverse primers—may also be successfully used in the assay. See Commentary for more detailed information.

Following the assay, the resulting raw data are analyzed using second-party software, usually Microsoft Excel or equivalent. The data analyses are dependent on the type of assay performed, and are outlined in detail as part of each protocol.

NOTE: General precautions for working with RNA are described in *UNIT 4.1* and other Chapter 4 units, and general precautions necessary for PCR are described in *UNIT 15.1*. In particular, the use of molecular-biology-grade water, RNase/DNase/nucleic acid–free tubes, aerosol-barrier pipet tips, and dedicated pipettors of all types (i.e., pipettors used only for RNA or PCR applications, which are kept out of areas used for plasmid or genomic DNA work) is strongly recommended for all steps in this unit. If dedicated pipettors are not available, the available pipettors should be thoroughly cleaned to remove nucleases and potential contaminants such as plasmid or genomic DNA. In addition, the use of gloves is required, since even a small amount of any contaminant can greatly impact the results of the assay.

STRATEGIC PLANNING

To begin performing a QPCR assay, design and validation of the appropriate primers and probes must first be completed (refer to Support Protocol 2). Second, the appropriate assay is selected based on the goal of the experiment and the desired data output (refer to Basic Protocols 1 and 2, and the Alternate Protocol, for guidelines used in making this determination). The chosen assay is then performed and the data are analyzed.

STANDARD CURVE METHOD FOR RELATIVE QUANTIFICATION

The relative standard curve method is used for determining the level of a gene of interest (GOI) relative to an endogenous reference RNA, and for calculating relative fold-changes of a GOI between experimental samples. The assay is useful for determining an "expression profile" of a single GOI within a group of samples. A dilution series of standard cDNA samples is constructed for the GOI and reference gene, and linear regression analysis is applied. The formulas resulting from the standard curves are used to interpolate the GOI and reference-gene quantities in the unknown samples. An endogenous

reference gene, often a housekeeping gene, is used as a control to normalize the amount of input template for each sample (see Commentary for parameters used in choosing the appropriate reference gene). The data are expressed as "normalized RNA level" in arbitrary units. The type of nucleic acid standard chosen depends upon the nature of the unknown samples and is discussed in the Commentary.

The relative standard curve assay does not require that the amount of the GOI or reference RNA in the standards be known. It depends on the linear regression formula produced by plotting the Ct versus the log nanogram (log ng) of input standard total RNA. It should be noted that cDNA concentrations are not typically determined following reverse transcription of the RNA. Here, quantity refers to total RNA input prior to reverse transcription. The standard curve–plotting function is available in most instrument software. If it is not, graphing software may be used instead. Since the input ng values refer to the input amount of total RNA, and not to a known amount of target molecules, the numbers generated are simply arbitrary and may not be compared with the numbers calculated for a different GOI. If comparison of the relative RNA levels between different RNA targets is desired, refer to the efficiency-corrected ΔCt method (Basic Protocol 2) or absolute quantification (Support Protocol 1). The standard curve method should be used instead of the $\Delta\Delta$Ct method (Alternate Protocol) to find fold-changes between samples when the amplification efficiencies of the primer sets are not 100% as determined by the template dilution assay (Support Protocol 2). If absolute levels of transcript are desired, synthetic RNA standards may be prepared through incorporation of radionucleotides (see Support Protocol 1) and used in standard curve analysis.

Materials

20 ng/μl experimental cDNA samples (concentration based on RNA input for cDNA synthesis; see UNIT 15.5)

Dilution series of standard cDNAs (see recipe) or dilution series of [^{35}S]RNA standards (see Support Protocol 1)

No-template control sample (NTC; prepared at the same time as the cDNA samples using molecular-biology-grade water instead of RNA; see Commentary)

No-reverse-transcriptase control samples (–RT; prepared at the same time as the cDNA samples using molecular-biology-grade water instead of reverse transcriptase; see Commentary)

2× SYBR Green or TaqMan mix containing ROX (Applied Biosystems, Bio-Rad, Invitrogen, Sigma, or see recipe for 2× SYBR Green mix)

Primer mixes, 1.25 μM each forward and reverse primer (see recipe and Support Protocol 2), for each reference gene and GOI to be tested

5 μM TaqMan probe (for TaqMan protocol only; see recipe and Support Protocol 2)

Molecular-biology-grade water (nucleic acid and nuclease free)

8-tube PCR tube strips (optional, but recommended; can be of low quality since they will only be used for mixing reaction components; ISC Bioexpress)

96-well PCR tube racks (optional, but recommended; ISC Bioexpress)

Digital multichannel pipettor, 8- or 12-channel, 5- to 100-μl capacity (recommended)

Centrifuge with swinging-bucket rotor and microtiter plate carriers

384-well optical reaction plates (Applied Biosystems)

Optical adhesive covers (Applied Biosystems)

Real-time thermal cycler: e.g., Applied Biosystems 7900HT

Microsoft Excel or spreadsheet program with equivalent statistical features

Set up plates

1. Plan the plate arrangement according to the number of samples and primer sets to be assayed (reference gene plus GOIs). For each primer set, include the standards,

Figure 15.8.1 Typical plate setup for the standard curve and efficiency-corrected ΔCt assays. (**A**) Organization of PCR tube strips on a 96-well PCR rack when preparing for any QPCR assay. It is advisable to premix the cDNA templates with the primer master mixes in tube strips before putting them into the reaction plate. This practice decreases the variability between replicate wells. A typical cDNA/primer mix setup is shown in relation to the final 384-well reaction plate (**B**). The plate arrangement shown represents an experiment in which 20 samples taken from experimental animals will be assayed for levels of one endogenous control RNA and three GOIs. The samples are plated in triplicate for each of the RNAs assayed, and a standard curve is required for each of the RNAs to be measured (the row before the unknown samples contains the standard samples, whose amounts are shown in ng total RNA). Abbreviations: GOI, gene of interest; NTC, no-template control; Ref, endogenous control gene; –RT, no-reverse-transcriptase control.

the NTC, and the –RT control as part of the sample group. See Figure 15.8.1 for an example of a typical plate setup.

All steps may be performed at room temperature if a vendor-supplied SYBR Green or TaqMan mix is used. Most vendor-supplied 2× mixes are stable at room temperature for a number of hours.

If the instrument is equipped with a plate-loading robotic arm, several plates may be prepared at once and stacked into the robotic arm queue. Alternatively, plates may be made several days in advance and stored at 4°C until ready to run, without a decline in the performance of the PCR enzyme. Advance preparation of plates is not recommended if SYBR Green or TaqMan mixes are prepared in the laboratory, since the home-made mixes lack additives present in the commercial mixes that confer stability to the reaction components.

High-Throughput Real-Time Quantitative Reverse Transcription PCR

15.8.4

Table 15.8.1 Master Mixes for the Standard Curve and Efficiency-Corrected ΔCt Assays

Components	Final concentration	Volume per well	Volume for each cDNA/primer mix (per sample in triplicate + 1 extra)	Master mix (no. of samples + 8 standards[a] + 1 extra)
SYBR Green assay				
2× SYBR Green mix	1×	5 µl	20 µl	
1:1 primer mix (1.25 µM each)	150 nM each primer	1.2 µl	4.8 µl	
Template cDNA	10-25 ng[b]	1.25 µl	5 µl	—
H$_2$O	N/A	to 10 µl	to 40 µl	
Total volume		10 µl	40 µl	Aliquot 35 µl into each tube containing cDNA
TaqMan assay				
2× TaqMan mix	1×	5 µl	20 µl	
1:1 primer mix (1.25 µM each)	300 nM each primer	2.4 µl	9.6 µl	
5 µM TaqMan probe	250 nM	0.5 µl	2.0 µl	
Template cDNA	10-25 ng[b]	1.25 µl	5 µl	—
H$_2$O	N/A	to 10 µl	to 40 µl	
Total volume		10 µl	40 µl	Aliquot 35 µl into each tube containing cDNA

[a]For this assay, the no-template control (NTC) and no-reverse-transcriptase (–RT) control are included as part of the standard sample set.
[b]Recommended amount of template for detection of both high and low levels of GOIs. If necessary, significantly less template may be used (picograms). Note that the cDNA template quantity is based upon the amount of total RNA input into the reverse transcription reaction.

2. Prepare primer master mixes according to Table 15.8.1, but without template cDNA.

   This can be done in advance, and the tubes may be put on ice or stored at 4°C for a few hours before preparing the reaction plate.

   The authors always use the same concentrations of PCR components because this greatly increases the high-throughput nature of the assay. Keeping the conditions of the master mixes constant allows not only universal mix conditions but universal cycling conditions. If the initial primer set does not perform well, new primers are designed (also see Support Protocol 2).

3. Place the appropriate number of 8-tube PCR strips into a 96-well PCR tube rack (see Fig. 15.8.1A). For convenience, use a different color tube strip for each different primer master mix that has been prepared. Label the side of each strip with the letter of the row of the reaction plate into which the samples will be placed.

   Alternatively, cDNA/primer mixes may be made in 0.65-ml microcentrifuge tubes or 0.2- or 0.5-ml PCR tubes.

   The cDNA may be put directly into the optical reaction plates followed by the primer master mix. However, this is not recommended because it introduces a potential source of experimental error, since the assay replicates are not premixed, but pipetted individually. The end result may be lower assay precision.

4. Using a multichannel pipettor, put 5 µl of cDNA (experimental samples, standards, and controls) into the bottom of the appropriate tubes in the strips.

The Polymerase Chain Reaction

15.8.5

5. Being careful not to touch the cDNA inside the tubes, use a multichannel pipettor to place a 35-μl aliquot of the appropriate primer master mix into each tube.

6. Cover the entire rack of tube strips with Parafilm and gently vortex to mix. Gently tap or briefly centrifuge the PCR tube racks (2 to 3 min at 1700 × g, 4°C or room temperature, in a swinging-bucket rotor with microtiter plate carriers) to get contents to the bottoms of the tubes.

7. Using a multichannel pipettor, dispense 10 μl of each cDNA/primer mix into the appropriate three wells of the optical reaction plate to generate each sample in triplicate (as planned in step 1; see Fig. 15.8.1B).

   If the multichannel pipettor has 8- or 12-channel dispensing capability, the triplicates can be dispensed at the same time for different rows. Note that due to the spacing between rows of a 384-well reaction plate, every other row can be added at once (i.e., A, C, E, G, I, K, M, O, and then B, D, F, H, J, L, N, P), thus greatly minimizing the pipetting time.

8. Cover the plate with the optical adhesive cover and then briefly centrifuge the plate as above to get contents to the bottoms of the wells.

Perform real-time PCR

9a. *For real-time PCR:* Transfer the plate to the real-time thermal cycler and run real-time PCR using the following program (consult the instrument manual for specific instructions):

1 cycle:	10 min	95°C (activates the hot-start *Taq* DNA polymerase)
40 cycles:	15 sec	95°C (collect data throughout)
	1 min	60°C (collect data throughout).

9b. *For melting (dissociation) curve analysis (for use with SYBR Green only):* Add these steps following the 40 cycles of the thermal cycling program.

   15 sec   95°C
   15 sec   60°C (collect data)
   Increase from 60° to 95°C at a 2% temperature ramping rate (collect data)
   15 sec   95°C (collect data).

   See Support Protocol 2 for a description of the use of melting curve analyses.

Analyze data

10. Analyze and export raw data (see instrument manual for detailed instructions about document setup, baseline, and threshold settings).

   Some instrument software applications contain a standard curve plotting feature. If this function is not available, use Excel or another graphing program to plot Ct versus the log nanograms (ng) of input total RNA for each standard, and apply a best-fit line to generate the linear regression formula $y = mx + b$, where y is Ct of the unknown sample, m is the slope, x is the quantity of the unknown sample (in log ng), and b is the y intercept for both the reference gene and each GOI. Interpolate the unknown sample quantities using the resulting formulas.

   The transformation of the fluorescence signal into Ct data, as well as methods for baseline and threshold settings, vary by instrument. The specific instrument manual should be consulted.

   In analyzing the raw data, it is important to adjust the cycle threshold (Ct) of the amplification plot to within the geometric phase of amplification. This is critical for proper analysis because the geometric phase represents the point of the reaction at which Ct is quantitatively related to the amount of initial PCR template. Note that a Ct decrease of 1 unit represents a two-fold increase in initial PCR template.

It is also important that the coefficient of determination, or R^2 value, for the linear regression formula be 0.99. If the R^2 value is less than 0.99, this suggests that one or more points of the standard curve are deviating significantly from the best-fit line. In this case, the accuracy of the data obtained from the linear regression formula of this standard curve may be compromised. The R^2 value will never be above 0.99.

11. Import data into Microsoft Excel or equivalent spreadsheet program with statistical features.

12. For each of the three replicates of a sample, calculate the average quantity (avg) of target cDNA interpolated from the standard curve, the standard deviation of the average (stdev), and the coefficient of variation (CV) according to the formula CV = stdev/avg.

13. Remove any outlier points (>17% CV). After removing the outlier point, recalculate avg, stdev, and CV.

    Only one point per replicate may be removed.

    A 17% CV correlates with the maximum allowable standard deviation that can distinguish a two-fold change with 99% confidence when samples are assayed in triplicate wells for both the endogenous reference and the GOI. If a 95% confidence interval is acceptable, a 21.8% CV may be used as the threshold for removing outliers. On the other hand, the Q-test (a test for rejection of discordant data) may be used to determine outlier points. Refer to Shoemaker et al. (1974) for a more in-depth description of this test.

14. For each sample, normalize the GOI quantity to that of the reference gene for the sample according to the following equation. Use the recalculated values if outlier points were removed in step 13.

$$\text{normalized value} = \frac{\text{avg GOI quantity}}{\text{avg reference quantity}}$$

15. Calculate the standard deviation (SD) of the normalized value according to the equation:

$$SD = (\text{normalized value}) \times \sqrt{(\text{CV of reference})^2 + (\text{CV of GOI})^2}$$

16. Plot the resulting values as a bar graph of normalized value versus sample name or experimental treatment group, with the error bars equal to the SD.

17. If desired, calculate fold-changes between samples by choosing a calibrator sample (usually vehicle-treated or wild-type control) and dividing all of the normalized values from step 14 and the SD calculated in step 15 by the normalized value of this calibrator.

    The resulting values are then expressed as fold-changes relative to the calibrator sample, which should now be equal to 1.

EFFICIENCY-CORRECTED ΔCt METHOD

The efficiency-corrected ΔCt method is used for determining the relative amounts of different GOIs that are normalized to an endogenous reference RNA (e.g., 18S cyclophilin). It may also be used to determine fold-changes of a specific RNA between samples, but may be excessive if the desired output is only fold-change (see Basic Protocol 1 or Alternate Protocol). Data obtained from the efficiency-corrected ΔCt method are expressed as "normalized RNA level" in arbitrary units, and the calculated levels may be compared to those of other GOIs when the same threshold setting and assay chemistry are used (i.e., SYBR Green or TaqMan chemistry). It should be noted that an assumption is made that the reverse transcription efficiency is equal for all RNA transcripts in a single sample

BASIC PROTOCOL 2

The Polymerase Chain Reaction

15.8.7

and for the same transcript between samples. In some cases, this may not be true (see Pfaffl, 2004, for further discussion); therefore, it is recommended that the RNA extraction method remain the same for all samples, and that all samples under study be reverse transcribed at the same time with the same reaction buffer.

The basis of quantitative PCR lies in the principle that for every additional thermocycle, a two-fold increase of template-specific product occurs. Several factors affect whether a change in one cycle truly represents a two-fold growth in product, in other words, whether the reaction is 100% efficient. To assess the reaction's efficiency, linear regression analysis is applied to a standard cDNA dilution series, just as in Basic Protocol 1. The slope of the resulting standard curve is used as a measure of PCR efficiency (E) according to the equation $E = 10^{(-1/\text{slope})}$. Note that different GOIs may produce different E values.

A slope of -3.3 produces an E value of 2, indicating that a perfect doubling of the template has occurred. Calculated E values of less than 2 imply that the template has not been perfectly doubled. Template, primer, and probe quality and quantity, sample complexity, and pipet calibration and buffer conditions like $MgCl_2$, salt, additives, and deoxynucleotide concentrations, all contribute to this efficiency (Pfaffl, 2004; see UNITS 15.1 & 15.5 for further details on these parameters). Given optimal buffer conditions and adequate primer, probe, and sample qualities, slight fluctuations in efficiency may still be observed between primer sets run on the same plate or between assay plates, even if they have been assembled and run on the same day by the same user. Efficiency correction is a means to account for inter- or intra-assay variability that is attributable to the aforementioned parameters.

The materials and setup of the assay are the same as in the relative standard curve method (see Basic Protocol 1), except that the linear regression formula produced by plotting the Ct versus the log ng of input standard total RNA is used only to determine PCR efficiency. This computed efficiency is then used to calculate the RNA levels (in arbitrary units) of the GOI and the endogenous control genes. The GOI RNA level in each sample is then expressed as a ratio relative to the endogenous control RNA level in that sample. Because the data are dependent upon Ct values and not a standard curve, the resulting values may be compared to those of another RNA.

1. Set up and run assay as described in Basic Protocol 1 (steps 1 to 9a). Refer to Figure 15.8.1 for an example of a typical plate setup and to Table 15.8.1 for master mix components.

2. Analyze and export raw data (see Basic Protocol 1, step 10), and then import into Microsoft Excel or equivalent spreadsheet program.

   The threshold values for all RNAs measured (including the endogenous reference) must be the same. It is important to determine a suitable threshold within the geometric phase of the amplification plots for all RNA transcripts to be compared.

3. Calculate PCR efficiency, $E = 10^{(-1/\text{slope})}$, for the endogenous control RNA and each GOI from the slopes of their corresponding standard curves.

4. Calculate the quantity of the endogenous control RNA and each GOI from their Ct values according to the formula quantity $= E^{-\text{Ct}}$.

   When the efficiency is 100% (i.e., slope $= -3.3$ and $E = 2$), the equation becomes quantity $= 2^{-\text{Ct}}$. This serves as the basis for the calculation performed in $\Delta\Delta Ct$ method (Alternate Protocol).

5. For each of the three replicates of a sample, calculate the average quantity (avg), the standard deviation of the average (stdev), and the coefficient of variation (CV), where CV = stdev/avg.

6. Remove outliers, normalize the GOI, calculate the SD, and plot the results (see Basic Protocol 1, steps 13 to 17).

COMPARATIVE OR ΔΔCt METHOD

The comparative Ct or ΔΔCt method is used for measuring the fold-changes in expression of a particular RNA transcript between experimental samples. Typically, this assay is used when investigating gene-expression differences between wild-type and knockout or transgenic animals, or between vehicle-control and drug-treated samples. The results are then expressed as "fold-changes" relative to a calibrator, such as an untreated or wild-type sample. The ΔΔCt method is only applicable when the primer sets for both the GOI and the endogenous reference gene have been shown to give perfect standard curve slopes (slopes $= -3.3 \pm 0.1$ with $R^2 = 0.99$) as assayed in Support Protocol 2.

1. Set up assay as described in Basic Protocol 1 (steps 1 to 9a), except refer to Figure 15.8.2 for an example of a typical plate setup and to Table 15.8.2 for master mix components.

   Standard cDNA samples are not needed in this method.

2. Analyze and export raw data (see Basic Protocol 1, step 10) and then import into Microsoft Excel or equivalent spreadsheet program.

3. For each of the three replicates of a sample, calculate the average (avg) cycle time (Ct) and then calculate the standard deviation (stdev).

4. Remove any outlier wells from the averaged Ct values (>0.3 stdev).

   Only one point per replicate may be removed.

   %CV may not be used, due to the logarithmic nature of both the Ct avg and Ct stdev. Instead, stdev must be used, where a stdev of 0.3 correlates with the maximum allowable standard deviation that can distinguish a two-fold change with 99% confidence; 0.4 stdev may be used for a 95% confidence interval.
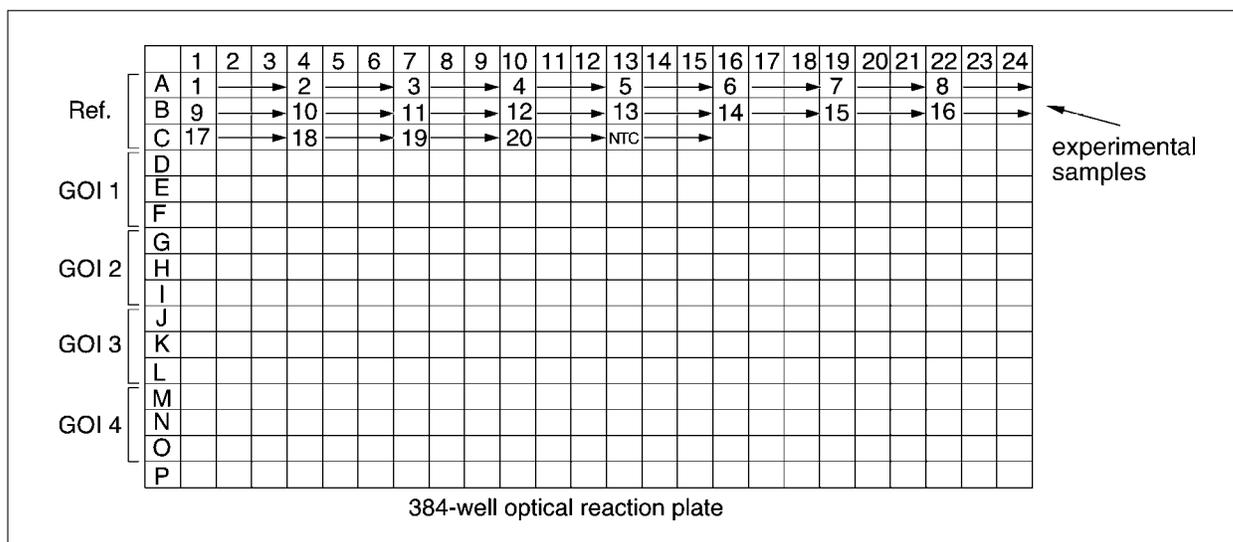
Figure 15.8.2 Typical plate setup for the ΔΔCt method. The plate arrangement shown represents an experiment in which 20 samples taken from experimental animals will be assayed for one endogenous control gene and four GOIs. The samples are plated in triplicate for each of the RNAs assayed. Abbreviations: GOI, gene of interest; NTC, no-template control; Ref, endogenous control gene.

Table 15.8.2 Master Mixes for the $\Delta\Delta$Ct Assay

Components	Final concentration	Volume per well	Volume for each cDNA/primer mix (per sample in triplicate + 1 extra)	Master mix (no. of samples + 1 NTC + 1 extra)
SYBR Green assay				
2× SYBR Green mix	1×	5 µl	20 µl	
1:1 primer mix (1.25 µM each)	150 nM each primer	1.2 µl	4.8 µl	
Template cDNA	10-25 ng[a]	1.25 µl	5 µl	—
H₂O	N/A	to 10 µl	to 40 µl	
Total volume		10 µl	40 µl	Aliquot 35 µl into each tube containing cDNA
TaqMan assay				
2× TaqMan mix	1×	5 µl	20 µl	
1:1 primer mix (1.25 µM each)	300 nM each primer	2.4 µl	9.6 µl	
5 µM TaqMan probe	250 nM	0.5 µl	2.0 µl	
Template cDNA	10-25 ng[a]	1.25 µl	5 µl	—
H₂O	N/A	to 10 µl	to 40 µl	
Total volume		10 µl	40 µl	Aliquot 35 µl into each tube containing cDNA

[a]Recommended amount of template for detection of both high and low levels of GOIs. If necessary, significantly less template may be used (picograms). Note that the cDNA template quantity is based upon the amount of total RNA input into the reverse transcription reaction.

5. For each sample, normalize the GOI Ct values to those of the reference gene for the same sample according to the equation:

$$\Delta Ct = avgCt_{GOI} - avgCt_{ref}$$

Calculate the standard deviation of ΔCt ($stdev_{\Delta Ct}$) as:

$$stdev_{\Delta Ct} = \sqrt{(stdev\ of\ reference)^2 + (stdev\ of\ GOI)^2}$$

6. Choose a calibrator.

   This will be the sample, tissue, gene, or control group to which the others will be compared.

7. Find the $\Delta\Delta Ct$, or calibrated value, for each sample, according to the equation:

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

   The stdev$_{\Delta\Delta Ct}$ will be the same as stdev$_{\Delta Ct}$, since the calibrator is arbitrarily set to be a constant.

8. Find the fold-change for each sample relative to the calibrator according to the equation:

$$fold\text{-}change = 2^{(-\Delta\Delta Ct)}$$

   For the sample that is chosen as the calibrator, the $\Delta\Delta Ct = 0$ and therefore the fold-change $= 2^{(-\Delta\Delta Ct)} = 1$.

9. Plot the resulting fold-changes on a bar graph of fold-change versus sample name or experimental treatment group. Determine the measure of experimental error as:

$$\text{SD}_{\text{fold-change}} = (\ln 2)(\text{stdev}_{\Delta\Delta Ct})(2^{(-\Delta\Delta Ct)})$$

GENERATION OF RNA STANDARDS FOR ABSOLUTE QUANTIFICATION BY REVERSE TRANSCRIPTION PCR

Absolute quantification of RNA molecules in unknown samples by reverse transcription PCR (RT-PCR) requires knowledge of the copy number of specific RNA molecules. These can be subjected to reverse transcription and PCR in the same manner as the experimental samples, thus accounting for the reaction efficiencies of both procedures. This protocol describes the production and quantification of synthetic RNA standards for use in Basic Protocol 1 to determine absolute amounts, instead of relative levels, of a GOI. RNA standards are produced via in vitro transcription in the presence of trace amounts of an ^{35}S-labeled ribonucleoside triphosphate (rNTP), which permits accurate quantification of transcripts by measurement of ^{35}S incorporation. This protocol encompasses template construction, in vitro transcription, DNase treatment, monitoring the efficiency of the reactions by agarose gel electrophoresis, and determination of yield. To determine yield, synthesized RNA can be quantitatively precipitated and purified away from unincorporated nucleotides by application to filters followed by trichloroacetic acid washes. This protocol describes a batch precipitation/washing method. For an individual filtration method, see *UNIT 3.4*. The preparation and use of RNA standards in quantitative RT-PCR assays have been described by Kramer and Coen (1995).

Materials

> cDNA or DNA fragment containing target sequence
> Vector containing T7, T3, or SP6 RNA polymerase promoter
> Appropriate restriction enzyme (*UNIT 3.1*) for linearizing plasmid
> 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol
> 49:1 (v/v) chloroform/isoamyl alcohol
> 3 M sodium acetate (*APPENDIX 2*)
> 100% ethanol
> Nuclease-free water
> 0.5 μg/ml sheared salmon sperm DNA
> 1 M Tris·Cl, pH 7.5 (*APPENDIX 2*)
> 1 M $MgCl_2$ (*APPENDIX 2*)
> 1 M DTT (*APPENDIX 2*)
> 500 μM 4NTP mix: 500 μM each ATP, CTP, GTP, and UTP
> 600 Ci/mmol (10 mCi/ml) [α-^{35}S]CTP or UTP
> Bovine serum albumin (BSA)
> Spermidine (for SP6 only)
> T7, T3, or SP6 RNA polymerase (*UNIT 3.8*)
> 1 U/μl RNase-free DNase I (e.g., Promega)
> RNeasy Mini Kit (Qiagen) or equivalent
> 10% trichloroacetic acid (TCA; see recipe), ice cold
> 100% methanol, ice cold
> Universal scintillation cocktail (preferably biodegradable, e.g., Ecoscint A, National Diagnostics)
>
> 1.5-ml screw-cap microcentrifuge tubes
> DE81 paper (Whatman)
> Filter paper

Glass fiber filters (Whatman GF/C 24-mm discs)
Transparent plastic wrap
Forceps
Heat lamp (optional)
250-ml glass or metal beaker
Liquid scintillation counter and vials

Additional reagents and equipment for subcloning (*UNIT 3.16*), plasmid minipreps (*UNIT 1.6*), digestion with restriction endonucleases (*UNIT 3.1*), agarose gel electrophoresis (*UNIT 2.5A*), purification of DNA (*UNIT 2.1A*), phage RNA polymerase reactions (*UNITS 3.4 & 3.8*), agarose-formaldehyde or glyoxal gel electrophoresis (*UNIT 4.9*), and drying and imaging of gels (*APPENDIX 3A*)

CAUTION: To minimize the risk of radioactive contamination, use screw-cap microcentrifuge tubes with cap gaskets and filtered pipet tips for all manipulations of radioactive solutions. Wear gloves and dispose of all ^{35}S-contaminated material properly. See *APPENDIX 1F* for more details on handling radioactivity.

CAUTION: Phenol, chloroform, and trichloroacetic acid are hazardous (see *APPENDIX 1H*).

NOTE: For all procedures involving RNA, use reagents and solutions that are free of contaminating RNases, DNases, and nucleic acids, and follow other guidelines for handling RNA (*UNIT 4.1*).

Construct and prepare template for in vitro transcription

1. Construct a transcription plasmid by subcloning (*UNIT 3.16*), placing the sequence contained in the RNA of interest downstream of the promoter for one of the three bacteriophage RNA polymerases (T7, T3, or SP6). Isolate the plasmid using a miniprep (*UNIT 1.6*), which will provide sufficient amounts of plasmid DNA for this protocol.

   There are numerous commercially available vectors that contain phage polymerase promoters on one or both sides of a multiple cloning site (see Table 2.10.1). Additional considerations for the construction of this plasmid are discussed in Critical Parameters and Troubleshooting.

2. Linearize 10 μg of plasmid with a restriction enzyme (*UNIT 3.1*) that will generate a template for run-off transcription. Run 5% of the linearization reaction volume on an agarose gel (*UNIT 2.5A*) alongside a control sample of uncut plasmid to confirm that the digestion is complete and yields the expected product size(s).

   Long transcription products from incompletely digested plasmids can lead to inaccuracies in quantification and extraneous products from RT-PCR.

3. Purify the linearized template from the reaction mixture using two or three organic extractions with 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol (*UNIT 2.1A*), one extraction with 49:1 (v/v) chloroform/isoamyl alcohol, and finally ethanol precipitation (*UNIT 2.1A*) in the presence of sodium acetate. Resuspend DNA in nuclease-free water.

   Acceptable results may also be obtained by using an enzymatic reaction clean-up kit (such as the QIAquick PCR Purification Kit, Qiagen) or by performing gel extraction (UNIT 2.6).

   If RNase contamination is a problem, the sample can be treated with 50 to 100 μg/ml proteinase K to destroy the RNases prior to purification.

   The concentration of linearized template does not need to be determined. The authors assume that most of the 10 μg used in the digest are recovered.

Synthesize RNA

4. For each transcription reaction, prepare four 1.5-ml screw-cap microcentrifuge tubes each containing 24 μl of 0.5 μg/ml sheared salmon sperm DNA: one pair to measure

input ^{35}S in duplicate and one pair to measure ^{35}S incorporation in duplicate. Also prepare one single tube as a control with no other additions. Set these tubes aside on ice.

The salmon sperm DNA will act as a carrier for the synthesized RNA during the acid precipitation step.

5. In another 1.5-ml screw-cap microcentrifuge tube, set up a 100-μl transcription reaction (minus the enzyme) as follows:

    40 mM Tris·Cl, pH 7.5
    10 mM MgCl$_2$
    5 mM DTT
    500 μM 4NTP mix
    1 μl 10 mCi/ml (600 Ci/mmol) [α-^{35}S]CTP
    2 to 5 μg DNA template (from step 3)
    5 μg BSA
    1 mM spermidine (for SP6 only).

    If the buffer contains spermidine, reaction components other than the enzyme should be at room temperature to avoid precipitation of the DNA.

    If multiple reactions are being performed, a master mix containing everything but the template should be prepared.

    In vitro transcription kits can be used for convenience, but they will increase the cost.

6. Mix reaction components well. Transfer 1 μl of the reaction to each of two tubes of salmon sperm DNA from step 4 (input ^{35}S samples). Set aside on ice.

7. Add 25 to 50 U RNA polymerase (T7, T3, or SP6) to the reaction mix (reaction volume should now be 98 μl). Incubate 1 to 2 hr at 37°C.

8. Transfer 5 μl of the reaction mixture to a separate tube and store on ice.

    This is the pre-DNase sample that will be assessed by gel electrophoresis.

9. Add 5 μl (5 U) RNase-free DNase I or similar enzyme and incubate at 37°C for 30 min.

    Transcription buffers typically contain sufficient magnesium ion concentrations (≥ 6 mM) to support DNase activity.

10. Purify the synthetic RNA using the RNeasy Mini Kit or similar product. Perform the final elution twice to maximize yield.

    RNA can also be purified using organic extraction and isopropanol precipitation (UNIT 4.1); however, this removes less of the unincorporated rNTPs and is especially not recommended for difficult templates where yields are low (e.g., GC-rich sequences).

11. Elute or resuspend RNA in 93 μl nuclease-free water.

    This volume is equal to the volume after step 8, thus making the gel samples from steps 8 and 13 directly comparable.

12. Transfer 1 μl of the purified RNA to each of two tubes of salmon sperm DNA from step 4 (incorporated ^{35}S samples). Set aside on ice.

13. Transfer 5 μl of the purified RNA to a separate tube and store on ice.

    This is the final RNA sample that will be assessed by gel electrophoresis.

14. Store the remainder of the RNA at −70°C in multiple aliquots to avoid repeated freeze/thaw cycles.

The Polymerase Chain Reaction

15.8.13

The size of the aliquots will depend on their intended use. It is convenient to prepare each aliquot with enough RNA for two or three samples.

Use low-retention/nonstick microcentrifuge tubes, if possible, because these prevent the adherence of RNA to the tube walls over time.

Confirm in vitro transcription product by gel electrophoresis

15. Electrophorese the pre-DNase (step 8) and final (step 13) samples through a formaldehyde or glyoxal agarose gel (see *UNIT 4.9*) of an appropriate concentration for the length of the RNA. Visualize synthesized products by staining with ethidium bromide (*UNIT 4.9*).

    Make the gel as thin as possible to hasten drying later. Verify that any plasmid DNA present in the pre-DNase sample (usually observed as a low-mobility band) is absent from the final sample. The successful removal of DNA should also be confirmed at the RT-PCR stage with a control lacking reverse transcriptase.

16. To dry the gel, first trim the gel to contain only relevant lanes. Stack one sheet of Whatman DE81 paper on top of five sheets of filter paper, all cut several inches larger than the gel. Center the gel on top of the DE81 paper and cover stack with a sheet of plastic wrap. Dry as for a polyacrylamide DNA gel (*APPENDIX 3A*), increasing the drying time as needed for the thickness of the agarose gel.

    The DE81 paper should retain most of the unincorporated [α-^{35}S]rNTP while the blotting paper will absorb excess moisture (and radioactivity).

    CAUTION: *Radioactive contamination of the gel dryer may occur. Be sure to conduct required surveys and decontamination as directed by the institutional radiation safety committee (also see APPENDIX 3F).*

17. Visualize labeled transcripts by autoradiography or by exposing to a phosphor screen (*APPENDIX 3A*).

    Confirm that there is only one major radiolabeled product of the expected size (see Commentary). Also confirm that the amount of synthetic RNA represented by the pre-DNase sample was efficiently recovered in the final sample. If the gel is not run too far, it will be possible to visualize the amount of unincorporated [α-^{35}S]rNTP removed by the purification procedure.

Quantify ^{35}S incorporation and RNA yield

18. Label glass fiber filters by cutting varied notches along the perimeter, and lay them out on a sheet of plastic wrap with forceps.

    Prepare four filters for the single tube of control salmon DNA (step 4), four filters for each of the tubes of input ^{35}S (step 6), and two filters for each of the tubes of incorporated ^{35}S (step 12). For the control salmon DNA and the input ^{35}S samples, two of each set of four replicate filters will be subjected to TCA precipitation and washing, while the other two will be put directly into scintillation vials as "unwashed" samples. For the incorporated ^{35}S samples, the two replicate filters will be subjected to TCA precipitation and washing.

19. Mix the tubes of salmon sperm DNA and spot 5 μl of each mixture onto the corresponding two or four replicate filters. Allow the spotted samples to air dry or use a heat lamp.

20. Use forceps to place duplicate filters from the control salmon DNA and the input ^{35}S samples in individual scintillation vials and set aside as unwashed samples.

21. Place the remaining duplicate filters from the control salmon DNA and the input ^{35}S samples, along with the duplicate filters from the incorporated ^{35}S samples, into a 250-ml glass or metal beaker with 50 ml of 10% ice-cold TCA.

    This will precipitate the nucleic acids and wash away unincorporated rNTPs. Up to 18 filters can be washed with 50 ml; for more filters, scale up the volumes proportionally.

22. Swirl the beaker on ice for 10 min, then pour off the TCA.

    Continual swirling ensures that filters do not clump together.

    CAUTION: *The TCA and the methanol wash will contain unincorporated rNTPs and should be disposed of as hazardous radioactive waste.*

23. Repeat steps 21 and 22 twice more.

24. Add 50 ml cold methanol to the filters, swirl on ice for 5 min, and pour off methanol.

25. Use forceps to spread out the washed filters on a sheet of plastic wrap and allow to dry.

    A heat lamp can be used to hasten this process.

26. Use forceps to transfer these filters to individual scintillation vials.

27. Add 5 ml scintillation cocktail to the vials of washed and unwashed filters and measure counts per minute (cpm) in a liquid scintillation counter.

    The washed and unwashed control filters and the washed input ^{35}S filters should contain only background levels of radioactivity (<200 cpm), confirming that unincorporated nucleotides were efficiently removed.

28. Average the cpm from duplicate filters and subtract background counts:

    input cpm = (unwashed input ^{35}S filters) – (unwashed control filters)
    incorporated cpm = (washed incorporated ^{35}S filters) – (washed control filters)

29. Determine the fraction of $[\alpha\text{-}^{35}S]$CTP that was incorporated according to the following equation using volumes at time of sampling:

$$\text{incorporation} = \frac{(\text{incorporated cpm}) \times (\text{final RNA volume at step 12})}{(\text{input cpm}) \times (\text{initial reaction volume at step 6}) \times (\text{NTP purity})}$$

    This calculation assumes a uniform product length. The NTP purity term refers to the fraction of ^{35}S present in intact NTP molecules. This amount is typically 0.90 to 0.99 in fresh radiochemical preparations. See Commentary for more details.

    At least 30% of radiolabel should be incorporated for simple templates and at least 10% for difficult templates with a high degree of secondary structure.

30. Multiply this fraction by the total amount (nmol) of radiolabeled plus unlabeled CTP in the reaction to obtain the amount in nmol of cytidine incorporated into product RNA.

31. Divide by the fraction of cytidine residues in the transcript to calculate the total nmol of ribonucleotides incorporated into product RNA.

32. Multiply this value by 10^{-9} mol/nmol and by Avogadro's number (6.022×10^{23} molecules/mol) to obtain the total number of incorporated ribonucleotides.

33. Divide by the transcript length to determine the number of transcripts composing the purified RNA.

34. Make duplicate serial dilutions of one or more transcripts quantified in this manner and process them through the DNase treatments and reverse transcription in parallel with the experimental RNA samples.

    By using duplicate standards, one can ensure that the protocols used to prepare cDNA are quantitative and reproducible. Five or six serial 10-fold dilutions are usually suitable. Exact quantities depend upon the anticipated range of expression of the GOI in the experimental samples.

The Polymerase Chain Reaction

15.8.15

These standard RNAs must be processed in as similar a manner as possible to that of the experimental samples to avoid introducing discrepancies in the efficiency of reverse transcription and PCR amplification of RNA standards versus experimental RNA samples. For example, mock-infected tissue or cell homogenates could be added to RNA standards used for assays of mRNAs from infectious agents. Unrelated RNA such as yeast or E. coli tRNA may be used (at concentrations that mimic the total RNA content of experimental samples) in cases where preparations free of the target RNA species are not available. If multiple RNA species will be assayed in single experimental samples, the different synthetic RNAs may be combined as a single, serially diluted RNA standard.

35. Employ the resulting duplicate set of standard cDNA samples in the standard curve method (see Basic Protocol 1) as the dilution series of standard cDNAs to obtain absolute quantification of RNA species in unknown samples.

    Generate standard curve by plotting Ct against the logarithm of input RNA copy number for the RNA standards. Linear regression performed on these points yields an equation from which the copy number of RNA in an unknown sample can be calculated from its Ct.

DESIGN AND VALIDATION OF SYBR GREEN AND TaqMan PRIMER/PROBE SETS

Considering that QPCR relies on the quality and the fidelity of the primers and probes that are used, very strict parameters for their design and subsequent validation are required. A common misconception in performing QPCR assays is that if the primer works for traditional end-point PCR, it is suitable for QPCR. In some cases this is true. However, the primer set must be tested in a QPCR validation assay before it can be used for RNA expression analysis. In keeping with the high-throughput capacity of QPCR, the thermocycling conditions are kept constant for all assays: 10 min at 95°C activates the hot-start *Taq* polymerase, followed by 40 cycles of the two-step 95°C melting and 60°C annealing. An extension step in the thermocycler program is not required, since all of the PCR products are 50 to 150 bp, thus making the run last only 1.5 to 2 hr. The concentrations of PCR reagents such as *Taq* DNA polymerase, $MgCl_2$, other salts, and dNTPs remain constant within the same chemistry (i.e., SYBR Green or TaqMan), and these so-called universal cycling conditions make primer and probe sequences the only point of flexibility in performing the assays.

The design of primer/probe sets requires the availability of reliable sequence information that may be obtained from databases like NCBI's GenBank or Ensembl, or from data produced by direct sequencing. The assay does not tolerate base mismatches between primer and template, especially in the probe sequence, a feature that allows for the detection of single-nucleotide polymorphisms (SNPs). Several primer/probe design software packages are available either for purchase or online. Otherwise, the user may design the primer sets by directly examining the sequence and choosing primers with the correct characteristics, as outlined in this protocol. The probe is labeled at the 5′ end with a fluorescent reporter such as 6-FAM or VIC, and at the 3′ end with a fluorescent or nonfluorescent quencher. The user should consult with the vendor that will synthesize the probe for the availability of each type of label.

The assay consists of a standard cDNA dilution series from which linear regression curves may be plotted. The slope of the resulting curve gives a measure of PCR efficiency, where -3.3 ± 0.1 with a coefficient of determination (R^2) of 0.99 indicates a reaction efficiency of 100%. Part of the initial SYBR Green validation also includes a melting (or dissociation) curve analysis. At the end of the repetitive cycles of the PCR, an additional melt-anneal-melt cycle is performed. The final melt occurs very slowly and the changes in both temperature and fluorescent signal are monitored over time. This decrease in fluorescence correlates with the dissociation of the double-stranded PCR product releasing the bound SYBR Green I fluorophores. The instrument software uses

an algorithm to transform and display the melting curve as the negative first derivative of the normalized fluorescence versus temperature (Applied Biosystems, 2001a). The presence of a single peak in the melting curve is indicative of a single PCR product, and occurs at the melting temperature of the product. Multiple peaks in this plot indicate that nonspecific products or primer dimers have been formed. Formation of a single product can be confirmed by running the PCR products on a 2% agarose or 10% polyacrylamide gel following the QPCR run. Occasionally, when two products are observed, the second product may have been formed during the plateau phase, which would not affect quantification. To confirm whether this has occurred, the QPCR run could be repeated and stopped during the exponential phase, and the reaction products run on an agarose gel. However, this is not feasible in practice because of the high-throughput nature of the assay. The best course of action when multiple products are observed in the dissociation curve is to redesign and validate a new primer set. Only primer sets that give a single peak in this curve should be used for experimental assays. Once a SYBR Green–based primer set has passed validation testing, the corresponding TaqMan probe is ordered and validated for PCR efficiency only. In rare cases, the SYBR Green assay conditions (e.g., primer concentration, Mg^{2+} concentration) will not be appropriate for TaqMan assays. This is observed as a decline in PCR efficiency. In this case, new primers may be designed to flank the probe sequence.

Additional Materials (also see Basic Protocol 1)

Primer/probe design software (Primer Express, Applied Biosystems)

Design primers

1. Retrieve the RNA sequence information from the appropriate source (e.g., Genbank or Ensembl).

2. Determine the locations of exon boundaries by aligning the mRNA sequence with its gene or by using NCBI's Entrez Gene Evidence Viewer (*http://www.ncbi.nlm.nih.gov*) or Ensembl's Genome Browser (*http://www.ensembl.org*).

   Some genes do not have introns, so this step may not be applicable.

3. Copy the sequence into the design software.

   Several design programs are available both commercially as stand-alone applications and as Web-based applications. Alternatively, primers and probes may be designed "by hand." Omit this step if designing by hand.

4. If using software other than Primer Express, use the following parameters:

   a. *QPCR primers:* Should have 40% to 60% GC content and melting temperatures around 60°C. Should not contain runs of the same nucleotide, repetitive sequences, or more than two G's and/or C's on the 3′ end (also called GC clamp).

   b. *PCR product (amplicon):* Should be 50 to 150 bases in length with an approximate melting temperature between 85° and 95°C.

   c. *TaqMan probe (anneals to sequence between primers):* Should have the same properties as the primers, except that the melting temperature should be around 70°C, and the sequence should not contain G's within a few bases of the 5′ end because of increased reporter quenching. In addition, the sequence must have more C's than G's, which can be accomplished by using the complementary strand sequence for the probe (Applied Biosystems, 2002b).

   Primer Express contains templates into which these parameters have been preloaded.

5. Choose a primer/probe set for which the primers anneal in different exons, or which have less than 5-bp overhangs into the adjacent exon on the 3′ end of the primer.

This step is necessary to avoid amplification of contaminating genomic DNA. Although the RNA is DNase-treated prior to reverse transcription, complete removal of genomic DNA is never achieved.

For intron-less transcripts or other primer sets that bind sequence within a single intron, the –RT controls are essential for each sample, to ensure that genomic DNA is not being amplified.

6. Perform a BLAST (or equivalent) search of both primers of the set together to verify that they will fully anneal to the correct sequence and only that sequence.

7. If the TaqMan probe will be used, run BLAST (*UNIT 19.3*) on the probe sequence to assess whether it binds to the correct sequence with 100% identity.

8. Order a small-scale synthesis of the primers from a suitable vendor. Standard desalting of the primers is sufficient, and no additional purification (e.g., HPLC) is required.

Once a primer set has been validated, large-scale synthesis may be more cost effective, especially for frequently used primers like the reference genes.

9. Validate the primer set according to the remaining steps of this protocol. If performing TaqMan-based assays, validate the primer set before ordering the probe. Once the primer set is validated, order the dual-labeled probe from an appropriate vendor, and validate according to the following steps.
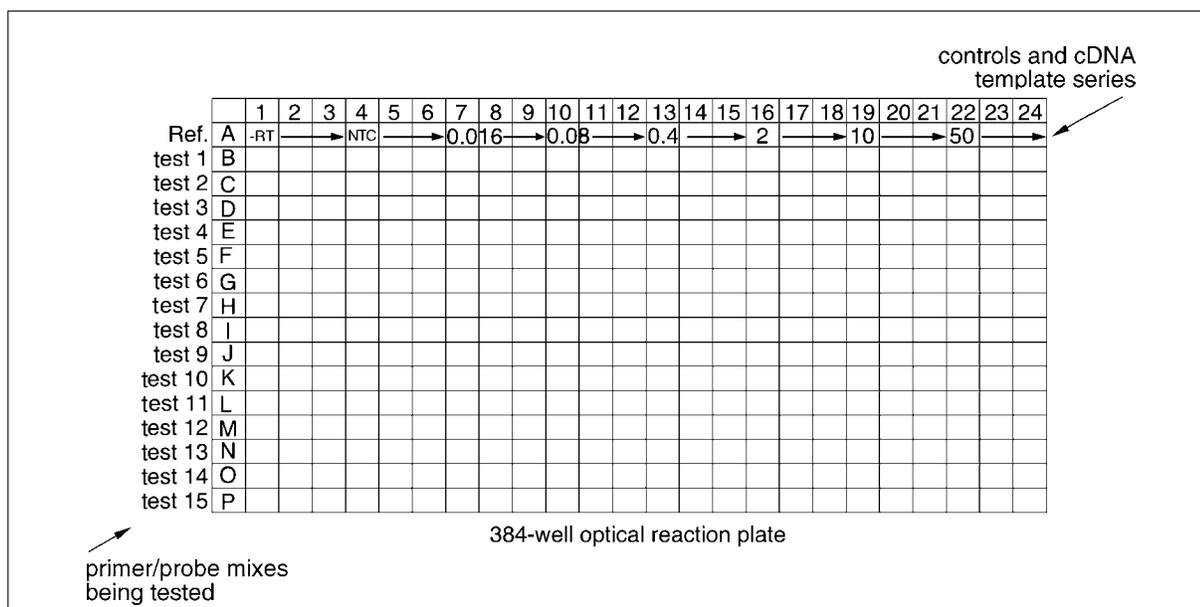
Figure 15.8.3 Typical plate setup for primer and probe validation assays. The plate arrangement shows a standard cDNA template dilution series that is being used to test 15 primer sets along with an endogenous control primer set that has already been validated. The same format is used when testing new probe sets. If different standard cDNAs are used to test primers/probes on the same plate (i.e., standards derived from different tissues), the validated endogenous reference primers/probes must also be run for that standard.

Table 15.8.3 Master Mixes for Primer/Probe Validation Assays

Components	Final concentration	Volume per well	Volume for each cDNA/primer mix (per sample in triplicate + 1 extra)	Master mix (for 8 standards[b] + 1 extra)
SYBR Green assay				
2× SYBR Green mix	1×	5 μl	20 μl	180 μl
1:1 primer mix (1.25 μM each)	150 nM each primer	1.2 μl	4.8 μl	43.2 μl
Template cDNA	0.016-50 ng[a]	1.25 μl	5 μl	—
H₂O	N/A	to 10 μl	to 40 μl	91.8 μl
Total volume		10 μl	40 μl	Aliquot 35 μl into each tube containing cDNA
TaqMan assay				
2× TaqMan mix	1×	5 μl	20 μl	180 μl
1:1 primer mix (1.25 μM each)	300 nM each primer	2.4 μl	9.6 μl	86.4 μl
5 μM TaqMan probe	250 nM	0.5 μl	2.0 μl	18 μl
Template cDNA	0.016-50 ng[a]	1.25 μl	5 μl	—
H₂O	N/A	to 10 μl	to 40 μl	30.6 μl
Total volume		10 μl	40 μl	Aliquot 35 μl into each tube containing cDNA

[a]Recommended template dilution series. The user may modify the range of cDNA concentrations based on the experimental system. Note that the cDNA template quantity is based upon the amount of total RNA input into the reverse transcription reaction.
[b]For this assay, the NTC and –RT controls are included as part of the standard sample set.

Validate primer set

10. Set up assay as described in Basic Protocol 1 (steps 1 to 9), except refer to Figure 15.8.3 for an example of a typical plate setup and to Table 15.8.3 for master mix components.

11. Test the new primer set using SYBR Green chemistry. If valid, test the TaqMan-based chemistry.

12. Following the instrument run, first check the dissociation curve. If more than one peak is present, the primer set is invalid and no other parameters are checked.

    It may be possible to eliminate the nonspecific product(s) detected in the dissociation curve analysis or gel electrophoresis by addition of enhancers such as betaine (UNIT 15.1). This approach may be warranted if the sequence constraints for a given GOI are limiting.

13. If a single peak is found in the dissociation curve, assess the PCR efficiency by calculating the slope of the linear regression curve as follows.

    a. Plot Ct (or crossing point, CP) versus log ng of the standard cDNA input for each concentration of standard as an *xy* scatter plot.

       This may be performed directly in the instrument software on the Applied Biosystems instrument, or can be done in Excel (or equivalent).

    b. Apply a best-fit curve and display the corresponding linear regression formula.

       If the slope of the curve is -3.3 ± 0.1 with $R^2 = 0.99$, the primer set amplifies at 100% efficiency, and the set is considered valid.

The Polymerase Chain Reaction

15.8.19

Efficiency is dependent upon several factors including pipet calibration, primer quality and dilution, and even variability in the instrument run. The slope, therefore, may not be exactly −3.3 ± 0.1. In this case, the slope of the test primer set must match that of a previously validated endogenous reference gene run for the same standard cDNA within ±0.1. For example, if the test primer gives a slope of −3.6 and the endogenous reference for the standard cDNA used to test the set gives a slope of −3.5, then the test set is valid. However, if the endogenous reference primer set gives a slope of −3.3 and the test primer has a slope of −3.6, the test set is invalid.

14. Repeat the linear regression analysis for the TaqMan probe/primer set to assess PCR efficiency.

> *Melting curve analysis cannot be performed for TaqMan-based assays, since cleavage of the probe releases the reporter that continuously fluoresces.*

REAGENTS AND SOLUTIONS

Use molecular-biology-grade (nucleic acid– and nuclease-free) or sterile-filtered double-deionized water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2***; for suppliers, see* **APPENDIX 4***.*

Primer mixes, 1.25 µM each forward and reverse primer

Mix small aliquots of 2.5 µM forward and reverse primer stocks (see recipe) in equal volumes (1:1). Store up to 1 to 2 months at 4°C in screw-capped tubes to prevent evaporation. Do not freeze, as repeated freeze-thaw cycles will degrade the primers.

Primer stocks, 100 and 2.5 µM

Purchase lyophilized oligonucleotides from any commercial source (synthesized at a 25-nM scale; standard desalting is sufficient, no additional purification is needed). Briefly centrifuge the tubes of powdered oligonucleotide to get contents to the bottom. Resuspend in molecular-biology-grade water to 100 µM. Before preparing primer mixes, dilute each stock (forward and reverse) to 2.5 µM. Store at –20°C. When stored properly and subjected to minimal freeze-thaw cycles, primer stocks can last >2 years.

Primer pairs produced at a 25-nM scale will yield enough reagent to test ∼4000 samples using SYBR Green, or ∼2000 samples using TaqMan assays.

Sterile-filtered double-deionized water may be used instead of purchased molecular-grade water. Do not use DEPC-treated water, because the slightly acidic pH may promote primer degradation. Tris buffer may be used instead of water, but should not contain EDTA, which acts as a Mg^{2+}-chelating agent and can inhibit the PCR.

Standard cDNAs, dilution series

DNase-treat (*UNIT 3.12*) and reverse transcribe (*UNIT 3.7*) a suitable RNA using 0.08 µg/µl random hexamer primers such that the final concentration of the standard is 40 ng/µl (based on RNA quantity; see *UNIT 15.5*). Include both a no-template control (NTC) for which water is used instead of RNA, and a no-reverse-transcriptase control (−RT). Following the reverse transcription, make a 5-fold dilution series of the 40 ng/µl standard to obtain working concentration standards of 40, 8, 1.6, 0.32, 0.064, and 0.0128 ng/µl. Store up to 1 month at 4°C or >2 years at –20°C (with minimal freeze-thaw cycles).

By using 1.25 µl/well of each of the standards in the final reaction plate, the resulting amount of starting template will be 50, 10, 2, 0.4, 0.08, and 0.016 ng. See Commentary for more information and suggestions about control and standard RNA samples.

Alternatively, if absolute RNA standards are being prepared via Support Protocol 1, these should be serially diluted first and then, in parallel with experimental RNA samples, DNase-treated and reverse transcribed (see Support Protocol 1, steps 34 to 35).

Table 15.8.4 Preparation of 2× SYBR Green Mix

Component	Volume (µl)				
25 mM MgCl$_2$	48	288	600	720	1200
10× Gold PCR buffer	40	240	500	600	1000
10× dNTP mix	40	240	500	600	1000
DMSO	40	240	500	600	1000
1:1000 SYBR Green I	20	120	250	300	500
50× ROX	8	48	100	120	200
AmpliTaq Gold polymerase (5 U/µl)	2	12	25	30	50
H$_2$O	2	12	25	30	50
Total volume in 2× buffer	200	1200	2500	3000	5000

SYBR Green mix, 2×

Combine the following components as indicated in Table 15.8.4:

25 mM MgCl$_2$, molecular biology grade (store at –20°C)

10× Gold PCR buffer (supplied with PCR enzyme; Applied Biosystems; store at –20°C)

10× dNTP mix: equal volumes of 2 mM dATP, dTTP, dCTP, and dGTP (store at –20°C)

Dimethylsulfoxide (DMSO), molecular biology grade (store at room temperature)

SYBR Green I dye (Molecular Probes; store at –20°C), diluted 1:1000 in water

50× ROX passive reference dye (Invitrogen; store at –20°C)

AmpliTaq Gold polymerase (5 U/µl; Applied Biosystems; store at –20°C)

Water, molecular biology grade

Prepare fresh and keep on ice prior to use in primer master mixes. Protect dyes and all prepared mixes from prolonged exposure to light by wrapping tubes in foil.

To maintain the high-throughput nature of the assay, all buffer conditions, including the concentrations of Mg^{2+}, dNTPs, and other additives, are kept constant. It is strongly recommended that a preformulated buffer be purchased from a reliable vendor, since these are stable at room temperature and have been quality-control tested to ensure optimal performance. The authors have obtained comparable results using mixes from Applied Biosystems, Bio-Rad, Invitrogen, and Sigma. However, it is important to compare lots, as there can be lot-to-lot differences in the commercial preparations.

TaqMan probe, 100 and 5 µM

Depending on the vendor, the probe may be supplied in a lyophilized form. In this case, resuspend to 100 µM in Tris·Cl, pH 8.0 (*APPENDIX 2*), prepared with molecular-biology-grade water and reagents. Before use, dilute a small amount of 100 µM stock to 5 µM with more Tris·Cl, pH 8.0. Store either concentration at –20°C. Avoid repetitive freeze-thaw cycles, and thaw on ice prior to use to preserve the integrity of the probe. Protect the probe from excessive exposure to light (e.g., using amber-colored screw-cap tubes) to prevent photobleaching of the fluorescent dyes and evaporation. See Support Protocol 2 and Commentary for additional considerations regarding the TaqMan probe.

The Polymerase Chain Reaction

15.8.21

Trichloroacetic acid (TCA) solution, 10% (w/v)

Prepare a 100% (w/v) TCA stock solution by dissolving the entire contents of a newly opened TCA bottle in water (e.g., dissolve the contents of a 500-g bottle of TCA in sufficient water to yield a final volume of 500 ml). Store up to 1 year at 4°C. Prepare 10% (w/v) TCA by dilution and store up to 3 months at 4°C.

CAUTION: *TCA is extremely caustic. Protect eyes and avoid contact with skin when preparing and handling TCA solutions.*

COMMENTARY

Background Information

Quantitative PCR is a rapid, robust, and highly sensitive polymerase chain reaction method used to quantify specific nucleic acid targets. Real-time quantitative PCR is different from end-point, or in-gel, analysis (see *UNIT 15.7*) in several ways. For real-time analysis, the increase in fluorescent signal resulting from PCR product synthesis is recorded during the course of the thermocycle. This allows the user to specify the point in the assay at which to "read" the data. Measurements are obtained from the geometric phase of the amplification reaction. This is the phase during which all of the components required for the PCR (e.g., dNTPs, primers, polymerase) are in excess, and therefore the deficit of an essential reaction component will not quench the efficiency of product synthesis. Following geometric amplification, the fluorescence curve reaches a plateau (i.e., the saturation point) as the reaction components begin to become limited and the kinetics of the reaction become unpredictable. At this stage, an increase of one thermocycle no longer correlates with a two-fold change in product (Applied Biosystems, 2002a). In contrast, in end-point PCR, quantification is often obtained by in-gel densitometry measurements at the end (or the saturation point) of the reaction where the reaction may be at a plateau, compromising quantification.

Real-time PCR depends both on a set of universal thermocycling and buffer conditions and on primer efficiency testing and correction where necessary. As a result, the accuracy and precision of the resolution (smallest detectable fold-change) is less than 2-fold, whereas resolution for end-point in-gel measurement is limited to about 10-fold (Applied Biosystems, 2002a). PCR assays in which samples are removed at measured cycle times and electrophoresed and possibly hybridized have better resolving power than end-point analysis. However, both in-gel methods suffer from the lengthy processing steps, compared to real-time PCR.

The two most widely used fluorescent detection methods, or chemistries, for QPCR are SYBR Green, a DNA-intercalating dye, and the fluorogenic probe. Several types of fluorogenic probes are currently available: the popular, dual-labeled hydrolysis probe (TaqMan probe) and the hybridization probes known as molecular beacons and scorpions. Both types of probes bind the sequence intervening the forward and reverse primer binding sites, and both rely on fluorescence resonance energy transfer (FRET) to silence the signal from the reporter dye while it is in proximity to the quencher dye. A hydrolysis probe is cleaved by the 5′ nuclease activity of the polymerase during the primer-extension phase of the reaction, and the reporter is released and becomes free to fluoresce continuously. Hybridization probes rely on a stem-loop structure to keep the reporter and quencher in proximity. Upon hybridization to the specific sequence, the distance between the reporter and quencher becomes too large to silence the reporter, and signal is detected (Tyagi and Kramer, 1996; Whitcombe et al., 1999).

There are advantages and disadvantages to each type of chemistry (SYBR Green versus TaqMan) in terms of sensitivity and specificity. SYBR Green I binds any double-stranded DNA and does not depend on a probe-cleavage event. Therefore, SYBR Green produces earlier Ct values, resulting in an apparent enhanced sensitivity (Whittwer et al., 1997; Morrison et al., 1998). TaqMan probes, on the other hand, supply another layer of sequence specificity in addition to the forward and reverse primers.

There are two methods of quantification that may be performed using real-time PCR: absolute and relative. Absolute quantification measures the copy number of a specific nucleic acid target in a sample. Relative quantification measures the difference in copy number between two samples that have each been normalized to an endogenous reference. Both can be used to compare the effects of different treatments on a particular RNA species or to

compare the levels of multiple RNA species in a single sample. The absolute method requires standards in which the copy number of the particular target has been carefully and accurately measured. From this standard sample, a dilution series is made and assayed for the target at the same time as the unknown samples. From the values obtained from linear regression analysis of the standard dilution series, the GOI copy number values may be interpolated. This will allow the user to assess the sensitivity (i.e., the lowest detectable copy number) of the GOI primer set. As an example, this type of analysis is used in both clinical and food science for the assessment of pathogen load and gene copy number (Pfaffl, 2004).

By contrast, relative quantification does not rely on the knowledge of a given transcript copy number in a standard sample. Instead, the changes in gene expression or the levels of a specified transcript may be measured and described as an arbitrary unit relative to some control sample ($\Delta\Delta$Ct or standard curve method), or to the level of some other control transcript in the same sample (standard curve or efficiency-corrected ΔCt method). For example, relative quantification allows for the measurement of the fold-change in expression for gene A in a treated sample versus an untreated sample, or for the assessment of the level of gene A in a sample relative to some housekeeping gene in the same sample.

Critical Parameters and Troubleshooting

Primer/probe design and validation

For RNA analysis, it is important to differentiate message from genomic DNA. In this respect, the use of amplicons that span an exon junction allows this requirement to be met. This is achieved by designing primer sets with the forward and reverse primers sitting in different exons. However, when the intervening intron is small, genomic DNA may still be amplified. This can be avoided by allowing a few base pairs on the 3′ end of either primer to overhang onto the neighboring exon.

To distinguish knockout or mutant samples using QPCR, primers/probes should be designed in the knocked-out or mutated region of the transcript. In this way, no amplification of the transcript will occur for the knockout, since the region recognized by the primers has been deleted or altered. Primers for transcripts that are rapidly degraded should be placed near the 5′ end of the RNA sequence, as degradation by ribonucleases generally occurs 3′ to

5′ (Brown, 2002). However, RNA transcripts that have undergone linear RNA amplification (for instance, RNA isolated from laser capture microdissected cells) are around 200 to 1000 bases in length and represent the 3′ ends of the transcripts. In this case, primers must be designed in a region near the poly(A)$^+$ tail.

It is absolutely mandatory to validate both primers and probes on the same instrument used to perform the experimental assay, i.e., when the instrument used to validate the primer sets is a different brand than the one that will be used for the experimental assays (e.g., Roche iCycler versus ABI 7900HT). Published primer/probe sets for which the PCR product is ≤150 base pairs and the annealing temperature is around 60°C, which have been validated properly, should be transferable to any system, but this must be empirically determined to ensure the reliability of the data.

In rare cases, the PCR efficiency of a validated primer set changes when switched from SYBR Green to TaqMan-based chemistry. This is due to the difference in primer concentration and/or $MgCl_2$ concentrations between the two buffers, and may often be overcome by redesigning the primers to recognize a sequence around the already synthesized probe.

Choice of chemistry

The selection between SYBR Green I and TaqMan-based assays depends upon the RNA sequence. If the RNA of interest has no polymorphisms or other variations in the region to which the primers bind, and the primers have been correctly designed and validated, then SYBR Green–based assays are adequate for gene-expression analyses. It is up to the user to decide if the addition of the often-costly TaqMan probe is worth the additional specificity it confers. In the case of polymorphisms or variants, differentiation between different RNA species may require the specificity of the probe, which can discriminate a single base difference.

Template quality

The quality of the cDNA template depends upon the integrity of the RNA. QPCR will tolerate some degradation of the RNA when random hexamers (or other -mers) are used to prime the reverse transcription reaction. However, it is not good laboratory practice to use degraded RNA, and the cause of the degradation should be addressed. Transcripts decay at different rates and have variable stability, so partial degradation of a sample at any point could lead to complete absence of detection of

The Polymerase Chain Reaction

15.8.23

the desired target RNA in a subsequent QPCR assay, with little to no change in the housekeeping gene used as a control.

Total RNA is used for QPCR to reduce the number of steps and potential sources of degradation during sample preparation. Purified messenger, or poly(A)$^+$, RNA can also be used. However, this subtractive purification could lead to the loss of transcripts that do not have a poly(A)$^+$ tail, or to the preferential enrichment of RNAs that have internal A tracts. The added processing reduces the recovery of material for subsequent use, and can cause degradation. If poly(A)$^+$ RNA is used, 50- to 100-fold less ($\sim$100 pg) sample template is required in the reaction mixture because mRNA represents 3% of total cellular RNA (Alberts et al., 1994).

Reverse transcription primers

Reverse transcription (RT) of RNA into complementary DNA (cDNA; refer to UNIT 15.5) may be performed using several different types of oligonucleotide primers. For QPCR, the preferred primer is a random hexamer, nonamer, or dodecamer oligo with 6, 9, or 12-base stretches of random sequences, respectively. Random primers have a much higher probability of efficiently amplifying all RNA transcripts, due to their indiscriminate nature (ABI, pers. comm.). This property also enables RNA secondary structure to be overcome, since the priming occurs in random places along the length of the transcript. mRNA-specific priming by oligo(dT)s at the poly(A)$^+$ tail and any internal poly(A) tract is another method of RT priming. This method will allow only polyadenylated RNAs to be converted to cDNA, thus limiting amplification of some GOIs or partially degraded samples. A mixture of a random oligo with an oligo(dT) primer may enhance detection of rare messages while still allowing for the detection of transcripts that lack polyadenylation. However, the use of this procedure may skew the measurement of relative RNA abundance towards intact, full-length mRNA over incomplete or rapidly degraded messages. Users should decide and test these parameters in their particular experimental systems. The third method of reverse transcription priming is the gene-specific reverse primer. The reverse PCR primer is used to specifically target the GOI for conversion to cDNA. This is often performed in the same QPCR plate as the PCR by adding RT enzyme to the PCR mix and adding an incubation step prior to the first step in the PCR cycling program (known as one-step RT-PCR).

While this may enhance detection of a specific RNA target, RNA secondary structure may not be overcome, depending on the priming site, and RT efficiency must be considered in addition to PCR efficiency.

An important assumption that is made when performing RT-QPCR is that RT efficiency is similar for the GOI between samples and for different GOIs. However, different tissues/sample types may contain variable levels of RT-inhibiting or -enhancing factors (Pfaffl, 2004). To control for these variables, it is recommended that all samples to be compared be prepared under the same conditions and at the same time (i.e., with regard to RNA extraction method and RT reaction).

Endogenous reference gene

Normalization of sample loading is essential in any quantitative comparative analysis to ensure that the measured differences between samples is not attributable to disproportionate amounts of starting material. For gene-expression assays, the normalizer must be an endogenous gene that is expressed at equal levels in all tissue or cell types and treatment conditions under study. Traditionally, one of the so-called housekeeping genes (e.g., GAPDH, cyclophilin, β-actin, HPRT, U36B4, 18S rRNA) is selected to serve this function. The choice is a point of controversy, since there are examples of fluctuations for most of the abovementioned genes under various treatment or physiological conditions (Schmittgen and Zakrajsek, 2000; Suzuki et al., 2000; Guo et al., 2001; Vandesompele et al., 2002; Dheda et al., 2004). The user should run a small pilot experiment to determine which endogenous reference is appropriate for the particular studies. A good method for doing this is to perform a ΔΔCt assay using several potential normalizer RNAs and a GOI that is not expected to show any fold-changes within a small set of samples. The GOI is then normalized to each of the reference RNAs individually for all of the samples. Finally, the housekeeping gene for which there is no detectable fold-change of the GOI between the test samples is chosen for use in experimental assays (Applied Biosystems, 2001b; Guo et al., 2001; Roche Applied Science, 2002).

Controls and relative RNA standards

Controls for the assay that are made alongside the cDNA standards and unknowns include a no-template control (NTC), made by substituting water for RNA, and a no-reverse-transcriptase (–RT) control, made by omitting

the reverse transcriptase. For primers that span an exon junction, a –RT control is not needed for every sample if, during the validation process, this control shows no amplification product. If the system under study is the result of introduction of an expression construct into cells, or if the GOI does not have introns or has a known processed pseudogene, then –RT controls should be made and assayed for every sample. An NTC should be run for every primer set of an assay to facilitate the detection of contaminants that contribute to fluorescent signal.

If amplification of the NTC occurs, primer-dimer or other nonspecific PCR products may have been formed, or contamination of a reagent or degradation of the primer mix may have taken place. If amplification occurs in the –RT control, this indicates the presence of genomic DNA if the primer/probe set does not span an exon junction, or the presence of primer-dimer or nonspecific PCR products, a contaminant, or degradation of the primer mix. Although all amplification products (including those that are nonspecific) contribute to the fluorescence signal, this fluorescence contribution can be considered negligible if the Ct values of the NTC and –RT are ≥ 7 cycles different from the experimental samples (Applied Biosystems, pers. comm.).

If the unknowns are RNA samples, the standard of choice is an RNA that is reverse transcribed in the same manner as the unknowns. A suitable standard RNA is one in which the expression of the GOI is at a moderate to high level and that has a similar composition to the unknown samples. The use of a plasmid or linear DNA standard is not advised for measurement of an endogenous tissue transcript, because these types of nucleic acids have different background compositions than RNA, are extracted differently, and are not reverse transcribed, and therefore may not have the same RT or PCR amplification efficiency as the experimental samples (Applied Biosystems, 2003; Pfaffl, 2004). Several vendors (e.g., Ambion, Clontech, Stratagene) supply total RNA preparations of many cell and tissue types collected from many different species. Both Stratagene and Clontech make total RNA pools termed "universal reference RNA," composed of mixtures of either various cell lines or whole tissues, respectively. These RNA pools represent $\geq 90\%$ gene coverage on microarrays (Novoradovskaya et al., 2000; Clontech, 2002), and are very useful as standards both for validation of primer/probe sets and for large-scale multigene studies.

Absolute RNA standards

The absolute quantification of RNA synthetic standards by radiolabel incorporation permits accurate determination of transcript amounts. Liquid scintillation counting of the filters typically yields incorporation values with errors of less than $\pm 5\%$. However, errors of up to 10% to 15% will not significantly affect quantification in most applications. The use of low-energy ^{35}S (compared to ^{32}P) at very low specific-activity levels minimizes potential radiolytic degradation of the synthetic RNA over time and minimizes personal radiation exposure. Very small amounts of standard RNA are typically needed for an RT-PCR assay (less than 10^8 copies), such that the amount of ^{35}S in each RT-PCR reaction will be extremely small.

One consideration is the purity of the radio-labeled ribonucleotide. Fresh lots are generally guaranteed by the manufacturer to be 90% to 99% intact NTP. The presence of other contaminating radiolabeled material is taken into account when calculating total input ^{35}S. Storage time and multiple freezing and thawing cycles will contribute to decomposition of the radionucleotide. If the integrity of the reagent is in doubt, consult the manufacturer, assess the fraction of intact NTP by thin-layer chromatography, or order a fresh lot.

While RNA can be quantified by optical density, care must be taken to eliminate unincorporated nucleotides (which will also absorb light at 260 nm) and take into account RNA secondary structure (which can reduce absorbance). A protocol for quantifying non-radioactive synthetic RNAs that accounts for secondary structure by measuring the absorbance of hydrolyzed RNA is described in Iyer and Struhl (1996). This alternative may be suitable for researchers who prefer not to handle radioactive compounds. RNA can also be quantified by comparison with mass standards on a gel; however, this method may be inaccurate due to the differential binding of ethidium bromide to single- and double-stranded RNA regions.

The most important concern is to avoid RNase contamination of equipment and reagents. If RNA yield from transcription is low or undetectable, RNA degradation is the most likely culprit. Gel electrophoresis of the RNA samples can distinguish transcription failure (no products in the pre-DNase sample) versus loss of RNA during subsequent manipulations (products in the pre-DNase sample but not in the purified sample). More frequent samples can be taken for gel analysis to help

The Polymerase Chain Reaction

15.8.25

pinpoint the troublesome step or reagents. A ribonuclease inhibitor such as SUPERase·In (Ambion) may be included in the transcription reaction and added to the purified RNA to preserve RNA integrity; however, this will merely mask RNase contamination, not eliminate it. Other possible solutions for poor incorporation or yield could be old reagents. Pay particular attention to the freshness of the [α-^{35}S]NTP, DTT, rNTPs, and polymerase.

A single RNA product species from the transcription is critical for the production of accurate RNA standards. Multiple product species of indeterminate content can cause inaccuracies in quantification and can also lead to extraneous PCR products. Product RNA species longer than expected indicate incomplete cleavage of the parent plasmid. Either increasing the efficiency of the plasmid digestion or purifying the linearized plasmid from an agarose gel will prevent this problem. Species smaller than expected could indicate that the RNA polymerases had difficulty transcribing full-length RNA. Use of a truncated template which eliminates problematic sequences may be required. Very minor contaminants may not affect quantification by more than a few percent. The researcher can try to estimate the amount of contaminating bands from the gel and account for these in the calculations. However, since the sequence of such contaminants is unknown, it is difficult to assess the effect during PCR amplification. Thus, the authors recommend striving to attain a single product species.

Additional considerations for constructing the transcription plasmid that will be used to provide the template for run-off transcription are as follows:

1. The cloned sequence from the gene of interest must include the sequence primed for reverse transcription and the PCR target sequence. Ensure that the clone does not include sequences, such as introns, that are not part of the RNA species. Full-length cDNA clones yield the most authentic synthetic RNAs. In some cases, truncated cDNAs may permit more efficient and consistent transcription of a single-length RNA product; however, obtaining a single-length RNA product species is critical.

2. If oligo-dT is used for reverse transcription, then the synthetic RNA will need a poly(A)$^+$ tail. In this case, construct a transcription plasmid using a vector such as pSP64 Poly(A)$^+$ (Promega), which contains a run of dA:dT residues at the 3$'$ end of the multiple

cloning site, allowing for the transcription of a synthetic poly(A)$^+$ tail.

3. Ensure that the orientation of the cloned cDNA will produce sense transcripts.

4. Engineer the plasmid such that cleavage by a restriction enzyme generates a linear piece of DNA that contains the phage polymerase promoter and the entire sequence of the desired synthetic RNA. This is ideally accomplished using an enzyme which linearizes the plasmid by cutting at only one site, at the end of the desired transcript sequence. Avoid including superfluous vector sequences in the transcribed region, since these will not be present in RNA from the experimental samples.

Avoid using restriction enzymes that create 3$'$ overhangs, because there is evidence that they cause RNA complementary to the intended transcripts to be generated (Schenborn and Mierendorf, 1985).

As an alternative to a plasmid, PCR products can also be designed for use as transcription templates by incorporating the polymerase promoter sequence into one of the primers (Mullis and Faloona, 1987).

Anticipated Results

The setup of the $\Delta\Delta Ct$ assay allows 64 samples (one of which should be the NTC) to be assayed for an endogenous reference gene and one gene of interest in triplicate; the standard curve and efficiency-corrected ΔCt assays will accommodate an endogenous reference gene and one gene of interest in triplicate for 56 unknowns, six standards, and two control samples. Assays performed on the ABI 7900HT are completed in 1.5 and 2 hr for TaqMan and SYBR Green-based assays, respectively. The results yield data that are highly reproducible and correlate well with traditional northern blotting and RNase-protection assays. Support Protocol 1 will yield 10 to 100 µg of single-length transcripts, equivalent to $\sim$10^{12} to 10^{13} RNA molecules, with a specific activity of $\sim$1 $\times$ 10^7 cpm/µg.

Time Considerations

In preparation for performing a QPCR assay, RNA and subsequent cDNA preparation may be carried out in advance. Prior to an actual experimental assay, primers and probes must be designed and validated. In most cases, design and validation may take several days to a few weeks. This includes the time to design, order, and synthesize the primers ($\sim$2 days, depending on the vendor), test the primers (a few hours), and, if desired, synthesize ($\sim$7 to

14 days, depending on the vendor) and test (a few hours) the corresponding probes.

Once the required primer/probe sets are validated, the experimental assays are performed. A single-plate assay may take 0.5 to 2 hr to prepare and 2 hr to run. The preliminary raw-data analyses on the instrument software may take less than half an hour. The time required for the following final analyses will depend upon the user's familiarity with both the mathematical and software applications. The typical workflow for an experimental assay is as follows. On the first day, prepare total RNA and determine concentration by UV or fluorescence spectroscopy (requiring 1 to 4 hr, depending on number of samples and method of preparation). Next, DNase-treat and reverse transcribe RNA to cDNA (requiring ∼3 hr for setup and incubations). On the second day, prepare master mixes for the assay (∼30 min to 1 hr). Next, prepare QPCR plate(s) (∼30 min to 1 hr per plate), run plate on instrument (1.5 to 2 hr), and collect and analyze data (1 to 3 hr).

For preparation of RNA standards, construction of the transcription plasmid can take anywhere from several days to several weeks; one additional day is needed to prepare the template for transcription. RNA synthesis, gel electrophoresis, and scintillation counting can be completed the following day. Following overnight gel drying, the autoradiography may require several hours to several days for sufficient exposure. During the incubation periods for transcription and DNase treatment, time becomes available to prepare the gel, filters and scintillation vials. Filters can be spotted and washed while the gel is running. If necessary, the samples for gel electrophoresis (dissolved in loading buffer) and/or the samples for scintillation counting may be stored at −20°C overnight and analyzed the following day.

Literature Cited

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D. 1994. The cell nucleus. *In* Molecular Biology of the Cell, 3rd ed., p. 370. Garland Publishing, New York.

Applied Biosytems. 2001a. ABI Prism 7900HT User Manual, rev. 4. Applied Biosystems, Foster City, Calif.

Applied Biosystems. 2001b. TaqMan human endogenous control plate: Protocol: Revision C. *http://docs.appliedbiosystems.com/pebiodocs/04308134.pdf*. Applied Biosystems, Foster City, Calif.

Applied Biosystems. 2002a. Real-time PCR vs. traditional PCR. *https://www.appliedbiosystems.com/support/tutorials/pdf/rtpcr_vs_tradpcr.pdf*. Applied Biosystems, Foster City, Calif.

Applied Biosystems. 2002b. Designing TaqMan MGB probe and primer sets for gene expression using Primer Express software. *http://www.appliedbiosystems.com/support/tutorials/pdf/taqman_mgb_primersprobes_for_gene_expression.pdf*. Applied Biosystems, Foster City, Calif.

Applied Biosystems. 2003. Creating standard curves with genomic DNA or plasmid DNA templates for use in quantitative PCR. *https://www.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf*. Applied Biosystems, Foster City, Calif.

Brown, T.A. 2002. How genomes function. *In* Genomes, 2nd ed. (S. Carlson, ed.) section 10.4. John Wiley & Sons, Hoboken, N.J.

Clontech. 2002. Control RNA for microarray experiments. *Clontechniques* XVII:6. *http://www.clontech.com/clontech/archive/APR02UPD/pdf/ControlRNA.pdf*. Clontech, Palo Alto, Calif.

Dheda, K., Huggett, J.F., Bustin, S.A., Johnson, M.A., Rook, G., and Zumla, A. 2004. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *BioTechniques* 37:112-119.

Guo, D., Henriksson, R., and Hedman, H. 2001. The iCycler iQ detection system for evaluating reference gene expression in normal human tissue, rev. A. *Amplification* 2804. *http://www.bio-rad.com/LifeScience/pdf/Bulletin_2804.pdf*. Bio-Rad, Hercules, Calif.

Iyer, V. and Struhl, K. 1996. Absolute mRNA levels and transcriptional initiation rates in *Saccharomyces cerevisiae*. *Proc. Nat. Acad. Sci. U.S.A.* 93:5208-5212.

Kramer, M.F. and Coen, D.M. 1995. Quantification of transcripts from the ICP4 and thymidine kinase genes in mouse ganglia latently infected with herpes simplex virus. *J. Virol.* 69:1389-1399.

Morrison, T.B., Weis, J.J., and Wittwer, C.T. 1998. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *BioTechniques* 24:954-962.

Mullis, K.B. and Faloona, F.A. 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol.* 155:335-350.

Novoradovskaya, N., Payette, T., Novoradovsky, A., Braman, J., Chin, N., Pergamenschikov, A., Fero, M., and Botstein, D. 2000. Pooled, high-quality reference RNA for human microarrays. *Strategies* 13:121-122. *http://www.stratagene.com/news/newsletter.aspx?iid=6*. Strategene, La Jolla, Calif.

The Polymerase Chain Reaction

15.8.27

Pfaffl, M.W. 2004. Quantification strategies in real-time PCR. *In* IUL Biotechnology Series, No. 5: A-Z of Quantitative PCR (S.A. Bustin, ed.) pp. 87-120. International University Line, La Jolla, Calif.

Roche Applied Science. 2002. Selection of housekeeping genes. Technical Note No. LC 15/2002. *http://www.roche-applied-science.com/lightcycler-online/lc_support/pdfs/lc_15.pdf.* Roche Applied Science, Indianapolis, Ind.

Schenborn, E.T. and Mierendorf, R.C. Jr. 1985. A novel transcription property of SP6 and T7 RNA polymerases: Dependence on template structure. *Nucl. Acid. Res.* 13:6223-6236.

Schmittgen, T.D. and Zakrajsek, B.A. 2000. Effect of experimental treatment on housekeeping gene expression: Validation by real-time, quantitative RT-PCR. *J. Biochem. Biophys. Methods* 46:69-81.

Shoemaker, J.P., Garland, C.W., and Steinfeld, J.I. 1974. Experiments in Physical Chemistry, pp. 34-39. McGraw-Hill, New York.

Suzuki, T., Higgins, P.J., and Crawford, D.R. 2000. Control selection for RNA quantitation. *BioTechniques* 29:332-337.

Tyagi, S. and Kramer, F.R. 1996. Molecular beacons: Probes that fluoresce upon hybridization. *Nat. Biotechnol.* 14:303-308.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Psepe, A., and Speleman, F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3:1-12.

Whitcombe, D., Theaker, J., Guy, S.P., Brown, T., and Little, S. 1999. Detection of PCR products using self-probing amplicons and fluorescence. *Nat. Biotechnol.* 17:804-807.

Whittwer, C.T., Herrmann, M.G., Moss, A.A., and Rasmussen, R.P. 1997. Continuous fluorescence monitoring of rapid cycle DNA amplification. *BioTechniques* 22:130-138.

Key References

Ambion. 2001. The top 10 most common quantitative RT-PCR pitfalls. Technotes Newsletter 8:8. Ambion, Houston, Tex.

A short, but useful checklist of critical considerations for performing any type of reverse transcription PCR.

Applied Biosystems. 1997. Relative quantitation of gene expression: ABI PRISM 7700 Sequence Detection System: User Bulletin #2: Rev B. Applied Biosystems, Foster City, Calif.

This bulletin outlines both the standard curve and $\Delta\Delta Ct$ methods and shows, by comparing data obtained using both calculations, that the resulting values are very similar regardless of the assay.

Applied Biosytems. 2001a. See above.

This instrument manual contains explanations about the transformation of fluorescence signal into Ct data in addition to outlining the proper method of baseline and threshold settings for ABI machines.

Users should consult their specific instrument manuals, since each type of instrumentation will require knowledge of slightly different terminology and parameters.

Livak, K.J. and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 25:402-408.

This article presents a detailed review of the derivation of the mathematical applications described in this unit.

Internet Resources

http://www.ncbi.nlm.nih.gov

NCBI Web site.

http://www.ensembl.org

Ensembl Web site.

http://www.gene-quantification.info/

The Gene Quantification Web site contains a host of information concerning QPCR.

http://pga.mgh.harvard.edu/primerbank/index.html

The Primer Bank database, hosted by Harvard University, contains user-submitted primer sequences for several mouse and human genes.

http://web.ncifcrf.gov/rtp/gel/primerdb/

The Quantitative PCR Primer Database (QPPD), maintained by the National Cancer Institute, contains primer and probe sequences for mouse and human genes collected from articles cited in PubMed.

http://www.ambion.com/techlib/index.html

Contains numerous, detailed articles and technical bulletins regarding transcription and general RNA handling issues.

http://www.promega.com/techserv/

Contains technical manuals with detailed protocol tips and troubleshooting for transcription applications

Other commercial vendor-sponsored technical support Web sites are also a very good resource for tips about RNA and QPCR applications.

Contributed by Angie L. Bookout,
   Carolyn L. Cummins, and
   David J. Mangelsdorf
Howard Hughes Medical Institute
University of Texas Southwestern
   Medical Center
Dallas, Texas

Jean M. Pesola and Martha F. Kramer
   (preparation of RNA standards)
Harvard Medical School
Boston, Massachusetts