Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR

Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR

Table of Contents

Section I

Introduction to Real-Time PCR and
Relative Quantitation of Gene Expression

1. Introduction

Real-time quantitative PCR offers researchers a powerful tool for the quantitation of target nucleic acids. To understand the value that real-time PCR provides over traditional PCR methods and to obtain information on chemistries and strategies, you can review:
Real Time PCR vs. Traditional PCR
Essentials of Real Time PCR

This tutorial guides you through performing relative quantitation of gene expression using real-time PCR technologies developed by Applied Biosystems. It assists you in understanding the foundations of relative quantitation and provides guidance for selecting assays, experimental strategies, and methods of data analysis. The information presented is relevant for instrumentation, reagents, and consumables provided by Applied Biosystems. This tutorial expands on many of the topics that are introduced in User Bulletin #2: Relative Quantitation of Gene Expression.

Throughout this tutorial there are many hyperlinks to external sites, documentation, and links to pages within this document. After you go to one of these hyperlinks, click the back button on your browser to return to your original location in the document.
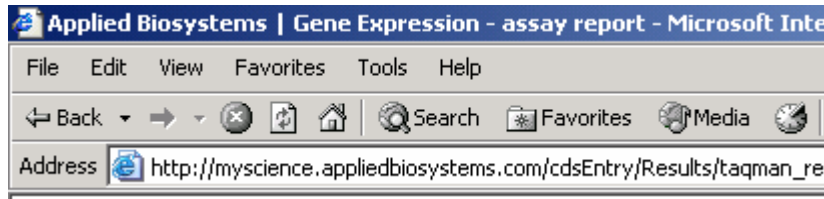
Applied Biosystems offers a variety of systems on which real-time quantitative PCR can be performed. These real-time PCR instruments are:

- Applied Biosystems 7300 Real-Time PCR System
- Applied Biosystems 7500 Real-Time PCR System
- Applied Biosystems 7900HT FAST Real-Time PCR System
- ABI® PRISM 7000 Sequence Detection System

2. What is Relative Quantitation?

Methods for relative quantitation of gene expression allow you to quantify differences in the expression level of a specific target (gene) between different samples. The data output is expressed as a fold-change or a fold-difference of expression levels. For

example you might want to look at the change in expression of a particular gene over a given time period in treated vs. untreated samples. For this hypothetical study, you can choose a calibrator sample (i.e. untreated at day 0) and an endogenous control gene to normalize input amounts. For all samples, levels of both target and endogenous control genes would be assessed by real-time PCR. The results (target levels normalized to endogenous control levels) would then be expressed in a format such as "At day 30, sample A had a 10-fold greater expression level of the target gene than at day 0".

If you want to obtain absolute quantities of gene targets you need to perform absolute quantitation, which is beyond the scope of this document.

3. Terms and Acronyms – The following terms and acronyms are used throughout this document. Additional information on specific definitions is available in the Appendix or by clicking the appropriate links.

Terms/ Acronyms	Definition
Active reference	An active signal used to normalize experimental results. Endogenous controls are an example of an active reference. Active reference means the signal is generated as a result of PCR amplification. The active reference has its own set of primers and probe.
Amplicon	A PCR product generated from a DNA or cDNA template.
Amplification efficiency	The rate at which a PCR amplicon is generated, commonly measured as a percentage value. If a particular PCR amplicon doubles in quantity during the geometric phase of its PCR amplification then the PCR assay is said to have 100% efficiency. The value assigned to the efficiency of a PCR reaction is a measure of the overall performance of a real-time PCR assay.
Baseline	The background fluorescence signal emitted during the early cycles of the PCR reaction before the real-time PCR instrument detects the amplification of the PCR product.
Calibrator	A sample used as the basis for comparative expression results
C_T	Threshold cycle. The C_T is the cycle number at which the fluorescence generated within a reaction crosses the threshold line. C_T values are logarithmic and are used either directly (comparative C_T method) or indirectly (interpolation to standard curves to create linear values) for quantitative analyses.
Custom TaqMan® Gene Expression Products[1]	Custom TaqMan® Gene Expression Assays are products designed, synthesized, and delivered as pre-mixed primers and TaqMan® MGB probe sets based on sequence information submitted by the customer.
Custom TaqMan® Genotyping Products[2]	Custom TaqMan® Genotyping Assays are products designed, synthesized, and delivered as a set of pre-mixed primers and TaqMan® MGB probes based on sequence information submitted by the customer.
Dynamic range	The range (maximum to minimum) of sample concentrations or input amounts that a given assay is capable of detecting.
Endogenous control	A gene sequence contained in a sample that is used to normalize target quantities. In addition to the target sequence, an endogenous control is quantified as a means of correcting results that can be skewed by input nucleic acid loading differences. Endogenous controls are an example of an active reference.
Experimental replicate	An amplification that uses the same PCR reagents as another amplification and that uses template preparations from similar but not identical samples. Experimental replicates provide information about the overall precision of the experiment. For example, if you want to examine the effect of drug treatment on the level of a mouse mRNA, you would treat multiple mice identically with the drug to determine the variation of response in the mouse population. A group of ten mice would represent ten experimental replicates.
Identical replicate	An amplification performed in multiple wells using the same template preparation and the same PCR reagents. Identical replicates provide: • Data preservation: If amplification fails in one well, replicates in other wells can potentially provide data. • Monitoring: Replicates can be used to monitor the precision of the PCR amplification and detection steps.
Passive reference	A dye that provides an internal fluorescence reference to which the reporter dye signal can be normalized during data analysis. The reference dye does not participate in the PCR reaction. This normalization corrects for fluorescence fluctuations that are caused by changes in reaction concentration or volume. Failure to use a passive reference dye can compromise accurate target quantitation. Applied Biosystems incorporates the internal passive reference dye ROX™ in all of its real-time PCR chemistries.

TaqMan® PDAR	TaqMan® Pre-Developed Assay Reagents (TaqMan® PDARs) are primer and probe sets designed to amplify specific target and endogenous control sequences in cDNA samples using the 5' nuclease assay.
Precision and Statistical Tests	**Amplification and Detection Step:** The degree to which identical replicates give similar values (degree of agreement). This type of precision can be used to monitor the accuracy of template and reagent pipetting, homogeneity of template, and instrument performance. **Experimental**: The degree to which experimental replicates give similar values. **Note:** For relative quantitation, better precision (identical and experimental) enables smaller fold differences in nucleic acid copy number to be distinguished with greater statistical confidence.
Rapid assay development guidelines	A series of design and experiment guidelines developed by Applied Biosystems that specify: • The use of Applied Biosystems Genomic Assays or automated primer and probe design using Primer Express® Software • The use of TaqMan® Universal PCR Master Mix or SYBR® Green I PCR Master Mix (provides standardized component concentrations and simplifies assay set-up) • Universal thermal cycling parameters (enables multiple assays to be run on the same plate) • Default primer and probe concentrations (to eliminate assay optimization).
Reference Gene	An active fluorescence signal used to normalize experimental results. Endogenous and exogenous controls are examples of active references. An active reference means the signal is generated as the result of PCR amplification using its own set of primers/probe.
Standards	A sample of known concentration used to construct a standard curve.
TaqMan® Gene Expression Assays[3]	TaqMan® Gene Expression Assays are biologically informative, pre-formulated gene expression assays for rapid, reliable detection and quantification of human, mouse and rat mRNA transcripts. Each product is delivered as pre-mixed primers and TaqMan® MGB probe at a 20X concentration
TaqMan® Genotyping Assays[4]	TaqMan® Genotyping Assays are biologically informative, validated primer and probe sets for detection of human SNPs. Each product is delivered as pre-mixed primers and TaqMan® MGB probes at a 20X concentration
TaqMan® MGB probes	Fluorogenic probes that are designed and synthesized as TaqMan® MGB probes contain a minor-groove-binding moiety that enhances the T_m differential between matched and mismatched probes. In addition, TaqMan® MGB probes contain a nonfluorescent quencher that provides enhanced spectral resolution when using multiple dyes in a reaction. TaqMan® MGB probes are ideal for use in both gene expression and SNP analysis assays using the 5' nuclease assay.
Target	An RNA or DNA sequence, or gene of interest.
Test sample	A sample compared against a calibrator as a means of testing a parameter change (for ex., the expression level of a gene) after an intervention such as a drug treatment, tumor transformation, growth factor treatment and so on.
Threshold	A level of normalized reporter signal that is used for C_T determination in real-time assays. The level is set to be above the baseline but sufficiently low to be within the exponential growth region of an amplification curve. The cycle number at which the fluorescence signal associated with a particular amplicon accumulation crosses the threshold is referred to as the C_T.

[1] Also referred to as TaqMan® Assays-By-Design® for Gene Expression Products.

[2] Also referred to as TaqMan® Assays-By-Design® for SNP Assays.

[3] Also referred to as TaqMan® Assays-on-Demand™ Gene Expression Products.

[4] Also referred to as TaqMan® Assays-on-Demand™ SNP Genotyping Products.

Applied Biosystems

4. Relative Quantitation of Gene Expression Requires Quantitation of Two Different Genes (Target and Endogenous Control)

To obtain accurate relative quantitation of a mRNA target, it is recommended to also evaluate the expression level of an endogenous control. By using an endogenous control as an active reference, you can normalize quantitation of targets for differences in the amount of total nucleic acid added to each reaction. For example, if you determine that a calibrator sample has a two-fold greater amount of endogenous control than a test sample you would expect that the calibrator sample was loaded with two-fold more cDNA than the test sample. Therefore, you would have to normalize the test sample target by two-fold to accurately quantify the fold-differences in target level between calibrator and test samples. Some factors that can cause total RNA sample loading differences are:

- Imprecise RNA measurement after extraction
- RNA integrity
- Inaccurate pipetting

For detailed information regarding endogenous controls, see
"Identification and Selection of Endogenous Controls for Relative Quantitation".

5. Factors Affecting Accurate Real-Time PCR Results

A variety of factors must be considered when setting-up real-time PCR reactions. During the initial set up it is important to include identical replicates for each input amount. The use of these replicates can help in identifying precision issues. After performing a real-time PCR run, you can gauge the accuracy of the results. If identical replicate samples have a C_T standard deviation >0.3 and/or a standard curve has a correlation coefficient (R^2 value) <0.99, the accuracy of the data is questionable. Some experiments may only tolerate low variation among identical replicates, for example, if you are looking for low-fold changes in target expression. It is important to appreciate that due to statistical distribution there is always a high level of C_T variation when target quantities approach single copy (C_T values of 34 - 40). Therefore, sample masses that yield C_T values in this range will unavoidably give rise to poorer precision and consequently less power to detect low-fold changes.

The following practices help to achieve accurate real-time PCR results.

a. Use high quality RNA
Poor quality RNA samples can lead to spurious real-time PCR results. Poor quality RNA preparation can be characterized by one or more of the following:

Table 1: Effects of a poor quality RNA sample on PCR results

Characteristics of a poor quality RNA sample	Potential impact on PCR results
Co-extracted proteins including RNases	PCR inhibition due to the presence of proteins and/or degradation of RNA template due to the presence of RNases
Carry-over chemicals (ex. Phenol)	PCR inhibition
Degraded RNA template	Loss of detection of rare transcripts
Co-extracted genomic DNA	Can serve as a PCR template and can confound RNA detection results

See Section II entitled **"RNA Preparation and Reverse Transcription"** for information on how to evaluate the quantity and quality of the RNA template and how to characterize the presence of PCR inhibitors and/or genomic DNA.

b. Test sample masses that yield results within the dynamic range of the assay
See "Determination of input RNA amounts to be used in a relative quantitation study"

c. Use the same pool of standards and/or calibrator through the whole study

Fore each study, it is recommended to prepare large pools of standard and calibrator cDNAs then aliquot these cDNAs into single-use tubes. Preparing and using the same pool of standard and calibrator cDNAs through the completion of a study can provide for consistent real-time PCR results.

d. Use reagents that contain the internal reference dye ROX^{TM}

Applied Biosystems software normalizes reporter dye signals to the passive reference dye ROX^{TM}. This normalization can compensate for minor variations in signal strength, which results in better precision. All Applied Biosystems real-time PCR reagents contain ROX^{TM} dye.

e. Use PCR master mixes and PCR reagent cocktails
The use of PCR master mixes and PCR reagent cocktails will help reduce the potential variability introduced from pipetting multiple reagents during setup.
(i) **Real-time PCR master mixes**: Applied Biosystems real -time PCR Master Mixes contain all of the components of the real time-PCR reaction except primers, probe and nucleic acid template. Use of these greatly reduces the chances of introducing pipetting errors during setup.

(ii) **PCR reagent cocktails**: Mix all of the components of a reaction into a reagent cocktail (PCR reagents, primers, probes, water, and so on) then dispense into the wells of a reaction plate. For an example of a real-time PCR reagent cocktail, refer to the tutorial <u>Reconstituting and Diluting Primers and TaqMan® Probes</u>, pages 3 and 4.

f. Perform accurate sample and reagent pipetting

Accurate pipetting with regularly calibrated pipettors is critical to obtaining accurate and precise data. Low volume pipetting (i.e., <5μl) can contribute to imprecision and pipetting of volumes less than this is not recommended, unless the pipettors are designed for these low volumes and are regularly calibrated. It is also recommended to briefly spin down the sealed plates, via low speed centrifugation, prior to running on the machine. The following table lists some of the consequences of inaccurate pipetting.

Table 2: Consequences of inaccurate pipetting

Pipetting problem	Consequence
Sample: Poor pipetting of identical replicates	High C_T standard deviations
Standards: Poor pipetting of standards	High C_T standard deviations (identical replicates), R^2 value <0.99
Standards: Consistent pipetting excess of diluent in serial dilution (ex. 100 μL instead of 90 μL)	Potentially good R^2 value $\geq$0.99, however slope of standard curve will be inaccurate; perceived lower PCR efficiency of assay
Standards: Consistent pipetting deficit of diluent in serial dilution (ex. 80 μL instead of 90 μL)	Potentially good R^2 value $\geq$0.99, however slope of standard curve will be inaccurate; perceived higher PCR efficiency of assay
Standards: Consistent pipetting excess of standard sample in serial dilution (ex. 12 μL instead of 10 μL)	Potentially good R^2 value $\geq$0.99, however slope of standard curve will be inaccurate; perceived higher PCR efficiency of assay
Standards: Consistent pipetting deficit of standard sample in serial dilution (ex. 8μL instead of 10 μL)	Potentially good R^2 value $\geq$0.99, however slope of standard curve will be inaccurate; perceived lower PCR efficiency of assay

g. Thoroughly mix PCR reagents

The various components within a real time PCR reaction can settle out and be distributed unevenly within a reaction set up and can have an effect on precision. It is important to thaw and thoroughly mix all reagents during the set-up procedure.

h. Use assays with high PCR efficiencies

PCR efficiency can be used to gauge the performance of a real-time PCR assay. Poor PCR efficiencies can result in poor quantitation. For information on estimating PCR efficiency, reference the section entitled "What is PCR Amplification Efficiency?".

Applied Biosystems TaqMan® Gene Expression Assays have amplification efficiencies of 100%. For information on the performance of TaqMan® Gene Expression Assays, reference Amplification Efficiency of TaqMan® Gene Expression Assays.

High PCR amplification efficiencies (near 100%) can be achieved if Applied Biosystems rapid assay development guidelines are followed. The guidelines recommend:

- The use of Applied Biosystems TaqMan® Gene Expression Assays or automated primer and probe design using the Primer Express® Software.
- The use of Applied Biosystems TaqMan® Universal PCR Master Mix or SYBR® Green I PCR Master Mix (provides standardized concentration of components that simplify assay set-up).
- Universal PCR thermal cycling parameters (enable multiple assays to be run on the same plate).
- Default primer and probe concentrations of 900 nM forward primer, 900 nM reverse primer, and 250 nM probe when using either cDNA or DNA as a substrate. A primer optimization study is recommended when using SYBR® Green I reagents due to the non-specific nature of SYBR® Green I reagents detection.

For additional information see Optimizing primer and TaqMan® probe concentrations.

Applied Biosystems

i. Set appropriate baselines and thresholds

To obtain accurate C_T values, it is critical to set appropriate baselines and thresholds. Some Applied Biosystems real-time PCR systems software packages can perform Auto C_T and Auto Baseline calculations. If your software does not have Auto C_T and Auto Baseline, or if you choose not to use this feature you need to set the baseline thresholds manually. For guidance on manual baseline and threshold settings, refer to the appropriate system's User's Guide. ABI PRISM® 7700 Sequence Detection System users can refer to "Data Analysis on the ABI PRISM® 7700 SDS: Setting Baselines and Thresholds".

6. What is PCR Amplification Efficiency?

Note: Accurate estimation of PCR efficiency depends on a variety of reagent, experimental set-up, sample quality, and analysis factors. For a list of factors affecting the quality of real-time PCR results, refer to Factors Affecting Accurate Real Time PCR Results.

PCR amplification efficiency is the rate at which a PCR amplicon is generated, commonly expressed as a percentage value. If a particular PCR amplicon doubles in quantity during the geometric phase of its PCR amplification then the PCR assay has 100% efficiency.

The slope of a standard curve is commonly used to estimate the PCR amplification efficiency of a real-time PCR reaction. A real-time PCR standard curve is graphically represented as a semi-log regression line plot of C_T value vs. log of input nucleic acid. A standard curve slope of –3.32 indicates a PCR reaction with 100% efficiency. Slopes more negative than –3.32 (ex. –3.9) indicate reactions that are less than 100% efficient. Slopes more positive than –3.32 (ex. –2.5) may indicate sample quality or pipetting problems. For additional information, see Identifying PCR Inhibition.

Note: A 100% efficient reaction will yield a 10-fold increase in PCR amplicon every 3.32 cycles during the exponential phase of amplification ($\log_2 10 = 3.3219$).
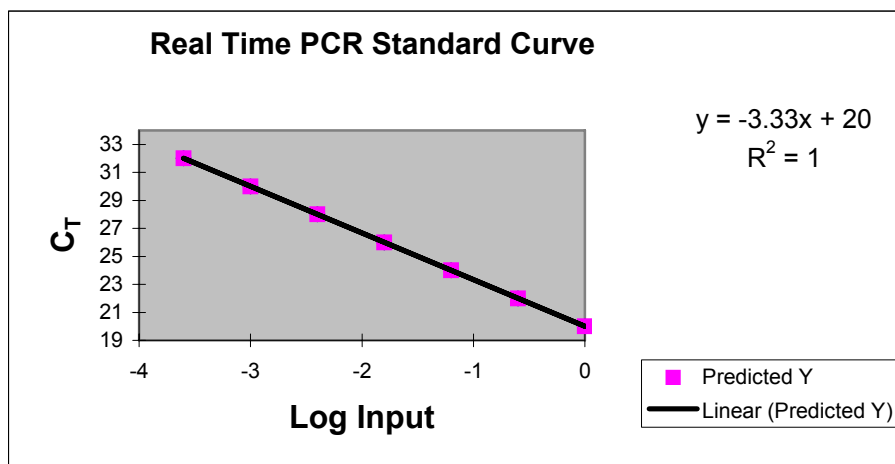
Figure 1: Real-Time PCR Standard Curve representing 100% PCR Efficiency

A calculation for estimating the efficiency (E) of a real-time PCR assay is:

$$E = (10^{-1/slope} - 1) \times 100$$

High PCR amplification efficiencies (near 100%) can routinely be achieved if you follow Applied Biosystems rapid assay development guidelines. PCR efficiency can be used to gauge the performance of a real-time PCR assay. Poor PCR efficiencies can result in poor sample replicate precision (identical replicates & experimental replicates) and as a consequence can result in poor quantitation.

For the comparative C_T method of relative quantitation (discussed later in this tutorial) to be valid, the efficiency of the target amplification and the efficiency of the active reference (endogenous control) amplification must be approximately equal. For more information see "A Validation Experiment is Necessary to Determine if your ΔΔCT Calculation is Valid".

Section II
RNA Preparation and Reverse Transcription

1. Introduction

Successful quantitation of gene expression requires input RNA of high quality. The isolation of high quality RNA is dependent on the selection of an extraction and purification system/method that provides for the highest quality product.

RNA is a very labile molecule. It is susceptible to degradation by RNases, which are highly stable and persistent proteins. Some RNA purification methods may co-extract high levels of proteins - including RNases. Co-extracted proteins and/or degraded RNA can result in spurious real-time PCR results. Extracted RNA that has an $A_{260/280}$ value $\geq$ 2.0 is considered relatively free of protein.

Handling, processing and storing RNA is beyond the scope of this document. For information refer to any standard molecular biology laboratory guide that discusses proper RNA handling.

After high-quality RNA is prepared, the reverse transcription (RT) procedure generates the cDNA used in the real-time PCR process. If you suspect that the RNA contains extracted proteins, (that is, the $A_{260/280}$ value < 2.0), it is recommended that RNase Inhibitor be added to the RT reaction at a final concentration of 1.0 U/µL. If you are using the ABI PRISM™ 6100 Nucleic Acid PrepStation and associated nucleic acid purification reagents you do not need to add RNase Inhibitor to the reverse transcription reactions.

Applied Biosystems has developed a system for the extraction and purification of RNA (also for separate DNA methods) that yields RNA (or DNA) that is of extremely high quality, and has minimal contaminating proteins. This system, the [ABI PRISM™ 6100 Nucleic Acid PrepStation](#), and the [associated chemistries](#), provide the researcher with the highest quality RNA for gene expression analysis (with $A_{260/280}$ values $\geq$ 2.0). The system also co-purifies less than 0.5% genomic DNA when using the PrepStation's RNA chemistries and less than 0.002% genomic DNA if performing the additional in-column DNase treatment with the [Absolute RNA Wash Solution](#).

For information on selecting the chemistry most appropriate for your sample type, see the guide ["ABI PRISM™ 6100 Nucleic Acid PrepStation: Selecting Appropriate Protocols, Reagents and Consumables"](#).

Important: The ABI PRISM™ 6100 Nucleic Acid PrepStation chemistries and consumables cannot be used independent of the instrument.

2. Quantifying Input RNA

Purified RNA in solution can be quantified using its absorbance at 260nm (A_{260}). Typically, a small amount of the RNA is diluted for reading on a spectrophotometer. Refer to the spectrophotometer user's manual for guidance on proper use of the instrument and associated consumables.

Note: If using the ABI PRISM^{TM} 6100 Nucleic Acid PrepStation, the elution solution should be used for diluting the RNA and blanking the spectrophotometer.

The absorption of 1 O.D. is approximately equivalent to 40 µg/mL of RNA. If the A_{260} value is not within the linear range of the spectrophotometer, the quantitation may not be accurate. Refer to the spectrophotometer user's manual for guidance on the linear range of quantitation.

RNA concentration is calculated using the equation:

RNA concentration (µg/mL) = A_{260} × 40 µg/mL × dilution factor

$A_{260/280}$ values can be used to characterize the presence of proteins in an RNA preparation. The $A_{260/280}$ value is calculated simply by dividing the A_{260} by the A_{280} value. If the $A_{260/280}$ value $\geq$ 2.0, the RNA sample is considered to be relatively free of protein. The RNA concentration is then used to calculate the volume of RNA to add to a reverse transcription reaction.

Example:
10 µL of RNA sample A was diluted in 90 µL of diluent. The entire volume of this dilution was pipetted into a 100 µL cuvette, and the spectrophotometer was programmed to read the diluted sample at A_{260} and A_{280}.

Table 1: RNA quantitation by UV absorbance

Sample	Stock volume of RNA preparation	Dilution	A_{260}	A_{280}	$A_{260/280}$	Concentration	Yield
RNA sample A	150 µL	1:10	0.58	0.29	2.0	232 µg/mL	34.8 µg

The <u>RNA concentration</u> of RNA sample A:

= 0.58 (OD_{260}) × 40 µg/mL × 10 (dilution factor) = 232 µg/mL

The <u>Yield</u> of RNA sample A:

= 232 µg/mL × 1 mL/1000 µL × 150 µL (stock volume) = 34.8 µg

The $A_{260/280}$ <u>value</u> of RNA sample A:

$$A_{260/280} = \frac{.58}{.29} = 2.0$$

3. Reverse Transcription for Relative Quantitation of Gene Expression

Reverse transcription is the process by which RNA is used as a template to synthesize cDNA. Among the first options to consider when selecting a method to perform the reverse transcription is whether to use a one-step RT or a two-step RT method. Also, the primer type for the reverse transcription must be selected.

Note: Applied Biosystems reverse transcription reagent kits recommend a range and/or a maximum amount of RNA per reaction. Refer to the appropriate protocol for recommendations of input RNA mass.

Tables 2 and 3 describes key features and benefits of one-step and two-step methods as well as a comparison of priming methods:

Table 2: Comparison of One-Step and Two-Step RT-PCR

Method	Features and Benefits
One-Step RT-PCR	• Requires single reaction mix because RT and PCR occur in the same tube • AmpErase® UNG cannot be used (TaqMan® EZ RT-PCR kit is exception) • May get better limit of detection with rare transcripts • Requires sequence-specific primer for cDNA synthesis.
Two-Step RT-PCR	• Requires two reaction mixes (RT reaction and PCR reaction) • cDNA can be stored for later use • AmpErase® UNG can be used if dUTP is not used in the RT step • By using random primers, can simultaneously reverse transcribe all mRNAs as well as 18S rRNA (that is, targets + endogenous controls) • Can use sequence-specific primer, random primers, or oligo $d(T)_{16}$ for cDNA synthesis.

Table 3: Comparison of primers for cDNA synthesis

Primers for cDNA synthesis	Considerations
Sequence-specific primer	• Use to reverse transcribe complementary RNA sequences only • Only primer type recommended for one-step RT-PCR • Can be used in two-step RT-PCR
Oligo $d(T)_{16}$	• Use to reverse transcribe only eukaryotic mRNAs and retroviruses with poly-A tails • Cannot reverse transcribe 18S rRNA • May have trouble transcribing long mRNA transcripts or transcripts containing hairpin loops • Tends to show bias towards 3' end of transcript
Random Primers (ex. hexamers)	• Can simultaneously reverse transcribe all mRNAs as well as 18S rRNA (i.e. targets + endogenous controls) • Try first for use with long transcripts or transcripts containing hairpin loops • Not biased to 3' end of transcript

Applied Biosystems

4. Selecting Reverse Transcription and Real-Time PCR Reagents

Applied Biosystems offers a variety of reverse transcription and real-time PCR reagents for relative quantitation applications. For information on the portfolio of reagents that are available, refer to the Applied Biosystems Real-Time PCR Systems Chemistry Guide (part# 4361966). The Chemistry Guide provides a comprehensive overview of reverse transcription as well as real-time PCR chemistries available from Applied Biosystems for quantitation studies. Also, you can visit the Applied Biosystems store to review reverse transcription and real-time PCR chemistries.

A common approach for two-step RT-PCR is used by the Applied Biosystems TaqMan$^®$ Gene Expression Assays. The TaqMan$^®$ Gene Expression Assays and Custom TaqMan$^®$ Gene Expression Assays are optimized for use with the High-Capacity cDNA Archive kit (reverse transcription) and the TaqMan$^®$ Universal PCR Master Mix (with or without AmpErase$^®$ UNG). Refer to Appendix A of the High-Capacity cDNA Archive Kit Protocol for an example of high-quality cDNA conversion performance.

Applied Biosystems recommends the use of random primers for performing the two-step RT-PCR method. With this approach, you can generate cDNA from a library of transcripts in a single reverse transcription reaction; therefore, you can perform real-time PCR quantitation of many different targets from one pool of cDNA.

5. Determination of Input RNA Amounts to use in a Relative Quantitation Study

The volume of sample that is needed in the reverse transcription (RT) reaction(s) can be determined by first calculating RNA concentration. Applied Biosystems reverse transcription reagent kits recommend a range and/or a maximum mass of RNA per reaction. Refer to the appropriate protocol for recommendations of input RNA mass. Although reverse transcription does not necessarily occur with 100% efficiency, if reverse transcription reactions are run under the same conditions, the RT efficiencies of each target are comparable across reactions. Refer to appendix A of the High-Capacity cDNA Archive Kit Protocol for an example of high quality cDNA conversion performance.

After RT, it is common not to quantify the resulting cDNA by UV absorbance. Instead the cDNA is assigned a concentration unit relative to the original concentration of RNA in the RT reaction. For example, if one loaded 10 μg of RNA into a 100 μL RT reaction, the designated concentration of the resulting cDNA would be 100ng/μL; which means 1 μL of sample contains the cDNA generated from 100 ng of RNA.

Each time you use a new source of RNA, especially when starting a new assay, it is important to verify that the cDNA mass tested yields real-time PCR results within the linear dynamic range of the real-time PCR assay.

To verify that a template mass yields results within the linear dynamic range of an assay, Applied Biosystems recommends running a relative standard curve. Use the appropriate primer/probe set, with an RNA (cDNA) input range of 100 ng to 10 pg (for example, 100 ng would be 1/10 of a 1μg RT reaction) in a series of ten-fold dilutions (in triplicate) for each dilution. The real-time PCR results (C_Ts) of a standard curve can help determine:

1. **Dynamic Range –** The range of input RNA (cDNA) the assay can detect. For example, in an assay with a high abundance of a particular target, suppose that the 100 ng input reaction yields C_Ts of low numerical values (that is, C_T 3 to10). This would indicate that the amount of input template is too high and that 10 or 1 ng of input material may represent the upper range to use. Conversely, a target with low expression levels (that is, 100 pg or 10 pg) may produce high numerical C_T values (that is, 35 - 40). If this were the case, you would want to run assays with a higher input quantity, for example 1 ng. Due to statistical distribution, a high level of C_T variation always occurs when target quantities approach a single copy (C_T values 35 to 40). Sample masses that yield C_T values in this range have poor precision, consequently low-fold changes are more difficult to accurately quantify.

2. **Assay Precision –** The replicate samples at each input amount will help in identifying potential precision issues that may need to be addressed. Replicate samples that have a C_T standard deviation >0.3 may indicate set-up concerns such as pipetting, mixing, or plate set-up factors. Some experiments may tolerate only low identical replicate variation, if the user is looking for low-fold change variations in target expression. When you evaluate the standard curve, a R^2 value <0.99 may indicate precision problems.

3. **Assay Efficiency –** This relative standard curve can assist in determining the efficiency of the assay. Review the section "What is PCR Amplification Efficiency" for more information.

6. Identifying PCR Inhibition

Note: Accurate real-time PCR results depend on a variety of reagent, experiment set-up, sample quality, and analysis factors. For a list of factors affecting quality real-time PCR results, refer to "Factors Affecting Accurate Real-Time PCR Results."

High protein levels as well as other chemical contaminants in RNA preparations can cause PCR inhibition. As discussed in the previous section, $A_{260/280}$ values < 2.0 may indicate high protein levels in an RNA preparation. An $A_{260/280}$ value < 2.0 does not predict that protein levels are high enough to cause PCR inhibition, but, the more the $A_{260/280}$ deviates from 2.0, the greater the chance of obtaining spurious results.

You can use real-time PCR data from standard curve plots to identify if inhibition is occurring at a level that causes spurious results. When used to characterize inhibition, these semi-log standard curves (page 20) are referred to as inhibition plots.

AB Applied Biosystems

Example: Inhibition Plot
In the two sample experiments that follow, RNA was purified using two different extraction methods. The RNAs were quantified by UV absorbance. RNA was taken from each preparation and reverse transcribed then a series of dilutions were prepared from each cDNA stock (Table 4).

Table 4: Dilutions of cDNAs used in Inhibition Plots

Dilutions	Mass of Sample 1 (μg)	Mass of Sample 2 (μg)
#1	1.0	1.0
#2	0.25	0.25
#3	0.063	0.063
#4	0.016	0.016
#5	0.0039	0.0039
#6	0.00098	0.00098
#7	0.00024	0.00024

The cDNAs were used for real-time PCR.

Results

The real-time PCR data generated from samples 1 and 2 are shown in table 5 and in Figures 2 and 3 on page 20.

Table 5: Real-time PCR data (C_T) from inhibition plot experiment

Mass of Samples (μg)	Log Input	Average C_T, sample 1	Differences between C_T values , sample #1 (ex. 22 minus 20)	Average C_T, sample 2	Differences between C_T values, sample #2 (ex. 27 minus 28)
1.0	0.0	20	N/A	28	N/A
0.25	−0.6	22	2.0	27	−1.0
0.063	−1.2	24	2.0	26	−1.0
0.016	−1.8	26	2.0	26	0.0
0.0039	−2.4	28	2.0	28	2.0
0.00098	−3.0	30	2.0	30	2.0
0.00024	−3.6	32	2.0	32	2.0
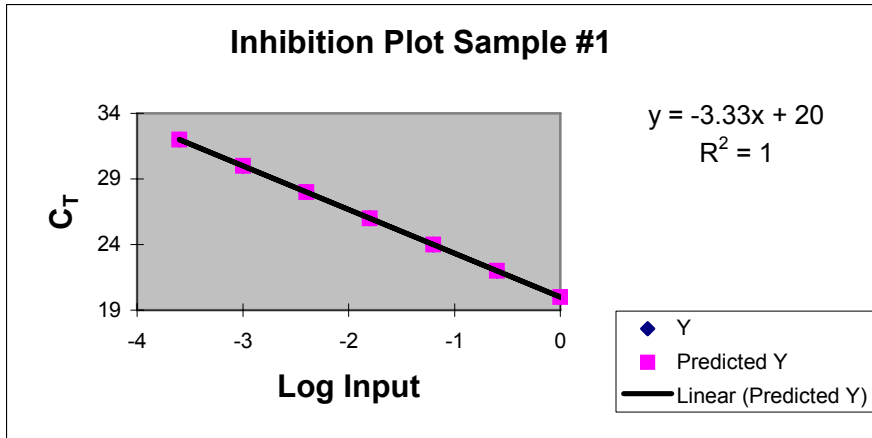
Figure 2: Inhibition Plot Sample #1 demonstrating no inhibition
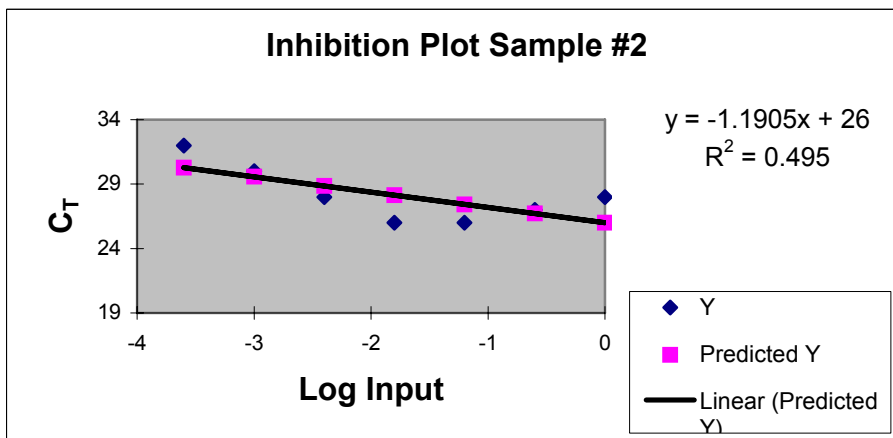
Figure 3: Inhibition Plot Sample #2 demonstrating presence of inhibitor

The data in Inhibition Plot #1 show a real-time PCR assay amplifying with 100% efficiency (slope = –3.3), without inhibition. Inhibition Plot #2 has a slope that is much more shallow, and the PCR efficiency is calculated to be greater than 100% (592%). A slope suggesting a PCR efficiency greater than 100%, may be an indicator of PCR inhibition. The slope in Figure 3 is caused by inhibitors that are highly concentrated in the less-diluted cDNA samples. As the cDNA is titrated, inhibitors are diluted as well. As a result, the less-diluted samples may have higher C_T values then the next dilution, and/or the predicted C_T difference is not achieved (i.e. in this example a four-fold change should yield a +2 C_T difference). This inhibition pattern can be less dramatic when the calculated efficiency is closer to, but still above, 100% efficiency. Closely examine the C_T differences between each result to observe inhibition. Because the inhibitors are most concentrated in the least-diluted samples, the most dramatic inhibition should be observed in the samples representing the standard curve points with the highest template concentration.

If the efficiency of the real-time PCR reaction is calculated to be above 100%, but there is still a consistent C_T difference between each standard point, then a consistent

pipetting error (standard sample or diluent) may be at fault rather than inhibitor effects. Refer to the "Perform accurate sample and reagent pipetting" section for additional information.

If you determine that PCR inhibitors are in your RNA sample(s), do one of the following.

1. Further purify the RNA preparation.
2. Perform RNA purification process on a sample using a new purification method.
3. Test your sample at a lower template concentration where it is known that PCR inhibition does not affect the real-time PCR results.

7. How Much Genomic DNA Contamination Can Be Tolerated in a Relative Quantitation of Gene Expression Assay?

Genomic DNA is often co-extracted with RNA and can serve as a template in downstream processes such as PCR. However, if your TaqMan® MGB probe spans an exon-exon junction, genomic DNA can be excluded as a template in a real-time PCR reaction.

In contrast, if both primers are designed within one exon, then genomic DNA could serve as a template for PCR amplification. In these cases, the user has to decide if the genomic DNA is sufficiently negligible.

RT reactions without reverse transcriptase (No RT controls) can be used to evaluate levels of genomic DNA in a RNA preparation. A No RT control is a reaction that has been prepared for reverse transcription (including RNA, dNTPs, buffer and so on) but no reverse transcriptase is added. One can estimate the amount of amplification in their samples that is attributable to genomic DNA templates by running No RT controls. For example, if a No RT control sample has a C_T value 10 cycles higher than an RT test sample, then the No RT control sample started out with approximately 1000-fold less target sequence (assuming 100% efficiency, 1 $C_T \approx$ 2-fold difference in initial template amount). Since the target template in this No RT control would exclusively be genomic DNA, one may conclude that 0.1% (1:1,000) of the amplification in the RT sample is attributable to the genomic DNA template. You will then have to determine if the PCR amplification attributable to the genomic DNA is sufficiently negligible compared to the amplification of the cDNA sequence.

If you determine that the amount of genomic DNA in your RNA samples is unacceptably high, do one of the following:

1. Purify the RNA using a new purification method.
2. Perform a DNase treatment, or repeat DNase treatment if one was already performed.

Note: The ABI PRISM 6100 Nucleic Acid PrepStation co-purifies less than 0.5% genomic DNA when using the PrepStation RNA chemistries and less than 0.002% genomic DNA when using the additional in-column DNase treatment with the AbsoluteRNA Wash Solution.

Applied Biosystems

21

Section III
Assay Selection and Design

Assay Selection and Design for Real Time PCR Quantitation

Among the factors to consider when designing a relative quantitation study are:

- Selection or design of primers and probes for the target gene(s).
- Identification and selection or design of primers and probes for appropriate endogenous control (housekeeping) gene(s).

Selecting or Designing Primers and TaqMan® Probes for Relative Quantitation of Gene Expression

Relative quantitation studies require optimally designed primer and probes sets. When all assays are designed under the same guidelines, all assays can be run at the same time. This universality of design, which is central to all the Applied Biosystems assays offerings, as well as the Primer Express® Software, requires little, if any optimization, provided the assays are run in the TaqMan® Universal Master Mix on an Applied Biosystems Real-Time PCR System. Applied Biosystems has a number of products available as well as a set of guidelines to follow when selecting or designing an assay.

1. TaqMan® Gene Expression Assays
2. Custom TaqMan® Gene Expression Assays
3. TaqMan® Pre-Developed Assay Reagents
4. Primer Express® software for custom designed assays
5. Design of Assays for SYBR® Green I reagent applications

Note: The use of primers that have been used in traditional PCR assays is not recommended. The criteria of successful design for real-time PCR may not have been used in the design of primers used in other applications. The chances of success are much higher if you re-design or select assays that have been designed using Applied Biosystems Rapid Assay Development Guidelines.

1. TaqMan® Gene Expression Assays (TaqMan® Assays-on-Demand™ Gene Expression Products)

TaqMan® Gene Expression Assays are biologically informative, pre-formulated gene expression assays used for rapid, reliable detection and quantification of human, mouse and rat mRNA transcripts. These assays have been designed from the annotation of genes from both the public sequence databases and Celera Genomics. Each assay contains pre-formulated primers and TaqMan® probes in a 20X concentration. Each tube supports 250 reactions at a 20 μL reaction volume or 100 reactions at a 50μL reaction volume. The TaqMan® Gene Expression Assays are designed to be used in two-step RT-PCR.

Note: TaqMan® Gene Expression Assays contain FAM™ dye-labeled probes and are optimized for singleplex reactions. Applied Biosystems recommends using the High-Capacity cDNA Archive Kit for reverse transcription and the TaqMan® Universal PCR Master Mix for PCR.

These products utilize TaqMan® MGB probes to provide for optimally designed assays. To obtain additional information regarding the processes that are used in the selection, design, and testing of these products see "TaqMan®Gene Expression Assays Design Schematics". For information on the performance of TaqMan® Gene Expression Assays, reference Amplification Efficiency of TaqMan® Gene Expression Assays.

TaqMan® Gene Expression Assays Nomenclature:

Each TaqMan® Gene Expression Assay is assigned an **Assay ID**.

The first two positions designate the species for which the assay was designed:

Hs = *Homo sapiens*   **Mm** = *Mus musculus*   **Rn** = *Rattus norvegicus.*

The second-to-last position in the Assay ID contains a letter that indicates the assay placement:

Assay Suffix	Assay Placement
_m	An assay whose probe spans an exon-exon junction of the associated genes and will not detect genomic DNA
_s	An assay whose primers and probes are designed within a single exon, such assays will detect genomic DNA
_g	An assay whose probe spans an exon-exon junction, but the assay may detect genomic DNA if present in the sample.
_mH, _sH, or _gH	The assay was designed to a transcript belonging to a gene family with high sequence homology. The assays are designed to give between 10 C_T and 15 C_T difference between the target gene and the gene with the closest sequence homology. An assay, therefore, will detect the target transcript with 1,000 to 30,000-fold greater discrimination (sensitivity) than the closest homologous gene.

Note: RNA samples should be free from genomic DNA when using an "s" or a "g" assay.

For example, the TaqMan® Gene Expression Assay for human GAPDH, **Hs99999905_m1**, indicates that this is a human multi-exonic gene assay that does not detect genomic DNA.

You can view, select, and purchase TaqMan® Gene Expression Assays through the Applied Biosystems web site.

The TaqMan® Gene Expression Assays: Product Quick Card describes how to search and order assays through the **myScience research environment** of the Applied Biosystem's website.

Additional information about the ordering process and appropriate documentation can be found in the Appendix section entitled "Ordering TaqMan® Gene Expression Assays".

AB Applied Biosystems

2. Custom TaqMan® Gene Expression Assays (TaqMan® Assays-by-Design℠ for Gene Expression Service)

Custom TaqMan® Gene Expression Assays are custom-designed assays for expression studies. Custom TaqMan® Genotyping Assays are used for SNP assays. These custom assay products (Gene Expression and Genotyping) provide the researcher the opportunity to select a specific sequence site to which the probe binds and is useful for the design of an assay that is not currently available through the TaqMan® Assays offerings. Applications that involve viral detection, species other than human, mouse, or rat, detection of specific pathogens, and splice junction variants are some examples of applications that would benefit from this custom design line of products. For gene expression assays on human, mouse, or rat samples, the TaqMan® Gene Expression Assays (TaqMan® Assays-on-Demand™ Gene Expression Products) are available. If a particular gene target is currently not available, you should consider a custom design. Note: Additional TaqMan® Gene Expression Assays are regularly added to the web site for ordering. Visit the [Applied Biosystems web site](#) for regular updates.

The Custom TaqMan® Gene Expression and Genotyping Assays (TaqMan® Assays-by-Design℠ Service) require the that you verify the integrity of the sequence you want to submit by using several bioinformatics searching tools. After you verify the sequence integrity, you can submit your order on-line. Applied Biosystems returns a QC-verified, all-in-one tube (primers + probe(s)), TaqMan® MGB genomic assay that is ready to use with TaqMan® Universal PCR Master Mix and ready to load on your Applied Biosystems real-time PCR platform. The Custom TaqMan® Assays enables you to collect significant experimental data without the labor and time required to design and test home-brew assays.

Currently, three different synthesis scales are available. For more information, see the Appendix section entitled ["Key Features of Custom TaqMan® Gene Expression Assays"](#).

The process of submission is very straight forward and requires the following steps:

a. Verify the Quality of your Sequence (important for all custom design including the use of the Primer Express® software).
Prior to submission, it is important to perform an upfront "bioinformatics check" on the sequence that you intend to submit. One tool that should be used is Repeat Masker, available at [RepeatMasker Home Page](#). Repeat Masker screens DNA sequences for interspersed repeats (ex. ALU sequences) and DNA sequences of low-complexity. The output is a modified version of your sequence in which all the repeats and low-complexity sequences are masked (replaced by Ns). The Custom TaqMan® Assays design software accepts sequences with A, C, T, G or Ns, and primers and probes are not designed over regions containing Ns.

Also, you should BLAST your sequence against appropriate databases (for example, genomic and EST databases) to identify regions of the sequence that have a high degree of identity with other portions of the genome, or high homology within a gene family. If you are working in a transgenic system, you must ensure that your primers and probes are unique to the species of interest if you want to differentiate between species. Regions of high identity can be masked (substituted) with Ns prior to BLAST submission. BLAST can be performed by visiting http://www.ncbi.nlm.nih.gov/BLAST.

AB Applied Biosystems

Refer to the Submission Guidelines protocol for more information on assessing sequence quality.

The sequencing of a variety of genomes and the extensive annotation tools developed by Celera Genomics provides a unique opportunity for subscribers of the Celera Discovery System™ Online Platform. Subscribers can verify sequence integrity, confirm SNPs, and also have access to a variety of other genomics tools.

b. Submit the sequence.

After you verify the sequence integrity, your sequence(s) is entered into the File Builder software and submitted for ordering. Download File Builder and view the demonstration of its use before submitting your sequence.

Sequence records require at least one coordinate site. For gene expression assay submissions, the coordinate identifies a target site for the assay. Specifically, coordinates are requested locations for probe design. Having a TaqMan® MGB probe designed over an exon-exon junction should exclude detection of genomic DNA in a real-time PCR reaction. However, if the gene of interest is a single exon gene, select multiple sites across the sequence to ensure optimal design.
Note: At least one coordinate is required for each sequence record. If multiple coordinates are submitted, the best assay is designed, synthesized and shipped. Customers receive only one assay back for each submitted sequence. If an assay could not be designed or did not pass the extensive QC analysis, the user is not sent the assay and is not charged.

You can upload your sequence submission file to the Applied Biosystems online store directly from the File Builder software. You must be a registered user to upload your TaqMan® Gene Expression Assay order. To register, go to the Applied Biosystems Store and select "Register Now".
After you complete the registration, Applied Biosystems Order Administration sends an e-mail within 48 hours confirming your registration. You then can submit your TaqMan® Gene Expression Assay orders via the web.

Additional product information and relevant documentation can be found in the Appendix in "Key Features of Custom TaqMan® Gene Expression Assays".

3. TaqMan® Pre-Developed Assay Reagents (TaqMan® PDARs)

TaqMan® Pre-Developed Assay Reagents (TaqMan® PDARs) are primer and probe sets designed to amplify specific target and control sequences in cDNA samples using the 5' nuclease assay. TaqMan® PDARs are available for human, mouse, and rat gene targets. In addition, TaqMan® PDARs are optimized for use with the TaqMan® Universal PCR Master Mix with (P/N 4304437) or without AmpErase® UNG (P/N 4324018) and use identical thermal cycling conditions as all other AB assays. As a result, multiple TaqMan® PDAR targets can be assayed on a single reaction plate. Certain TaqMan® PDAR endogenous control formulations are available as primer limited and contain probes labeled with the VIC® reporter dye. This allows multiplexing of TaqMan® PDAR controls with TaqMan® target primer and probe sets, provided that the control gene is more abundantly expressed than the target gene.

Additional information on these products is found in the Appendix in [TaqMan® Pre-Developed Assay Reagents (PDAR), Protocols and Ordering](#).

4. Use of Primer Express® Software for the Design of Primer and Probe Sets for Relative Quantitation of Gene Expression.
Note: Verifying the quality of the sequence used for primer and probe design is very important. For additional information please refer to [Verifying the Quality of your Sequence](#).

If an assay does not exist for a particular application, a manual design is necessary. When a design must be at a very specific location that is not amenable to automated design, then a manual design may be necessary, i.e., allele specific PCR, pathogen detection assays that need to be comprehensive for various strains, etc. The Primer Express software is a primer and probe design software that allows you to custom design primer and probe sets for a variety of assay types – including relative quantitation. Applied Biosystems has a number of design guidelines that are featured within the Primer Express software program. The Primer Express software is included with the purchase of any Applied Biosystems real-time PCR system. Full utilization of Primer Express software is beyond the scope of this document, however, the reader is referred to several web tutorials to assist in the practical usage of Primer Express software for the design of primers and probes.

[**Designing TaqMan® MGB Probe and Primer Sets for Gene Expression Using Primer Express® Software Version 2.0**](#)
This tutorial details how a TaqMan® MGB probe can be designed over a specific region of a template sequence such as an exon-exon junction (intron splice-site).

[**Primer Express® Tabs and Annotation Tools: An Overview of the Features of Primer Express® v2.0**](#)
This tutorial provides an overview of the Primer Express Software.

5. Design of Assays for SYBR® Green Reagent Applications

SYBR® Green I is a fluorescent dye that binds to the minor groove of double-stranded DNA (i.e., amplicons) and fluoresces when bound to DNA and excited by a light source. Because no probe is involved in these reactions, there are some disadvantages to its use; specifically:

- SYBR® Green I dye also binds and fluoresces when bound to primer dimers and non-specific amplification products. As a result, additional optimization steps are needed to ensure robust amplification and accurate quantitation.
- SYBR® Green reagents cannot be used in multiplex assays. In gene expression assays, the endogenous control would have to be run in a separate well. Also, use of SYBR® Green reagents for SNP (allelic discrimination assays) is not supported by Applied Biosystems.

However, SYBR® Green reagent is useful as a screening tool if you want to quickly assess the relative expression levels of a variety of genes in a variety of sample types. After you obtain preliminary data, it is recommended to perform a TaqMan® probe-based assay to achieve more highly accurate quantitative results.

For SYBR® Green reagent assay development it is recommended to design the assay using the Primer Express® software. Submit only the primers for synthesis. However, should a TaqMan® probe-based assay be run in the future, the probe sequence compatible with the primer set would already have been designed.

For more information on the use of SYBR® Green reagent applications and products available from Applied Biosystems, refer to the following protocols and tutorial:

SYBR® Green PCR and RT-PCR Reagents

SYBR® Green PCR Master Mix and RT-PCR Reagents

Primer Express® Software v2.0: Designing Primers and TaqMan® TAMRA™ Probes for 5' Nuclease Assays and Primers for Real Time PCR Assays Using SYBR® Green Dye.
Note: This tutorial does not cover strategies on how to design primers to exclude genomic DNA as a PCR template.

Section IV
Identification and Selection of
Endogenous Controls for Relative Quantitation

One of the most important steps in relative quantitation experimental design is the selection of an appropriate endogenous control. Normalization to an endogenous control (often referred to as a housekeeping gene) allows you to correct results that can be skewed by differing amounts of input nucleic acid template. Any gene shown to be expressed at the same level in all study samples can potentially be used as an endogenous control.

Selection of an endogenous control requires three criteria:

1. Uniformity of Endogenous Control Expression Levels

Because the endogenous control is used to normalize differences in the amount of cDNA that is loaded into PCR reaction wells, endogenous control expression levels must be similar in all samples in the study. So, it is critical to determine if the study treatment or intervention is affecting the expression level of your candidate endogenous control gene(s). Using real-time PCR, you can test endogenous controls for uniform expression by comparing the endogenous control C_T levels in several or all study samples. In the test, you must load identical amounts of cDNA for each test sample. It is important to normalize RNA loading by some external measurement, such as UV absorbance. Then, load equivalent amounts of RNA into RT reactions and take equivalent volumes of the cDNA for the real-time PCR reactions. Endogenous control gene expression levels should vary only slightly.

Note: RNA purity and integrity are also critical for this analysis. High levels of co-extracted protein can result in spurious results due to PCR inhibition. Highly degraded RNA can also result in spurious results. See **"RNA Preparation and Reverse Transcription"**.

Sample masses should span the target gene's expected range of expression in the study. It is important to demonstrate that while the target's expression levels may vary widely, expression of the endogenous control remains constant.

In summary, to verify the uniform expression of endogenous controls:

a. Select candidate endogenous controls and obtain assays (primer and probe sets).

b. Purify RNA from samples in the study.

c. Use an external method (such as UV absorbance) to quantify RNA levels.

d. Verify RNA integrity (gel electrophoresis) and quality ($A_{260/280}$).

e. Load equivalent amounts of RNA from study samples into RT reactions.

f.   Load equivalent volumes of cDNA from RT reactions into real-time PCR reactions for all candidate endogenous controls.

g.   Analyze and interpret results*.

h.   Select endogenous control(s) to use in the study.

*For assistance in the interpretation of results, see section 5 of the [TaqMan® Human Endogenous Control Plate Protocol](#).

The Applied Biosystems® [TaqMan® Human Endogenous Control Plate](#) (P/N 4309920) allows users to test several human samples simultaneously for the expression of 11 candidate human endogenous controls that cover a broad range of biological functions. This 96-well plate is pre-loaded with lyophilized primers and probes for 11 different housekeeping genes. In addition, one column of wells is dedicated to the detection of an artificial IPC (internal positive control) sequence to help interpret aberrant results caused by PCR inhibitors. After interpreting the results of the control plate, you can order the primers and probes for the selected human housekeeping gene(s) as a TaqMan® Gene Expression Assay (see ["TaqMan® Gene Expression Assays"](#)).

A variety of mouse and rat endogenous controls are also available as TaqMan® Gene Expression Assays and can be viewed on the [Applied Biosystems web site](#).

2.   **Validation of Target and Control Genes' Amplification Efficiency for the Comparative C_T Method**

If performing the comparative C_T method (also known as the $\Delta\Delta C_T$ method), ensure that the target(s) and endogenous control have similar or relatively equivalent PCR efficiencies. See ["A Validation experiment is necessary to determine if your ΔΔCT calculation is valid"](#) for an explanation of the method commonly used to evaluate the relative PCR efficiencies of target and endogenous control reactions. The validation experiment is also discussed on page 14 of [User Bulletin #2: Relative Quantitation of Gene Expression](#).

3. Multiplexing Endogenous Controls and Target Genes

When you undertake a multiplex assay, ensure that:

- The expression level of the endogenous control gene is greater than that of the target gene.

- The gene that is more highly expressed (i.e. endogenous control) is set-up with its primers at a limiting level. This ensures that the amplification of the less abundantly expressed target is not compromised by the amplification of the more abundant target. The exclusion of this competition between targets is required for accurate quantitation of both genes. To obtain additional information regarding multiplexing, refer to User Bulletin #5: Multiplex PCR with TaqMan$^®$ VIC$^®$ Probes.

  Certain TaqMan$^®$ PDAR Endogenous Control formulations are available as primer-limited assays that contain probes labeled with the VIC$^®$ reporter dye. This allows multiplexing of TaqMan$^®$ PDAR controls with TaqMan$^®$ target primer and probe sets, provided that the control gene is more abundantly expressed than the target gene.
  Note: TaqMan$^®$ Gene Expression Assays are optimized for singleplex reactions only.

To test that the endogenous control is more abundantly expressed than the target gene, use test samples that span the expected range of target gene expression. Run separate control and target reactions for each sample and ensure that all endogenous control C_Ts are lower than target C_Ts. If this is not the case, you can do either of the following:

- Repeat using another endogenous control that is consistently expressed in the sample set to be tested.

- Conduct the study by running the control and the target in separate wells (singleplex).

Additional product and documentation information is available in "TaqMan® Human Endogenous Control Plate Assays and Protocols".

AB Applied Biosystems

Section V

Customized and **Pre-configured**
Relative Quantitation Products

Applied Biosystems offers plates and low density arrays that are either pre-configured with specific primer and probe sequences or that allow a researcher to configure the product to have specific target sequences. These products streamline the workflow process, reduce the cost of reagents and eliminate the design and optimization steps often necessary when using non-customized products. Currently, four products are available:

- TaqMan® Low Density Arrays
- TaqMan® Low Density Immune Profiling Array
- TaqMan® Cytokine Gene Expression Plate
- TaqMan® Human Endogenous Control Plate

1. TaqMan® Low Density Arrays (7900HT Micro Fluidic Card)

TaqMan® Low Density Arrays are an efficient, flexible technology that enables real-time PCR in a low-density array format and streamline gene expression using the ABI PRISM® 7900HT Sequence Detection System. When you perform studies on whole genome arrays, such as the Applied Biosystems Expression Array System, the TaqMan® Low Density Arrays provide an excellent approach for microarray validation. TaqMan® Low Density Arrays are designed for custom assay configuration using TaqMan® Gene Expression Assays. The low-volume configuration reduces the cost of reagents. The wide selection of formats allows the selection of different numbers of targets, and offers flexibility in the number of samples and replicates run on each low density array.

After you receive your configured TaqMan® Low Density Arrays, combine your cDNA sample(s) with TaqMan® Universal PCR Master Mix, load cDNA(s) into the 8 ports, centrifuge into the 384 micro chambers, seal, and run on the 7900HT system (equipped with a TaqMan® Low Density Array upgrade) to receive high quality real-time PCR results. This process eliminates labor-intensive pipetting steps and allows you to receive high throughput, gold standard 5'-nuclease assay results without liquid-handling robotics.

Ordering TaqMan® Low Density Arrays

For instructions on configuring and ordering TaqMan® Low Density Arrays, see "How to Order TaqMan® Low Density Arrays."

For additional product and documentation information, see "Applied Biosystems TaqMan® Low Density Arrays".

2. Pre-Configured TaqMan® Low Density Arrays (Immune Profiling)

The [Pre-Configured TaqMan®Low Density Immune Profiling Array](#) contains 96 TaqMan® Gene Expression Assays for the gene expression analysis of targets known to have implications in the immune response in humans.

3. TaqMan® Cytokine Gene Expression Plate

The [TaqMan®Cytokine Gene Expression Plate](#) is a 96-well reaction plate pre-loaded with lyophilized primers and probes for 12 human cytokine targets and 18S rRNA (as an endogenous control). The primers and probes are dried down in the bottom of the wells, and each target primer and probe set is located in 8 wells. The 18S rRNA control assay is present in each well in a primer-limited format to allow for multiplex analysis of target and control. To use the plate, simply add TaqMan® Universal PCR Master Mix and cDNA to the wells and run the plate on a Applied Biosystems Real-Time PCR System. The user can generate relative expression profiles in which expression levels are calculated relative to a calibrator sample, using the Comparative C_T Method ($\Delta\Delta C_T$ Method).

Cytokine targets:	IL1α	IL-4	IL-10	IL-15
	IL1-β	IL-5	IL-12p35	IFN-γ
	IL-2	IL-8	IL-12p40	TNF-α

Additional key features as well as appropriate documentation is available in "[TaqMan® Cytokine Gene Expression Plate Product Information and Protocols](#)".

4. TaqMan® Human Endogenous Control Plate

Refer to ["Identification and Selection of Endogenous Controls for Relative Quantitation"](#).

Section VI

Ordering Real-Time PCR Reagents

To order Applied Biosystems reagents including custom primers and TaqMan® probes, go to the Applied Biosystems Store.
Note: You must be registered to order Applied Biosystems reagents via the web. To register, go to the Applied Biosystems Store Registration. After you complete the registration, Applied Biosystems sends an e-mail within 48 hours confirming your registration. You can then place an order.

To order Real-Time PCR reagents and consumables in the Applied Biosystems Store:

1. Select SDS/Real Time PCR in the Store Catalog, then locate the items you want to purchase.
2. Select the boxes for the products to be ordered (on the left side of the Product Names).
3. After you select all the products, select Add to Basket.
4. To process the order, select the Shopping Basket link at the top of the page. Select Proceed to Checkout, then fill out the requested information to complete the order.

To order Sequence Detection Primers and TaqMan® probes:

1. Select ABI Prism® Primers/Probes in the Store Catalog.
2. Select TaqMan® Primers & Probes and locate the items you want to purchase.
3. Select the boxes for the products to be ordered (on the left side of the Product Names).
4. After you select all the products, select Add to Basket.
5. Select the Shopping Basket link at the top of the page.
6. The products will now be itemized in the shopping basket.
   a. Select the "customize" button next to the product name for each custom primer and TaqMan® probe.
   b. Follow the instructions to enter the sequence of the primer or TaqMan® probe.
   c. Repeat this process for all custom primers and TaqMan®probes.
7. To process the order, select Proceed to Checkout, then fill out the requested information to complete the order.

If you have questions on placing an order, contact your local Applied Biosystems Sales Representative. You may locate your regional office or contact person through our Contact Us page on the Applied Biosystems website.

Section VII

Relative Quantitation of Gene Expression
Experimental Design and Analysis:
Relative Standard Curve Method and
Comparative C$_T$ Method (∆∆Ct)

1. Introduction
Applied Biosystems supports two experimental design and analysis methodologies for relative quantitation of gene expression.

- Relative Standard Curve Method
- Comparative C$_T$ Method (∆∆Ct)

Before you begin your experimentation, consider which method suits your needs. Depending on a variety of factors, one method may be more appropriate than the other. The advantages of each method and factors to consider are described below.

Relative Standard Curve Method
Advantage: This method requires the least amount of validation because the PCR efficiencies of the target and endogenous control do not have to be equivalent.
Considerations (and Applications): This method requires that each reaction plate contain standard curves, and requires more reagents and more space on a reaction plate. This approach gives highly accurate quantitative results because unknown sample quantitative values are interpolated from the standard curve(s).
Consider this method when testing low numbers of targets and small numbers of samples and if you are looking for very discrete expression changes.

Comparative C$_T$ Method (∆∆C$_T$)
Advantage: Standard curves are not required to run on each plate. This can result in reduced reagent usage.
Considerations (and Applications): TaqMan® Gene Expression Assays have amplification efficiencies of 100%, and because of this, when using TaqMan® Gene Expression Assays, calculated fold change values correlate better to expected fold change values when the comparative C$_T$ method is used. If you are using custom primers and probes, an initial validation relative standard curve is recommended to validate the PCR efficiencies of the target and endogenous control(s), particularly when you are looking for low-expression-level fold changes.
The comparative C$_T$ method is useful when a high number of targets and/or number of samples are tested. Consider this method when using a high throughput strategy and when validating microarray results. For additional information on validating microarray results, see 5' Nuclease Assays for Validating Hits from Fluorescent Microarrays.
Applied Biosystems offers real-time PCR software products that perform the ∆∆C$_T$ calculations in addition to real-time PCR plate set-up and analysis. This software, called RQ Study, is available for most Applied Biosystems real-time PCR systems.

The information that follows will detail how data can be analyzed using either methodology.

2. The Relative Standard Curve Method

The relative standard curve method uses a set of relative standards from which unknown samples are quantitated. Standard curves for relative quantitation are easy to prepare because quantity is expressed relative to some basis sample, such as the calibrator. A **calibrator** is a sample used as the basis for comparing results. For example, in a study of drug effects on gene expression, an untreated control would be an appropriate calibrator. For all experimental samples, target quantity is determined by interpolating from the standard curve and then dividing by the target quantity of the calibrator. The calibrator, then, becomes the 1X sample, and all other quantities are expressed as an *n*-fold difference relative to the calibrator.

Because the sample quantity is divided by the calibrator quantity, the unit from the standard curve is cancelled out. Thus, all that is required of the standards is that their relative dilutions be known. For relative quantitation, therefore, any stock cDNA, RNA, or DNA containing the appropriate target can be used to prepare standards.

For the proper use of relative standard curves:

1. Stock cDNA, RNA, or DNA must be accurately diluted, but the units used to express the dilutions are not important. If two-fold dilutions of a reverse transcription product (cDNA) from a control cell line are used to construct a standard curve, the units could be the dilution values 1, 0.5, 0.25, 0.125, and so on. By using the same stock cDNA, RNA, or DNA to prepare standard curves for multiple plates, the relative quantities determined can be compared across the plates.
   Note: AB recommends that you prepare large pools of standard and calibrator cDNAs and then aliquot these cDNAs into single-use tubes. Using the same pool of standard and calibrator cDNA through-out a study helps improve the accuracy of real-time PCR results.
2. A DNA standard curve can be used for relative quantitation of RNA. For example, you can use genomic DNA (if primers are designed within a single exon) or plasmid DNA containing the target of interest. This assumes that the reverse transcription efficiency of the target is the same in all samples, but the exact value of this efficiency need not be known. To obtain additional information on the generation of standard curves review "Creating Standard Curves with Genomic DNA or Plasmid DNA Templates for use in Quantitative PCR".

Because quantitation should be normalized to an endogenous control, standard curves are prepared for both the target and the endogenous reference. For each experimental sample, you determine the amount of target and endogenous reference from the appropriate standard curve.

a. Example of the Standard Curve Method (Singleplex): Using an Independent Sample for a Standard Curve

Overview
In this example Raji cell cDNA is used to create a relative standard curve to determine the difference in target quantity between a test sample and a calibrator sample. Raji RNA, extracted from a human B-cell lymphoma cell line, is considered here to be an independent sample because it is not an experimental sample, but has utility as a standard curve simply because it expresses both the target and endogenous control genes. There are advantages to using an independent sample for your standard curve. Because the calibrator sample is not used to create a standard curve, not as much calibrator nucleic acid is used, making it easier to use the same "lot" of calibrator(s) throughout the study. This can be particularly useful if a calibrator sample is in short supply. Additionally, you can commonly find a variety of independent samples (ex. cell lines) that provide an abundance of nucleic acid. Because of the abundance of nucleic acid, large stocks of standard curves can be prepared from the same exact source of nucleic acid. By using the same stock cDNA, RNA or DNA to prepare standard curves for multiple plates, the relative quantities determined can be reliably compared across multiple plates.

Example
In this example, three reverse transcription reactions were performed. In each reaction tube, 1 µg of total RNA from Raji cells, calibrator sample, and test sample were reverse-transcribed (Figure 4).
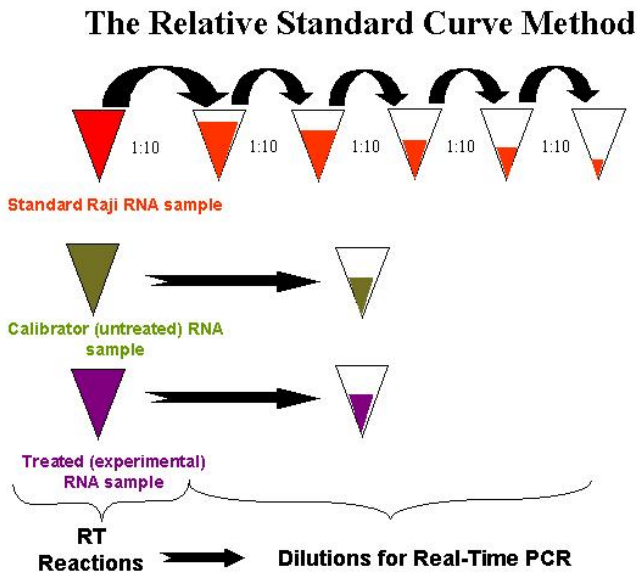

Figure 4: Dilutions of cDNA for real-time PCR Reactions in the relative standard curve example. These dilution series for standard, calibrator, and treated samples are done in duplicate to PCR amplify the target sequence (ex. IL-4) and endogenous control (ex. 18S rRNA).

For the real-time PCR reactions, one-tenth of the Raji RT product was used as the highest template mass point for the standard curve. Therefore, the 100 ng sample represents 1/10 of the RT product. The other standard curve points were prepared from subsequent serial dilutions. Therefore, the 10 ng sample represents 1/100 of the RT product, the 1 ng sample represents 1/1000 of the RT product, and so on.
Within each PCR run, 1/1000 of the calibrator and test sample RT products (or 1 ng) were tested versus the standard curve.

Table 6: √ = sample masses tested by real-time PCR in the relative standard curve example. Note: Masses simply represent proportion of RT reaction and mass units (ex. ng) cancel-out in relative quantitation calculations.

Mass		100 ng	10 ng	1 ng	0.1 ng	0.01 ng
Sample Type	Standards	√	√	√	√	√
	Calibrator			√		
	Test			√		

Two series of real-time PCR reactions were prepared to quantify both the target and endogenous control sequences. Therefore, the Raji standard curve, the calibrator sample, and the test sample were subjected to PCR amplification to quantify both the target and endogenous control sequences.
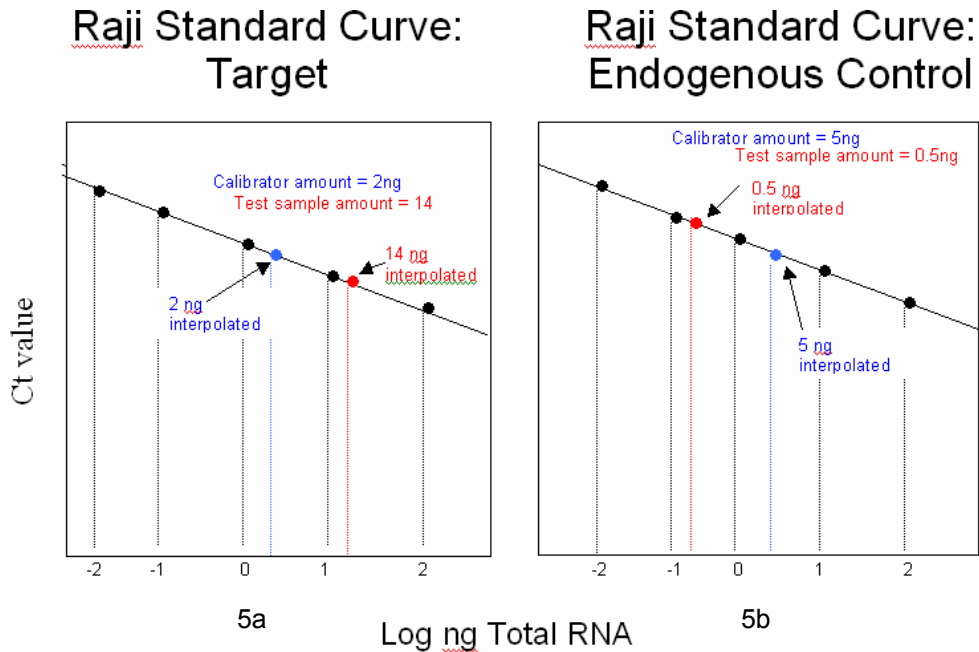
Figure 5: Raji Standard Curve Plots: log of input versus C_T value

If the amount of target in both the calibrator and test samples were equivalent, the calibrator sample and the test sample real-time PCR amplifications would yield equivalent C_T values. However, in this example, using the test sample target C_T value, interpolation from the standard curve yielded a mass of 14 ng. Using the calibrator sample target C_T value, interpolation from the standard curve yielded a mass of 2 ng (Figure 5a).

However, you also need to factor in the quantity of endogenous control. The test sample endogenous control result yielded a mass of 0.5 ng, whereas the calibrator sample endogenous control result yielded a mass of 5 ng (Figure 5b).

Table 7: Relative standard curve example results from interpolation of unknown C_T values to standard curves.

Experiment Results (interpolation of unknowns to standard curves)		
	Test sample	**Calibrator sample**
Target	14 ng	2 ng
Endogenous control	0.5 ng	5 ng

The test sample and calibrator sample target masses can be normalized by their respective endogenous control results to calculate a normalized target value. To calculate the normalized target, divide the target result by the endogenous control result (see below).

$$\text{Normalized target (test sample)} = \frac{\text{Target}}{\text{Endogenous Control}} = \frac{14\,\text{ng}}{0.5\,\text{ng}} = 28$$

$$\text{Normalized target (calibrator sample)} = \frac{\text{Target}}{\text{Endogenous Control}} = \frac{2\,\text{ng}}{5\,\text{ng}} = 0.4$$

The normalized target values are divided by one another to calculate the fold-difference in target quantity between the test sample and the calibrator sample.

Fold difference in target = $\dfrac{\text{Normalized target (test sample)}}{\text{Normalized target (calibrator sample)}}$ = $\dfrac{28.0}{0.4}$ = 70-Fold

The calculations above show that the test sample has seventy-fold more target RNA than the calibrator sample. Note that the mass units cancel in this relative quantitation calculation. This example demonstrates conceptually the standard curve method using an independent sample to create a standard curve.

It is recommended to run replicates (identical and experimental) of samples to gain a higher level of confidence in the data. Replicate target and endogenous control quantity values can be averaged and standard deviations can be calculated. These standard deviation results are then reported with the fold-difference result. The next section provides an explanation of how to calculate these standard deviation values.

Applied Biosystems

b. Standard Deviation Calculations Using the Relative Standard Curve Method
Note: This example is taken from User Bulletin #2 pages 10 and 34.

The number of experimental replicates run in a study will have a direct impact on the downstream data analysis (i.e. are the observed fold-differences in nucleic acid statistically significant?). Careful consideration must be exercised when determining the number of experimental replicates to be tested in a relative quantitation study. Refer to the appendix for information on experimental replicates, precision and statistical tests. When you run replicates, results are generally expressed as averaged values ± standard deviations.

Experimental Example: Expression Levels of c-*myc* Normalized to GAPDH

In this example, the standard curve method is used to evaluate the relative expression levels of c-*myc* between kidney (test sample) and brain samples (calibrator sample). GAPDH is used as the endogenous control. The standard curve was prepared from dilutions of a cDNA sample prepared from Raji total RNA. Target and control were run in separate wells.

Replicates of each sample were PCR-amplified to evaluate both c-*myc* and GAPDH. Table 8 summarizes data from the example experiment:
- -Replicate quantity values (ng)
- -Replicate quantity averages ± standard deviations (ng)
- -Normalized c-*myc* values (normalized to GAPDH),
- -Fold-difference values (normalized c-*myc* relative to brain).

Table 8: Relative expression of c-*myc* and GAPDH in brain and kidney as calculated using the relative standard curve method.

Tissue	c-*myc* ng Total Raji RNA	GAPDH ng Total Raji RNA	c-*myc*$_N$ Normalized to GAPDH	c-*myc*$_N$ Relative to Brain
Brain	0.033	0.51		
	0.043	0.56		
	0.036	0.59		
	0.043	0.53		
	0.039	0.51		
	0.040	0.52		
Average	**0.039±0.004**	**0.54±0.032**	**0.072±0.009**	**1.0 ±0.12**
Kidney	0.40	0.96		
	0.41	1.06		
	0.41	1.05		
	0.39	1.07		
	0.42	1.06		
	0.43	0.96		
Average	**0.41±0.016**	**1.02±0.052**	**0.402±0.025**	**5.5±0.35**

Calculations Example

This section will detail how to calculate the values in Table 8.

- Step 1: Calculate the mean and standard deviations of the replicate sample results (ex. c-*myc* ng total Raji RNA and GAPDH ng total Raji RNA).
- Step 2: Calculate the normalized target value (ex. c-*myc*$_N$ normalized to GAPDH)
  - a) calculate the normalized target value
  - b) calculate the standard deviation of the quotient
- Step 3: Calculate the fold-difference between test sample normalized target and calibrator sample normalized target (ex. c-*myc*$_N$ relative to brain)

Step 1: Calculate the mean and standard deviation values of the replicate sample (ex. c-*myc* ng Total Raji RNA and GAPDH ng Total Raji RNA).

Note: The quantity mean and standard deviation values can be calculated by the ABI PRISM® Sequence Detection System software. The software must be set up defining the standard samples as standards, assigning quantity values to the standard samples, and setting-up your replicate unknown samples as replicates. Consult the appropriate User Guide for information on setting up the plate document.

Alternatively, you can perform step 1 within Microsoft® Excel:
1. Export Results (analyzed data) from instrument software.
2. Arrange the input quantity values in a column as shown in Table 8.
3. Select **Tools > Add-Ins > Analysis ToolPak** in Microsoft® Excel software, then click **OK**.
4. Select **Tools** > **Data Analysis**. A window will appear.
5. Select **Descriptive Statistics** and then click **OK**.
6. Click the field to the right of **Input Range**, then select the wells that contain the quantity values.
7. Click the box to the left of **Summary Statistics,** then click **OK**.
8. A new page displays descriptive statistics with mean (average) and standard deviation values.

Step 2: Calculate the normalized target value (ex. c-*myc*$_N$ normalized to GAPDH).

a) Calculate the normalized target value.

A normalized target value is calculated by dividing the average target value (mean of replicate target results) by the average endogenous control value (mean of replicate endogenous results). For instance, calculate c-*myc*$_N$ normalized to GAPDH for the kidney sample.

$$\text{Normalized target value} = \frac{\text{kidney c-}myc\text{ quantity mean}}{\text{kidney GAPDH quantity mean}} = \frac{0.41}{1.02} = 0.40$$

b) Calculate the standard deviation of the quotient.

The standard deviation of the quotient is calculated from the standard deviations of the target and endogenous control mean quantities (calculated in step 1).

In order to do this, first calculate the coefficient of variation (cv) for both c-*myc* and GAPDH.

$$cv = \boxed{\dfrac{s}{X} = \dfrac{std\ dev}{mean\ value}}$$

$cv_1 = \dfrac{kidney\ c\text{-}myc\ std\ deviation}{kidney\ c\text{-}myc\ quantity\ mean} = \dfrac{0.016}{0.41} = 0.039$

$cv_2 = \dfrac{kidney\ GAPDH\ std\ deviation}{kidney\ GAPDH\ quantity\ mean} = \dfrac{0.052}{1.02} = 0.051$

Next, calculate the cv of the quotient

$$\boxed{cv = (cv_1^2 + cv_2^2)^{1/2}}$$ where $X^{1/2}$ is the square root of X

$cv = (0.039^2 + 0.051^2)^{1/2} = 0.064$

Finally, since the cv and mean are known, calculate the standard deviation of the quotient by solving for s.

$$\boxed{cv = \dfrac{s}{X} = \dfrac{standard\ deviation}{mean\ value}}$$

$s = (cv)(X)$ where X is c-*myc*$_N$ (c-*myc* normalized to GAPDH)

$s = (0.064)(0.40) = 0.025$

Step 3: Calculate the fold-difference between test sample normalized target and calibrator sample normalized target (ex. c-*myc*$_N$ relative to Brain)

a) Divide the normalized target quantity by the calibrator sample normalized target quantity. Note that when brain (calibrator) is divided by itself, the value is 1.0.

$\dfrac{normalized\ target\ quantity\ (kidney)}{normalized\ target\ quantity\ (brain)} = \dfrac{0.402}{0.072} = 5.5$

b) Calculate the standard deviation

Since the cv and mean are known, calculate the standard deviation of the quotient by solving for s.

$$\boxed{cv = \dfrac{s}{X} = \dfrac{standard\ deviation}{mean\ value}}$$

AB Applied Biosystems

Note: The calculation of c-myc$_N$ relative to brain involves division by the calibrator value. This is division by an arbitrary constant, so the cv of this result is the same for the cv for c-myc$_N$ (calculated in step 2b).

cv = (0.064) $\longrightarrow$ This cv is the same cv for c-myc$_N$ (calculated in step 2b).

s = (cv)(X)

s= (0.064)(5.5) = 0.35

The fold-difference in the level of normalized c-*myc* in kidney relative to brain would then be reported as 5.5 ± 0.35.

3. The Comparative C$_T$ Method (ΔΔ C$_T$ Method)

The Comparative C$_T$ Method, also referred to as the ΔΔC$_T$ Method, is similar to the Relative Standard Curve Method, except it uses arithmetic formulas to achieve the result for relative quantitation. It is possible to eliminate the use of standard curves and to use the ΔΔC$_T$ Method for relative quantitation as long as the PCR efficiencies between the target(s) and endogenous control(s) are relatively equivalent. This is discussed in greater detail in this section.

Applied Biosystems offers relative quantitation calculation and analysis software for many of its real-time PCR Systems. Once a particular assay as been validated, these software packages perform all the calculations and final fold change determinations that are presented in this section. Contact your local Applied Biosystems sales representative for information about these software packages.

Arithmetic Formula:
The amount of target, normalized to an endogenous reference and relative to a calibrator, is given by:

$$2^{-\Delta\Delta C_T}$$

For the derivation of this formula, refer to pages 11 to 13 of User Bulletin #2: Relative Quantitation of Gene Expression.

a. A Validation Experiment Is Necessary to Determine If Your ΔΔC$_T$ Calculation Is Valid.
Note: TaqMan® Gene Expression Assays have amplification efficiencies of 100%, Therefore, when using TaqMan® Gene Expression Assays, it is not necessary to measure efficiency. AB has extensively tested the design parameters and is confident that the resulting assays will have 100% efficiency (±10%) when measured over a 6-log dilution range, in samples that are free of PCR inhibitors.
Note: The experimental values presented are taken from User Bulletin #2, page 14.

Guidelines for a Validation Experiment

Note: Accurate real-time PCR results depend on a variety of reagent, experimental set-up, sample quality, and analysis factors. For a listing of factors involved in quality real-time PCR results, see Factors Affecting Accurate Real Time PCR Results.

The guidelines for running an effective validation experiment are:

1. The input of cDNA should ideally span 5 to 6 logs (i.e.100 ng to 10 pg) and span the expression levels of your target(s). The initial results will help you in assessing the dynamic range of each assay. Additional details on assessing the dynamic range are in "Determination of input RNA amounts to be used in a relative quantitation study". **Note**: It may not be possible to include all datapoints in your validation experiment analysis as some dilution points may be outside the dynamic range of the assay(s).
2. Run at least 3 replicates for each standard-curve point.
3. Run the target and endogenous control reactions in separate wells (singleplex reactions).
4. The primer and probe concentrations should be at the recommended levels of 900 nM for each primer and 250 nM for each probe. If optimization experiments yield alternative optimum primer and/or probe concentrations, adjust the concentrations accordingly.

For a valid $\Delta\Delta C_T$ calculation, the efficiency of the target amplification and the efficiency of the reference amplification must be approximately equal. To determine if the two amplification reactions have the same PCR efficiency, you can look at how the ΔC_T ($C_{T\ target} - C_{T\ reference}$) varies with template dilution. Assessing the relative efficiencies of the target amplification and the reference (endogenous control) amplification is achieved by running standard curves for each amplicon utilizing the same sample. The sample in the validation experiment must express both the target and reference genes. For example, a sample that ultimately is evaluated during your experimentation (such as the calibrator) could be used. The C_T values generated from equivalent standard curve mass points (target vs. reference) are used in the ΔC_T calculation ($C_{T\ target} - C_{T\ reference}$). These ΔC_T values are plotted vs. log input amount to create a semi-log regression line. The slope of the resulting semi-log regression line can be used as a general criterion for passing a validation experiment. In a validation experiment that passes, the absolute value of the slope of ΔC_T vs. log input is < 0.1.
Note: If the efficiencies of the two PCR reactions are equal, the plot of log input amount versus ΔC_T has a slope of approximately zero.

AB Applied Biosystems

In the following example, replicates of each standard curve point were evaluated by real-time PCR. The mean and standard deviations of the replicate sample C_T values are presented in the following table.

Table 9: ΔC_T calculations for the validation experiment.

Input Amount of Total RNA (ng)	c-*myc* Average C_T	GAPDH Average C_T	ΔC_T c-*myc* – GAPDH
1.0	25.59 ± 0.04	22.64 ± 0.03	2.95 ± 0.05
0.5	26.77 ± 0.09	23.73 ± 0.05	3.04 ± 0.10
0.2	28.14 ± 0.05	25.12 ± 0.10	3.02 ± 0.11
0.1	29.18 ± 0.13	26.16 ± 0.02	3.01 ± 0.13
0.05	30.14 ± 0.03	27.17 ± 0.06	2.97 ± 0.07
0.02	31.44 ± 0.16	28.62 ± 0.10	2.82 ± 0.19
0.01	32.42 ± 0.12	29.45 ± 0.08	2.97 ± 0.14

For information on calculating the standard deviations of ΔC_Ts, see "Calculating the standard deviation of the ΔC_T and $\Delta\Delta C_T$ values."

b. Plotting the Results of the Validation Experiment

Export the analyzed data to Microsoft Excel® software then re-tabulate the data as shown below.

Table 10: Log input RNA and ΔC_T of targets for validation experiment

| Input Amount of Total RNA (ng)	Log of Input Amount: ng Total RNA	ΔC_T c-*myc* – GAPDH
1	0.0	2.95
0.5	–0.3	3.04
0.2	–0.7	3.02
0.1	–1.0	3.01
.05	–1.3	2.97
.02	–1.7	2.82
.01	–2.0	2.97

To plot this data in Microsoft Excel® software:
1. Click any cell below the table.
2. Select **Tools > Add-Ins > Analysis ToolPak** then click **OK**.
3. Select **Tools > Data Analysis**.
4. Select **Regression** then click **OK**.
5. In the regression window that appears, click in the field to the right of **Input Y range** then select the cells that contain the "ΔC_T c-*myc* – GAPDH" values. A flashing dashed line borders these cells.
6. Click the field to the right of **Input X range** then select the cells that contain the "Log of Input Amount: ng Total RNA" values. A flashing dashed line borders these cells.
7. Under **Output options**, select the **New Workbook** radio button.
8. Under **Residuals**, select the box next to **Line Fit Plots** then click **OK**.

A Summary Output table and a Line Fit plot appear within a new workbook.

9. Click on one of the **Predicted Y** data points (in the graph). The data points are highlighted with a different color.
10. Right click on one of the highlighted points while the Predicted Y data points are highlighted. In the new pull-down menu that appears, select **Add Trendline**. An "Add Trendline" window opens.
11. Verify that the "straight/linear trend/regression type" (default) is selected.
12. Select the **Options** tab. Select the box to the left of **Display equation on chart**, then click **OK**. The equation of the regression line is displayed on the chart. The slope of the line is "m" in the equation

$$Y = mx + b$$

Therefore, the slope of the line in this example is 0.0472 (see Figure 6). This would be considered a passing validation experiment because the absolute value of the slope of ΔC_T vs. log input is < 0.1.
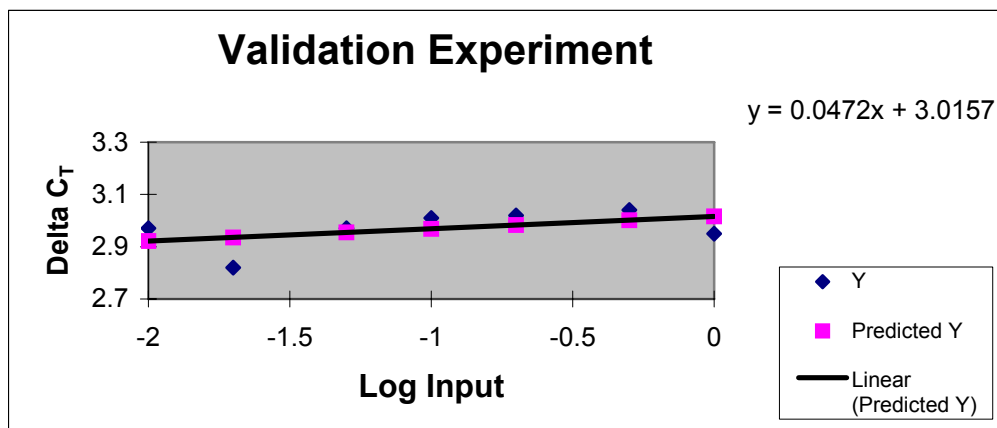
Figure 6: Validation plot of ΔC_T vs. log input amount of RNA

c. Validation Experiment Results

Ideally, the absolute value of the slope of the validation experiment should be <0.1. If it is not, re-assess the data. When examining the validation experiment results, it is important to assure that rigorous analysis is done on the data. Analysis of the two real-time PCR standard curves may indicate that some data points are anomalous. For example, you should look at the outer points of the standard curves (high input and low input). If inhibitors are present, they can affect the higher concentration points. If they are removed, the slope of the standard curve may improve. If low levels of target are present, the lower concentration points may need to be removed as the lower limits of detection may have been reached. This secondary analysis can assist in re-defining the dynamic range for a particular assay. It is also important to look at the precision among the replicates of each dilution set. An outlier in a replicate sample can alter the efficiency calculation. Remove any outliers and re-analyze. Ensure proper setting of the baseline and threshold for each run. If the absolute value of the slope of the validation experiment is still > 0.1, do one of the following:

1. Optimize or Re-design Assay(s)

The Comparative C_T method requires that the target and endogenous controls have relatively similar efficiencies – but the efficiencies do not have to be 100%. If one of the assays has a relatively higher efficiency than the other, one may need to look at re-designing, or re-selecting the less optimal assay and performing the validation experiment again. In addition, a primer optimization strategy may be needed for assays designed using the Primer Express® software to determine the optimum primer concentration for a particular target. Additional details are in the Appendix in: Optimizing Primer and TaqMan® Probe Concentrations.

2. Determine if the Error Introduced by the Differing PCR Efficiencies is Tolerable for Your Application

When you evaluate fold changes, be aware that unequal PCR efficiencies will correlate to a decrease in the accuracy of the calculated fold change. When you look for only high fold change levels (i.e., 100-fold, 1000-fold) a slightly less accurate calculated fold change may not necessarily impact the interpretation of the results as there may be a greater tolerance for efficiency variation. However, if one is looking at relatively small fold differences (i.e., 2-fold, 4-fold) the tolerance for efficiency variation may be less. In this case, it may be prudent to optimize or re-design assay(s) to achieve a passing validation experiment, or pursue the use of the relative standard curve method. If you want to quantify the error introduced by the differing PCR efficiencies, compare the results of the comparative C_T method with results from a relative standard curve method. Because the accuracy of the relative standard curve method is not dependent on the relative efficiencies of the target and reference assays, you can use this comparison to gauge the amount of error in your comparative C_T method experiment. Then, determine if the error can be tolerated in your relative quantitation study.

d. The Comparative C_T Method ($\Delta\Delta C_T$ Method): Data Analysis Example

The following table presents data from an experiment where the expression levels of a target (c-*myc*) and an endogenous control (GAPDH) are evaluated. The levels of these amplicons in a series of drug-treated samples are compared to an untreated calibrator sample. A validation experiment previously demonstrated that the efficiency of the target amplification and the efficiency of the reference (endogenous control gene) amplification are approximately equal. Therefore, the $\Delta\Delta C_T$ method can be used to calculate the fold-differences in c-*myc* between samples.

The number of experimental replicates run in a study directly affects the downstream data analysis (i.e. are the observed fold-differences in nucleic acid statistically significant?). Careful consideration must be exercised when determining the number of experimental replicates that will be tested in a relative quantitation study. See the appendix for more information on experimental replicates, precision and statistical tests. Mean C_T values and standard deviations are used in the $\Delta\Delta C_T$ calculations. In this example, each sample type was run in triplicate. Each sample C_T mean was calculated and standard deviations were calculated for each mean C_T value.

Table 11: Fold change expression of c-myc after treatment, calculated by $\Delta\Delta C_T$ method

Sample	c-*myc* Average C_T	GAPDH Average C_T	ΔC_T c-*myc*-GAPDH	$\Delta\Delta C_T$ ΔC_T treated -ΔC_T untreated	Fold difference in c-*myc*$_N$ relative to untreated
untreated	30.49±0.15	23.63±0.09	6.86±0.19	0.00±0.19	1 (0.9-1.1)
Drug treatment A	27.03±0.06	22.66±0.08	4.37±0.10	−2.4±0.10	5.6 (5.3-6.0)
Drug treatment B	26.25±0.07	24.60±0.07	1.65±0.10	−5.11±0.10	37 (34.5-39.7)
Drug treatment C	25.83±0.07	23.01±0.07	2.81±0.10	−3.95±0.10	16.5 (15.4-17.7)

<u>**Step 1**</u>**: Calculate the mean and standard deviation values of the replicate sample results.**
Note: The mean C_T and standard deviation values can be calculated by ABI PRISM® Sequence Detection System software (with the exception of the ABI PRISM® 7700 SDS software).The software must be setup appropriately defining the standard samples as standards, assigning quantity values to the standard samples, and setting-up the replicate unknown samples as replicates. Consult with the appropriate User Guide for information on setting up the plate document.

Mean C_T values and standard deviations can be calculated in Microsoft® Excel software:
1. Export analyzed results from the instrument software.
2. Select **Tools > Add-Ins > Analysis ToolPak** within Microsoft® Excel software, then click **OK**.
3. To calculate means and standard deviations in Microsoft® Excel software, first tabulate the C_T values for a given sample in a column. Select **Tools > Data Analysis**. A window opens.
4. Select **Descriptive Statistics**, then click **OK**.
5. Click the field to the right of **Input Range**, then select the wells that contain the C_T values.
6. Click the box to the left of **Summary Statistics**, then click **OK**. A new page opens displaying descriptive statistics with a mean (average) and standard deviation values.

<u>**Step 2: Calculate the ΔC_T value.**</u>

The ΔC_T value is calculated by:

$$\Delta C_T = C_{T\ target} - C_{T\ reference}$$

For example, subtraction of the average GAPDH C_T value from the average c-*myc* C_T value of the untreated sample yields a value of 6.86.

ΔC_T untreated = 30.49 – 23.63 = 6.86

AB Applied Biosystems

Step 3: Calculate the standard deviation of the ΔC_T value.

The standard deviation of the ΔC_T is calculated from the standard deviations of the target and reference values using the formula:

$s = (s_1^2 + s_2^2)^{1/2}$; where $X^{1/2}$ is the square root of X

and s= standard deviation

For example, to calculate the standard deviation of the untreated sample ΔC_T value:

$s_1 = 0.15$ and $s_1^2 = 0.022$
$s_2 = 0.09$ and $s_2^2 = 0.008$
$s = (0.022 + 0.008)^{1/2} = 0.17$

Therefore, ΔC_T untreated = (30.49 ±0.15) – (23.63 ±0.09) = 6.86 ±0.17

Step 4: Calculate the $\Delta\Delta C_T$ value.

The $\Delta\Delta C_T$ is calculated by:

$$\Delta\Delta C_T = \Delta C_T \text{ test sample} - \Delta C_T \text{ calibrator sample}$$

For example, subtracting the ΔC_T of the untreated from the ΔC_T of Drug Treatment A yields a value of –2.5.

$\Delta\Delta C_T = 4.37 - 6.86 = -2.5$

Step 5: Calculate the standard deviation of the $\Delta\Delta C_T$ value.

The calculation of $\Delta\Delta C_T$ involves subtraction of the ΔC_T calibrator value. This is subtraction of an arbitrary constant, so the standard deviation of the $\Delta\Delta C_T$ value is the same as the standard deviation of the ΔC_T value.

Therefore, $\Delta\Delta C_T$ Drug Treatment A sample =

$\Delta\Delta C_T = 4.37±0.10 - 6.86±0.17 = -2.5±0.10$

Standard deviation of the $\Delta\Delta C_T$ value is the same as the standard deviation of the ΔC_T value

Step 6: Incorporating the standard deviation of the ΔΔC$_T$ values into the fold-difference.

Fold-differences calculated using the ΔΔC$_T$ method are usually expressed as a range, which is a result of incorporating the standard deviation of the ΔΔC$_T$ value into the fold-difference calculation.

The range for target$_N$, relative to a calibrator sample, is calculated by: $2^{-\Delta\Delta Ct}$ with ΔΔC$_T$ + s and ΔΔC$_T$ – s, where s is the standard deviation of the ΔΔC$_T$ value.

For example, the drug-treatment A sample has a 5.3 to 6.0-fold difference in expression of the target$_N$ relative to the untreated (calibrator) as indicated below.

$\quad$ ΔΔC$_T$ + s = –2.5 + 0.1 = –2.4

$\quad$ $2^{-\Delta\Delta Ct} = 2^{-(-2.4)} = 5.3$

and

$\quad$ ΔΔC$_T$ + s = –2.5 – 0.1 = –2.6

$\quad$ $2^{-\Delta\Delta Ct} = 2^{-(-2.6)} = 6.0$

e. What If a ΔΔC$_T$ Value Is Positive?

ΔΔC$_T$ calculations express data in the context of test sample versus calibrator. If the ΔΔC$_T$ calculation is positive (for ex. +2.0), then:

$\quad$ $2^{-\Delta\Delta Ct} = 2^{-(2.0)} = 0.25$

In this example the test sample has 0.25 or 1/4 the amount of target RNA as the calibrator.

Appendix A
Definitions

Baseline

To determine the C_T for an amplification plot, AB real-time PCR software uses data (background signal) collected from a range of PCR cycles called the "baseline" (the default baseline for some software packages occurs between cycles 3 and 15). This baseline signal is most commonly fluorescence that is attributed to background, before significant accumulation of the target amplicon. First, the software calculates a mathematical trend based on the baseline cycles' normalized reporter (R_n) values to generate a baseline subtracted amplification plot of ΔR_n versus cycle number. Next, an algorithm searches for the point on the amplification plot at which the value crosses the threshold setting. The fractional cycle at which the intersection occurs is defined as the threshold cycle (C_T) for the plot.

Note: It may be necessary to adjust the baseline and threshold settings to obtain accurate data. For help on setting baselines and thresholds, refer to your instrument user guide. ABI PRISM® 7700 Sequence Detection System users can refer to "Data Analysis on the ABI PRISM® 7700: Setting Baselines and Thresholds".

Calibrator

The calibrator is a sample that is used as the basis for comparative expression results. As an example, if a study were looking at the effect of a particular drug on the expression level of a gene of interest, the calibrator sample might be the untreated sample. Large preparations of the same calibrator stock RNA or DNA can be made to last through the extent of the study.

Experimental Replicates

Experimental replicates are amplifications that share the same PCR reagents, while the template preparations come from similar, but not identical samples. Experimental replicates provide crucial information about the overall precision of the experiment. For example, if a researcher wishes to examine the effect of a drug treatment on the relative level of a mouse mRNA, it is inappropriate to drug treat just one mouse. A number of mice must be treated similarly with the drug to determine the variation of response in the mouse population. A group of ten mice would represent ten experimental replicates.

In general, the fewer the number of replicates, the greater the potential for deviation from the true variance of the system. The number of experimental replicates to run in a relative quantitation study should be based on the following considerations:

- The fold-difference value to be used in your statistical hypothesis (for example 5-fold, 10-fold and so on).
- The statistical test to be used (for example t-Test).
- The significance level (α) to be used for the test statistic.

AB Applied Biosystems

Precision and Statistical Tests

Precision is the degree to which repetitions of a test produce similar quantitative values. Although precision can be measured in many ways, the most common method is to calculate the standard deviation.

Standard deviations are used to perform statistical tests on experimental results. In the case of relative quantitation, standard deviations are required to evaluate whether the observed fold differences in nucleic acid are statistically significant or merely the variation inherent in the assay.

What Is Acceptable Precision?

The desired limits of precision in a quantitative experiment are primarily determined by the statistical comparisons being attempted with the data, whether the experiment uses absolute quantitation or is strictly relative. You must decide:

- On the relative quantitative objective of the assay, which is often described as a fold difference. For example, you may want to distinguish five-fold differences, but someone else may wish to distinguish ten-fold differences.
- How many replicates will be run, what the level of statistical confidence should be, and which statistical test should be used.

Number of Replicates and Variability

In general, the fewer the number of replicates, the greater the variability in the standard deviation. As the number of replicates increases, individual variations are averaged out, and the standard deviation approaches the actual variance of the system.

Number of Replicates and Statistical Confidence

If you do not want to perform the requisite 30 replicates to determine the true variance of a system, you can use the t test (also called Student t-test). Statistical programs are available, but t values can also be obtained from the t Table in statistics textbooks. To determine the t value from a table, cross-index the degrees of freedom, which equals the number of experimental replicates minus one, with the desired confidence interval.

Causes of Low Precision

Many factors can cause loss of precision. Examine each factor below if a loss of precision occurs within a particular assay.

   a. **Improper Threshold/Baseline Setting**: If you manually adjust the baseline and threshold for an assay, make sure you set the values appropriately. For help on setting baselines and thresholds, refer to your instrument user guide. ABI PRISM® 7700 Sequence Detection System users can refer to ["Data Analysis on the ABI PRISM® 7700: Setting Baselines and Thresholds"](#).
   b. **Imprecise pipetting**: Accurate pipetting with calibrated pipettors is critical in obtaining accurate and precise data. Minor volume variations can adversely impact the precision of the results, especially at low-volumes (<5μl).

Applied Biosystems

c. **Incomplete mixing**: Components in a real-time PCR reaction can settle out and be distributed unevenly within a set up reaction and can have an effect on precision. Thoroughly mix all reagents through the set-up procedure.

d. **Master Mixes**: The use of PCR master mixes will help reduce the variability of pipetting multiple reagents.

e. **Other**: Air bubbles in the reaction wells, writing on the top of the caps/sheets, drops on the sides of the wells, can contribute to precision errors.

Reference Gene

An active signal used to normalize experimental results. Endogenous and exogenous controls are examples of active references. Active reference means the signal is generated as the result of PCR amplification. The active reference has its own set of primers/probe.

Endogenous control – An RNA or DNA that is present in each experimental sample as isolated. By using an endogenous control as an active reference, you can normalize quantitation of a messenger RNA (mRNA) target for differences in the amount of total RNA added to each reaction.

Exogenous Control – A characterized RNA or DNA spiked into each sample at a known concentration. An exogenous active reference is usually an in vitro construct that can be used as an internal positive control (IPC) to distinguish true target negatives from PCR inhibition. An exogenous reference can also be used to normalize for differences in efficiency of sample extraction or complementary DNA (cDNA) synthesis by reverse transcriptase.

Standards

In relative quantitation of gene expression studies, a standard is commonly derived from a cDNA pool. cDNA standards can be generated from a biological sample (ex. cell line) that simply expresses the target and the reference (endogenous control) genes. So, the standard nucleic acid does not necessarily have to be derived from the same source (tissue or cell) as the test sample. For example, the cDNA standard used in the User Bulletin #2 relative standard curve example (pp 4 to10) is derived from Raji cells, a B-cell Lymphoma cell line. Whereas the test sample cDNAs are all derived from tissue samples. The ability to use an unrelated sample type (unrelated to your test sample) as the source of a relative standard is particularly useful when the nucleic acid from study samples is limited. By choosing a cell line that can be grown in large quantities, large amounts of standard nucleic acid can be prepared. The advantage of having large preparations is that by using the same stock RNA or DNA to prepare standard curves for multiple plates (aliquots of each standard curve point), the normalized quantities can be compared across plates.

Plasmid or genomic DNA standards could also be used. Using a DNA (plasmid or genomic DNA) standard curve for relative quantitation requires the assumption that the reverse transcription efficiency of the target is the same in all samples, but the exact value of this efficiency need not be known. You can eliminate the use of standard curves, and use the Comparative C_T ($\Delta \Delta C_T$) Method for relative quantitation.

AB Applied Biosystems

For additional information about generating plasmid DNA or genomic DNA standard curves, review [Creating Standard Curves with Genomic DNA or Plasmid DNA Templates for Use in Quantitative PCR](#).

TaqMan® MGB Probes

TaqMan® MGB probes consist of an oligonucleotide containing a reporter dye (i.e. FAM™ or VIC®) at the 5′ end, and a minor-groove-binder moiety and a nonfluorescent quencher dye at the 3′ end. The minor-groove binder acts as a probe T_m enhancer. Probes designed as TaqMan® MGB probes have much shorter length probes, which enhances the T_m differential between matched and mismatched probes. A probe sequence with a single mismatch is more likely to be displaced by the DNA polymerase, AmpliTaq Gold®, than it is to be cleaved by the enzyme during amplification. In addition, TaqMan® MGB probes contain a nonfluorescent quencher that provides for enhanced spectral resolution when using multiple dyes in a reaction. TaqMan® MGB probes are applicable for either gene expression assays or for SNP genotyping assays. In SNP assays, when two probes with a single base pair mismatch are competing for the same target, the minor-groove binder provides a greater T_m differential between the two probes and therefore increases the discrimination ability in these assays. In gene expression assays, TaqMan® MGB probes enhance specific probe binding and also lends itself better to multiplex assays.

R = Reporter Dye (FAM™ and VIC® Dyes)
NFQ = Non-Fluorescent Quencher
MGB = Minor Groove Binder (T_m enhancer)

Optimizing Primer and TaqMan® Probe Concentrations

Applied Biosystems reagent protocols describe how to optimize primer and probe concentrations for a 5′ nuclease assay or primer concentrations for a real-time PCR assay using SYBR® Green I dye. Protocols can be obtained from the Applied Biosystems [Products and Literature Service](#) web site.

For most quantitative 5′ nuclease assays designed and performed using Applied Biosystems rapid assay development guidelines, a final concentration of 900 nM forward primer, 900 nM reverse primer and 250 nM probe provides a highly reproducible and sensitive assay when using cDNA or DNA as a template. Due to the nonspecific nature of its detection, SYBR® Green I primer optimization should be eliminated only with caution. However, if all guidelines are followed, 50 nM to 300 nM each of forward and reverse primer should provide robust amplification with a good level of specificity when using cDNA or DNA as a substrate.

Appendix B
Reagents, Protocols, and Supporting Documentation

I. TaqMan® Gene Expression Assays and Custom TaqMan® Gene Expression Assay Reagents, Protocols, and Ordering Information

Item	Reagent Part Number	Protocol Part Number
High-Capacity cDNA Archive Kit	4322171	4322169
TaqMan® Universal PCR Master Mix (with AmpErase UNG)	4304437	4304449
TaqMan® Universal PCR Master Mix (no AmpErase® UNG)	4324018	4304449
TaqMan® Fast Universal PCR Master Mix, No AmpErase® UNG	4352042	4351891
Real-Time PCR Systems Chemistry Guide	N/A	4348358
TaqMan® Gene Expression Products: Protocol	N/A	4333458
RNase Inhibitor	N808-0119	N/A

A. Ordering TaqMan® Gene Expression Assays:

1. Searching for Assays:

The TaqMan® Assays-on-Demand™ Gene Expression: Product Quick Card, describes how to search for and order the assays. The following highlights some of the search steps covered in the Quick Card.

1. Login to the Applied Biosystems Online Store.

2. In Store Catalog, click on Genomic Products > TaqMan® Gene Expression Assays.

3. Click on TaqMan® Gene Expression Assays.

4. In the Search & Order box, click on the Go button.

Note: Alternatively, you can start the search process by selecting the MyScience environment button in the Applied Biosystems header. Then, select Search next to TaqMan® Gene Expression Assays.

You can search by:

Keyword
Find assays by searching for keywords such as gene name, gene symbol, RefSeq accession number, public accession number, etc.
Batch ID
Find assays by searching for multiple IDs including Celera ID, AB ID, or GenBank accession number.
Classifications
Find assays by molecular function (e.g., kinase) or biological process (e.g., apoptosis).

The search output yields a variety of information about the assay including: Assay ID, Public ID (ex. RefSeq), LocusLink Gene Name and symbol, Gene Function (ex. Interleukin Receptor) and Biological Process (ex. Cytokine and chemokine mediated signaling pathway). You can double-click on the Assay ID to access more detailed information about the assay including Celera Panther system and Gene Ontology Category information. Assay information also includes exon boundary and assay location information.

2. Placing an Order for TaqMan® Gene Expression Assays
When you locate your assay, select the checkbox to the left of the Assay ID. You must be logged into the store to place the assay(s) into your shopping basket. Once logged-in, move your mouse to the pull-down menu on the right-hand side of the web page. Select <u>My Basket</u> to order the Assays, then, click the <u>add to</u> button. You must be a registered user to place TaqMan® Gene Expression Assays orders. To register, go to http://store.appliedbiosystems.com. Once the registration has been filled-out, Applied Biosystems order administration sends an e-mail within 48 hours confirming your registration. You then can place your TaqMan® Gene Expression Assays products into your shopping basket.

B. TaqMan® Gene Expression Assays Documentation

Document Title	Document Part Number
TaqMan® Assays-on-Demand™ Gene Expression Products: Protocol: Rev A	4333458
TaqMan® Assays-on-Demand™ Gene Expression: Product Quick Card	127MI07-01
TaqMan® Universal PCR Master Mix Protocol	4304449
TaqMan® Fast Universal PCR Master Mix, No AmpErase® UNG: Protocol	4351891
High-Capacity cDNA Archive Kit Protocol	4322169
User Bulletin #2 Relative Quantitation of Gene Expression	4303859

C. Key Features of Custom TaqMan® Gene Expression Assays

- Eliminates the manual and technology-specific task of designing primers and probes
- Cost per data point comparable to "build your own" assay
- Preformulated custom, one-tube assays eliminate the steps of optimizing reaction conditions. Ready to use with rapid assay guidelines

D. Custom TaqMan® Gene Expression Product Part Numbers

Part Description	Part Number	Number of reactions at 50μL reaction volume	Number of reactions at 20μL reaction volume
GX V-scale	4331348	140	360
GX S-scale	4332078	300	750
GX A-scale	4332079	1,160	2,900

E. Custom TaqMan® Gene Expression and Genotyping Documentation

Document Title	Document Part Number
TaqMan® Assays-By-DesignSM Service Protocol Submission Guidelines	4334672
TaqMan® Assays-By-DesignSM Service Protocol For Gene Expression Assays	4334429
TaqMan® Assays-By-DesignSM Service Quick Reference Card For Gene Expression Assays	4335636
TaqMan® Universal PCR Master Mix Protocol	4304449
TaqMan® Fast Universal PCR Master Mix, No AmpErase® UNG: Protocol	4351891
High-Capacity cDNA Archive Kit Protocol	4322169

Applied Biosystems

II. TaqMan® Pre-Developed Assay Reagents (PDAR), Protocols, and Ordering Information

A. PDAR Targets

Part Description
TaqMan® PDAR target reagents – to view part numbers for all PDAR targets

B. TaqMan® Pre-Developed Assay Reagents Protocols and Documentation

Document Title	Document Part Number
Pre-Developed TaqMan® Assay Reagents Gene Expression Quantification Protocol	4310255
TaqMan® Universal PCR Master Mix Protocol	4304449
TaqMan® Fast Universal PCR Master Mix, No AmpErase® UNG: Protocol	4351891
User Bulletin #2 Relative Quantitation of Gene Expression	4303859

Applied Biosystems

III. TaqMan® Human Endogenous Control Plate Assays and Protocols

A. Available Assays

The human endogenous control gene targets from the TaqMan® Human Endogenous Control Plate are available as TaqMan® Gene Expression Products (TaqMan® Assays-on-Demand™ Products)

Part Number	Gene	Assay ID	Accession #
4331182	Eukaryotic 18S rRNA	Hs99999901_s1	X03205
4331182	Ribosomal protein, RPLPO	Hs99999902_m1	XM_007036
4331182	ACTB (beta actin)	Hs99999903_m1	NM_001101
4331182	PPIA (cyclophilin A)	Hs99999904_m1	NM_021130
4331182	GAPD (GAPDH)	Hs99999905_m1	NM_002046
4331182	PGK1 (phosphoglycerate kinase 1)	Hs99999906_m1	NM_000291
4331182	beta 2-microglobulin	Hs99999907_m1	NM_004048
4331182	GUSB (beta glucuronidase)	Hs99999908_m1	NM_000181
4331182	HPRT1 (HGPRT)	Hs99999909_m1	NM_000194
4331182	TBP (TATA-box binding protein)	Hs99999910_m1	NM_003194
4331182	TFRC (CD71)	Hs99999911_m1	NM_003234

B. TaqMan® Human Endogenous Control Plate Product Part Numbers

Part Description	Part Number
TaqMan® Human Endogenous Control Plate with TaqMan® Universal PCR Master Mix and Control Total RNA	4309920
TaqMan® Human Endogenous Control Plate with TaqMan® Universal PCR Master Mix	4309921
The human endogenous control gene targets from the TaqMan® Human Endogenous Control Plate are also available as TaqMan® PDAR Endogenous Controls	

.

C. TaqMan® Human Endogenous Control Plate Product Documentation

Document Title	Document Part Number
TaqMan® Human Endogenous Control Plate Protocol	4308134
Pre-Developed TaqMan® Assay Reagents Gene Expression Quantification Protocol	4310255
User Bulletin #5 Multiplex PCR with TaqMan® VIC Probes	4306236
User Bulletin #2 Relative Quantitation of Gene Expression	4303859

AB Applied Biosystems

IV. Applied Biosystems TaqMan® Low Density Arrays

A. Documentation

Document Title	Document Part Number
Applied Biosystems 7900HT Micro Fluidic Card: Getting Started Guide	4319399
How to Order TaqMan®Low Density Arrays	106701

V. TaqMan Cytokine Gene Expression Plate Product Information and Protocols

A. Key Features
- Easy-to-use kit contains all components needed to study a panel of 12 Human cytokines.
- Pre-loaded primers and TaqMan® probes provide an easy-to-use solution for cytokine gene-expression studies.
- Pre-optimized multiplex assays eliminate assay design and optimization time
- All primer and probe concentrations are optimized for use with the TaqMan® Universal PCR Master Mix, requiring only specific cDNA to be added before loading and running the plate.
- All assays have equivalent efficiencies that allow relative quantitation studies using the Comparative C_T method to increase throughput.
- Because assays are designed to exclude the detection of genomic DNA, they are tolerant to moderate levels of DNA contamination.

B. TaqMan® Cytokine Gene Expression Plate Product Part Numbers

Part Description	Part Number
TaqMan® Cytokine Gene Expression Plate 1 with TaqMan® Universal PCR Master Mix and Control Total RNA (2 x 96-well plates)	4307266
TaqMan® Cytokine Gene Expression Plate 1 with TaqMan® Universal PCR Master Mix	4307265

C. TaqMan® Cytokine Gene Expression Plate Product Documentation

Document Title	Document Part Number
TaqMan® Cytokine Gene Expression Plate 1 Protocol	4306744
TaqMan® Universal PCR Master Mix Protocol	4304449
User Bulletin #2 Relative Quantitation of Gene Expression	4303859

Applied Biosystems

AB Applied Biosystems