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

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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 basic information on chemistries and strategies, you can review the following tutorials:
Real Time PCR vs. Traditional PCR
Essentials of Real Time PCR

This document 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 all 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 StepOne™ Real-Time PCR System
- Applied Biosystems StepOnePlus™ Real-Time PCR System
- Applied Biosystems 7300 Real-Time PCR System
- Applied Biosystems 7500 Real-Time PCR System
- Applied Biosystems 7500 Fast 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 a treated vs. untreated samples.

For this hypothetical study, you can choose a calibrator (reference) 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. Please see the following for more information about setting up and analyzing absolute quantitation (standard curve experiments):

- Applied Biosystems 7300/7500/7500 Fast: Absolute Quantitation, Using Standard Curve Getting Started Guide
- Applied Biosystems 7900HT: Absolute Quantitation, Using Standard Curve Getting Started Guide
- Applied Biosystems StepOne™: Standard Curve Experiments Getting Started Guide

3. Terms and Acronyms –
The following terms and acronyms are used throughout this document. Additional information on specific definitions is available in Appendix A or by clicking on 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. See also reference sample.
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	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	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 (Technical 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.

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.
Reference Sample	A sample used as the basis for comparative expression results. See also calibrator.
Standards	A sample of known concentration used to construct a standard curve.
TaqMan® Endogenous Controls	TaqMan® Endogenous Controls are assays for housekeeping genes that are commonly used as endogenous controls. Several are available in with VIC® TaqMan® MGB probes in primer limited format for multiplexing purposes. Organisms include: human, mouse, rat, and eukaryotic 18S.
TaqMan® Gene Expression Assays	TaqMan® Gene Expression Assays are biologically informative, pre-formulated gene expression assays for rapid, reliable detection and quantification of mRNA transcripts from several model organisms. Each product is delivered as pre-mixed primers and TaqMan® MGB probe at a 20X concentration
TaqMan® Genotyping Assays	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® miRNA Assays	TaqMan® miRNA Assays include a miRNA specific RT primer and a 20X mix of a TaqMan® MGB probe and primers. Assays are available for several model organisms.
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.

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 cDNA or 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 (eg. 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 "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

For more information 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 consistent real-time PCR results.

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

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

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(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 as well as variations in formulation.

(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 [Reconstituting and Diluting Primers and TaqMan® Probes](#), 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 ≥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 ≥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 ≥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 ≥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.

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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% when using high quality RNA. If your RNA is of poor quality (example: degraded, high protein co-purification) the assay efficiency can be greatly compromised. For more information about RNA quality please see "Assessing RNA Quantity and Quality." For information on the performance of TaqMan® Gene Expression Assays, reference the Application Note "Amplification Efficiency of TaqMan® Gene Expression Assays."

High PCR amplification efficiencies (near 100%) can additionally 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.

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".

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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, and is 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 ($\log_2 10 = 3.3219$).
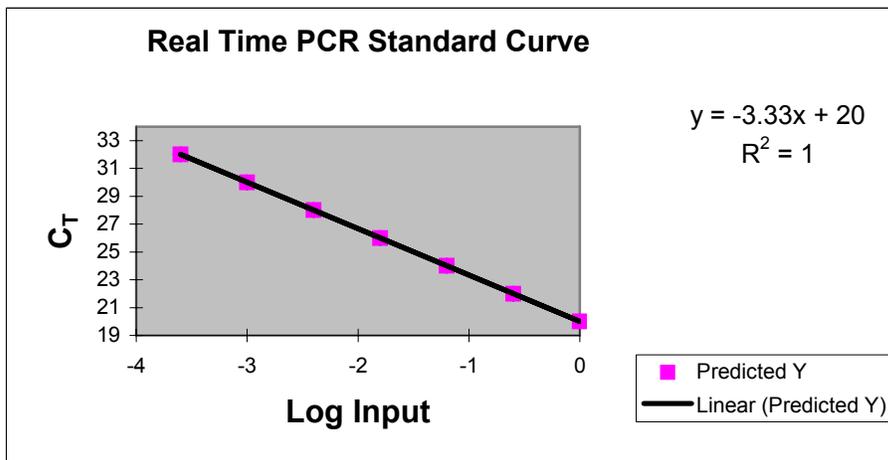
Figure 1: Real-Time PCR Standard Curve representing 100% PCR Efficiency

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 and use high quality starting RNA/cDNA template. 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

A: RNA Preparation: Obtaining High Quality RNA

Tissue or Cell Sample Collection and Disruption

Isolating high quality, intact RNA is the first and often the most critical step in performing many fundamental molecular biology experiments including RT-PCR. Proper treatment and handling of tissues and cells prior to RNA isolation and storage of the isolated RNA are crucial components of obtaining high quality RNA.

I. Appropriate Sample Storage and Processing

1. **Fresh:** Most animal tissues can be processed immediately after harvest. It is important to keep fresh tissues on ice and to process them within 30 minutes of dissection.
2. **Frozen:** Samples are usually frozen on dry ice or in liquid nitrogen when:

   a) they are collected over a period of time and cannot be processed simultaneously,
   b) there are too many samples to process at once,
   c) samples are collected in the field, or
   d) the mechanical processing of fresh samples is insufficient for thorough disruption.

While freezing allows the researcher to postpone RNA isolation, it is time consuming and makes subsequent RNA isolation a more laborious process.

3. **Tissue Storage Solutions:**

   a) [Ambion® RNA*later*® Tissue Storage:RNA Stabilization Solution](#) allows the researcher to postpone RNA isolation for days, weeks, or even months after tissue collection without sacrificing RNA integrity, and potentially without freezing the sample. Dissected tissue or harvested cells are simply dropped into the RNA*later*® solution at room temperature. The solution permeates the cells and stabilizes the RNA. The samples are then stored at 4°C. Samples can be shipped on wet ice or even at room temperature if shipped overnight. Figure 1 below shows the integrity of RNA isolated from tissues stored in RNA*later* solution at 4°C, room temperature, and at 37°C**.** Samples stored at 4°C generate intact RNA even after storage for one month. Tissue and cells stored in RNA*later*® Solution are compatible with most RNA isolation procedures.
   b) [Ambion RNA*later*®-ICE Frozen Tissue Transition Solution](#) is a novel reagent for transitioning frozen tissue to a state that is easily processed for extraction of high quality RNA. Frozen tissues are simply submerged in RNA*later*®-ICE and allowed to

thaw overnight at -20°C. Once thawed, the tissues can be processed like fresh tissues using standard RNA isolation procedures.
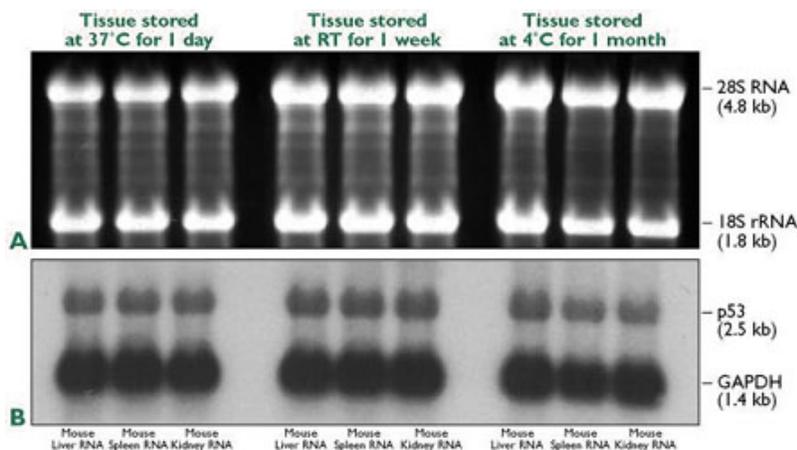

Figure 1. High Quality RNA Isolated From Tissue Stored in RNA*later*®
Tissue Storage:RNA Stabilization Solution. RNA was purified from the tissues using TRI Reagentl® (Ambion®). Five µg of each RNA sample was separated on a denaturing agarose gel. **Panel A** shows ethidium bromide-staining of the gel. **Panel B** shows a Northern blot of the same gel hybridized with 10^6 cpm/mL of an RNA probe to p53 and 10^4 cpm/mL of an RNA probe to GAPDH, using the Ambion **NorthernMax® Kit**.

II. Complete, Quick Sample Disruption

Fast and thorough sample disruption is another essential component of RNA isolation and affects RNA yield and quality. Slow disruption by placing cells or tissues in a denaturant lysis solution without any additional physical shearing may result in RNA degradation by endogenous RNases that are inaccessible to the lysis solution. Incomplete disruption may also result in decreased yield because some of the RNA remains trapped in intact cells and is unavailable for purification.

1. **Mechanical Disruption of fresh and frozen tissue, cultured cells**

    a) Fresh: When disrupting fresh tissue, all cells need to be sheared immediately at the time the lysis solution is added. Samples should never be left undisrupted in lysis solution. Mechanical methods for disrupting fresh tissue and cells include homogenization by the following methods: using a Dounce or mechanical homogenizer, vortexing, sonication, use of a French press, and bead milling.

    b) Frozen: Frozen samples are disrupted by grinding in liquid nitrogen with a mortar and pestle. During this process, it is important that the equipment and tissue remain at cryogenic temperatures—the tissue should be dry and powdery after grinding. Grinding should be followed by thorough homogenization with a Dounce or mechanical homogenizer in a lysis buffer. Samples stored frozen in the Ambion® RNA*later*® solution or in the Ambion® RNA*later*®-ICE solution can be processed like fresh tissue as described earlier.

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c) Cultured Cells: Cells grown in suspension are collected by centrifugation, washed, and resuspended in a lysis solution. Lysis is completed by immediately vortexing or vigorously pipetting the solution. Attached cells can be lysed directly on the culture plate. Lysis solution is added directly to the plate or flask and cells are scraped into the solution. The lysate is then transferred to a tube and vortexed or pipetted to ensure complete cellular disruption. Alternately, cells can be detached, and processed as suspension cultures. Placing the flask or plate on ice while washing and lysing the cells will further protect the RNA from endogenous RNases during these steps.

2. **Enzymatic Disruption**

a) Lysozyme, zymolase, and lysostaphin digestion are used to disrupt structures that are not easily sheared by mechanical methods alone. Enzymatic treatment is usually followed by sonication, homogenization, or vigorous vortexing in a lysis buffer. Enzymatic methods may also be used for specific eukaryotic tissues, e.g. collagenase is used to break down collagen prior to cell lysis.

III. Options for Total RNA Isolation

Ambion® provides several options for isolation of total eukaryotic RNA that are compatible with a variety of cells and tissues, including those from animals, plants, and yeast. A few of those ideal for RT-PCR applications are described below:

1. Ambion® TRI Reagent® is a phenol-based reagent that contains a unique combination of denaturants and RNase inhibitors and is used in a single-step disruption/separation procedure. The tissue or cell sample is homogenized or disrupted in the Ambion® TRI Reagent, chloroform is mixed with the lysate, and the mixture is separated into three phases by centrifugation. The RNA is then precipitated from the aqueous phase with isopropanol. The entire procedure can be completed in under one hour.

2. Ambion®-RNAqueous® Technology is a rapid, filter-based RNA isolation system that does not require the use of phenol, chloroform, or other toxic organic chemicals. The entire procedure can be completed in 20–30 minutes, depending on the time required for tissue disruption. The Ambion® RNAqueous-4PCR Kit provides RNA that is free of genomic DNA contamination from samples as small as 1 mg or 100 cells. There are additional RNAqueous kits available for other sample sizes.

3. The TaqMan® Gene Expression Cells-to-CT™ Kit makes it possible to skip RNA purification from cultured cells and go straight to real-time PCR in as little as 7 minutes. Gene expression can be assessed from as few as 10 to as many as $1x10^5$ cells. The procedure is geared for high throughput gene expression analysis—lysis can be performed directly in 96- and 384-well culture plates—and is amenable to automation. The procedure includes an optional digestion of genomic DNA.

4. Ambion®-RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE Tissues is designed to extract total nucleic acid from formaldehyde or paraformaldehyde-fixed, paraffin-embedded (FFPE)

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tissues. The kit procedure requires about 45 minutes of hands-on time and can easily be completed in under 1 day when isolating RNA. The recovered nucleic acids are suitable for downstream applications such as microarray analyses and qRT-PCR.

For more information about how to choose a RNA isolation chemistry appropriate for your needs, please see the Ambion® RNA Isolation Decision Tree.

IV. RNA storage

Once isolated, the purified RNA pellet is dissolved in an RNase-free solution for storage. Ambion produces several solutions for this purpose including:

- Ambion® RNA Storage Solution (1 mM sodium citrate, pH 6.4 ± 0.2)
- 0.1 mM EDTA (in DEPC-treated ultrapure water)
- TE Buffer (10mM Tris-HCl, 1 mM EDTA, pH 7.0)
- Ambion® RNAsecure™ Resuspension Solution is a unique non-enzymatic reagent for the irreversible inactivation of RNases in enzymatic reactions. To inactivate RNases, the RNA pellet is resuspended in the RNAsecure™ Resuspension Solution and heated to 60°C for 10 minutes. Reheating after the initial treatment will reactivate the RNase-destroying agent to eliminate any new contaminants.

V. Assessing RNA Quantity and Quality

The quality of isolated RNA is crucial for the success of downstream applications, but also varies with the subsequent experimental procedures.

1. RNA yield

When isolating RNA, it helps to have a rough idea of how much total RNA can be recovered from a given type of sample. Figure 2 provides general guidelines for estimating RNA yields from a variety of tissues.
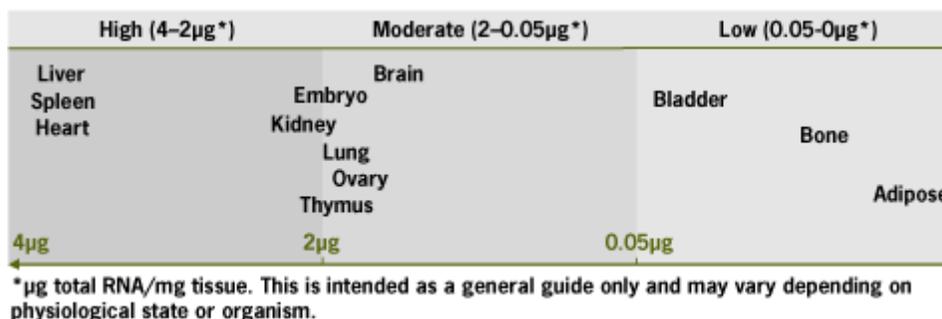

Figure 2: Total RNA Yields from Different Tissues

RNA quantitation can be done using one of the following methods:

a) ***Fluorescent Dyes*** (e.g. RiboGreen®, Invitrogen) Fluorescent dyes are used to quantitate RNA in solution. The fluorescence emitted by the RNA sample is compared to a standard curve. This technique is more sensitive than spectrophotometry and is less sensitive to protein and free nucleotide interference although DNA contamination can cause inaccurate quantitation. The procedure also requires a fluorometer.

b) ***Spectrophotometry:*** Spectrophotometry measures the absorbance of an RNA sample at a wavelength of 260 nm. It is a simple assay and uses equipment that is available in most laboratories but it is not a sensitive technique and readings are affected by any molecule that absorbs at 260 nm.

2. RNA Integrity
Although some applications are tolerant of partially degraded RNA (e.g. RT-PCR), it is still important to assess RNA integrity to ensure that samples being compared are of similar integrity.

a) ***Denaturing Agarose Gel Electrophoresis*** For total RNA samples, discrete, though thick 28S:18S ribosomal RNA (rRNA) gel bands and an approximate mass ratio of 2:1 are indications of high integrity (Figure 3, lane 3). Smeared 28S and 18S rRNA bands and an 28S:18S rRNA ratio less than 2:1 indicate RNA degradation (Figure 3, lane 2). Note that some tissues and species yield total RNA with a 28S:18S rRNA ratio of less than 2:1. Messenger RNA or small RNAs require Northern blotting to assess integrity if using this technique.
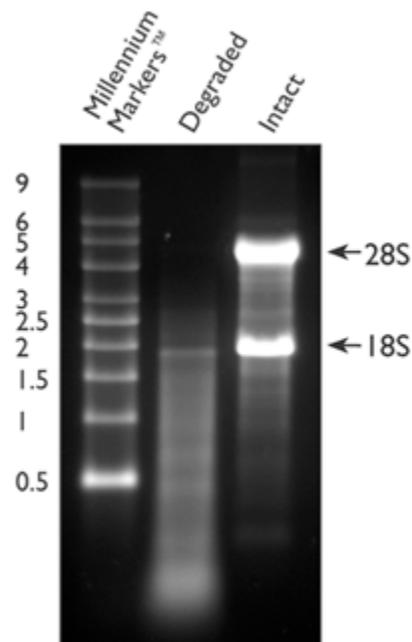

Figure 3. Intact vs. Degraded RNA. Two µg of degraded total RNA and intact total RNA were separated alongside the Ambion® RNA Millennium Markers™ on a 1.5% denaturing agarose gel. The 18S and 28S ribosomal RNA bands are clearly visible in the intact RNA sample (lane 3). The degraded RNA appears as a lower molecular weight smear (lane 2).

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b) ***Agilent 2100 Bioanalyzer*** This instrument uses capillary electrophoresis (microfluidics) to analyze nucleic acids. It requires only 25 ng of input RNA. A 2:1 ratio in the area under the peaks for 28S and 18S rRNA indicates intact total RNA (Figure 4). Degradation is indicated by less pronounced peaks for 28S and 18S rRNA and a 28S:18S rRNA ratio significantly less than 2:1.
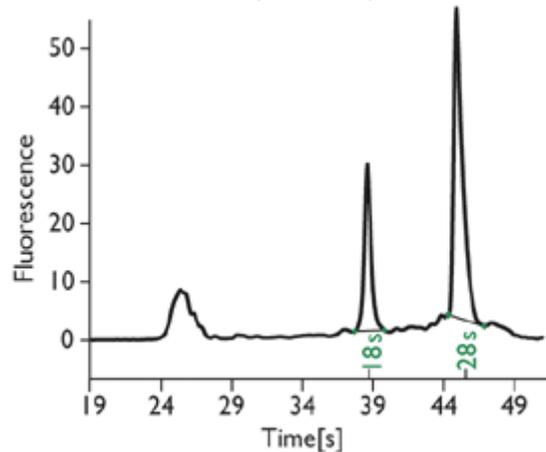
Figure 4. Agilent® 2100 Bioanalyzer Scans of High Integrity Total RNA. The 18S and 28S peaks are clearly visible at 39 and 46 seconds, respectively.

3. RNA purity

RNA samples may sometimes be contaminated with chemicals, proteins, and other molecules that can inhibit or interfere with downstream applications. Pure RNA has an $A_{260}:A_{280}$ ratio of ~2.0. RNA with a significantly lower $A_{260}:A_{280}$ ratio should be further purified by phenol-chloroform extraction, LiCl precipitation, or washing to remove residual salt.

Genomic DNA contamination can lead to inaccurate RNA quantitation and can cause problems in downstream applications such as RT-PCR. The presence of ethidium bromide-stained material in the wells or smeared just below the wells after denaturing agarose gel electrophoresis indicates genomic DNA contamination. Another easy way to check for DNA contamination is to perform PCR on an RNA sample that has not been reverse transcribed (minus RT control). The presence of an amplified product indicates DNA contamination. Treating RNA samples with DNase is a simple method to alleviate this problem. The Ambion® TURBO DNA-*free*™ kit provides protocols and reagents for both removing contaminating DNA, and for subsequently removing the DNase contamination.

Summary

Follow these recommended steps to obtain high quality RNA and improve the success of downstream applications:

I.   Store samples carefully prior to RNA isolation
II.   Disrupt samples completely to prevent RNA degradation and to increase RNA yield
III.   Choose an appropriate RNA isolation kit
IV.   Store RNA in RNase-free solutions
V.   Accurately quantitate RNA and confirm that RNA is of high integrity and purity

B. Reverse Transcription for Relative Quantitation of Gene Expression

Reverse transcription (RT) is the process by which RNA is used as a template to synthesize cDNA. This cDNA then serves as the template in the real-time reaction. Among the first options to consider when selecting a method to perform the reverse transcription is whether to use a one-step or a two-step RT method. Choosing an appropriate RT primer can significantly impact real-time results.

Tables 2 and 3 describe key features and benefits of one 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 the exception) - May get better limit of detection with rare transcripts - Requires sequence-specific RT primer for cDNA synthesis. - RNA is probed only for specified transcripts and the rest is lost after the reaction is complete.
Two-Step RT-PCR	- Requires two discrete 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 also use sequence-specific primers, random primers, or oligo d(T)$_{16}$ for cDNA synthesis.

Table 3: Comparison of RT primers for cDNA synthesis

Primers for cDNA synthesis	Considerations
Sequence-specific primer	• Use to reverse transcribe gene specific RNA sequences only • Only primer type recommended for one-step RT-PCR • Can also be used in two-step RT-PCR
Oligo d(T)$_{16}$	• Use to reverse transcribe only eukaryotic mRNAs and retroviruses with poly-A tails • Not all genes have polyA-tails. Cannot reverse transcribe 18S rRNA • May have trouble transcribing long mRNA transcripts (> 1500 bp) or transcripts containing hairpin loops (secondary structure) • May not efficiently reverse transcribe degraded RNA due to loss of intact poly-A tail. • 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, transcripts containing hairpin loops, and degraded RNA • Not biased to 3' end of transcript

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.

I. Evaluating Performance of the Reverse Transcription Chemistry

Whether you choose to use a one-step or two-step method, you must carefully consider the performance of the reverse transcription chemistry. C_t values are derived directly from cDNA, not RNA, but if the RT step is performing sub optimally, the C_t values may no longer accurately reflect the original quantities of RNA target present. Not all RT kits have the required degree of performance to accurately quantify RNA in real-time PCR.

When evaluating a reverse transcription kit, you must consider the following parameters: capacity, linearity, and efficiency. One can test these three factors by performing a dynamic range test.

A dynamic range test consists of a serial dilution of an RNA sample which has been extracted using the same method as other samples in the experiment.   Each RNA standard dilution point is then reverse transcribed using the same volume. These

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standards are then used to evaluate the capacity, linearity, and efficiency of the RT chemistry.

Capacity

Capacity is the maximum mass of RNA that may be added to the reverse transcription reaction without a loss of RT efficiency. Exceeding the capacity of the chemistry may disturb the quantitative relationships between RNA species and lead to errors in RNA quantitation. To test the capacity, the above prepared RNA standards are used.

*Note: A common practice of two-step users is to run serially diluted cDNA standards. As seen in the following figure, running cDNA serial dilutions does **NOT** evaluate the RT performance.*

Figure 1 (below) shows that while the cDNA standards did not reveal poor chemistry performance at high concentrations of input, the corresponding RNA standards show that this particular RT chemistry is not efficient at higher RNA concentrations ("tailing effect.")
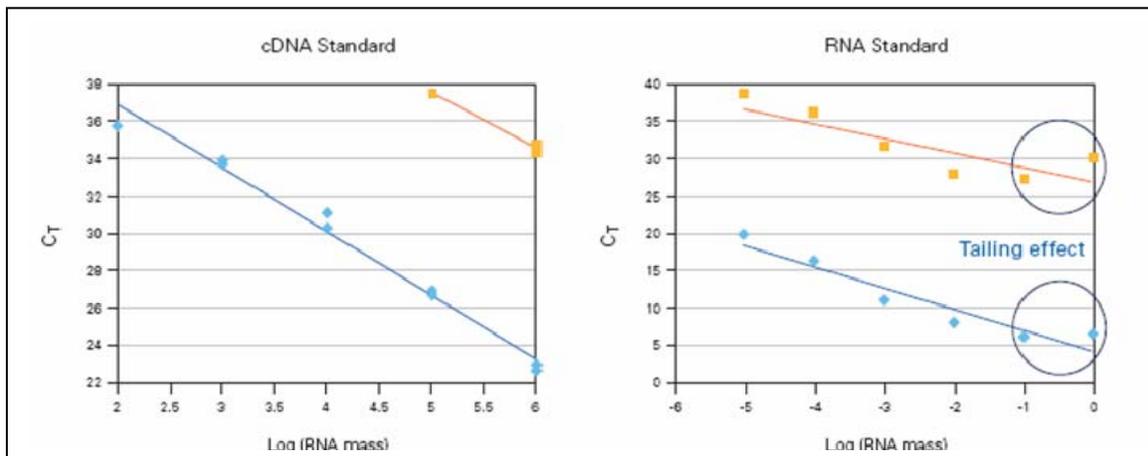
Figure 1: Two-step RT PCR Chemistry using random priming. The standard curves are made from either a serial dilution of cDNA or a serial dilution of RNA standards with each concentration independently reversed transcribed. The lower curve is the normalizing gene while the upper line is the target gene.

Consequences of poor capacity: Exceeding the capacity of the RT chemistry can lead to significant errors in RNA quantitation. The ΔCt for each RNA standard dilution point was calculated by subtracting the Ct of the normalizing gene from the Ct of the target gene. As seen in Figure 2 (below), at higher RNA concentrations, the ΔCt is quite large (points 5 and 6) as compared to the ΔCt at lower RNA concentrations (points 2-4). Once the capacity of the RT chemistry has been exceeded, accuracy of the quantitative results will be significantly compromised.
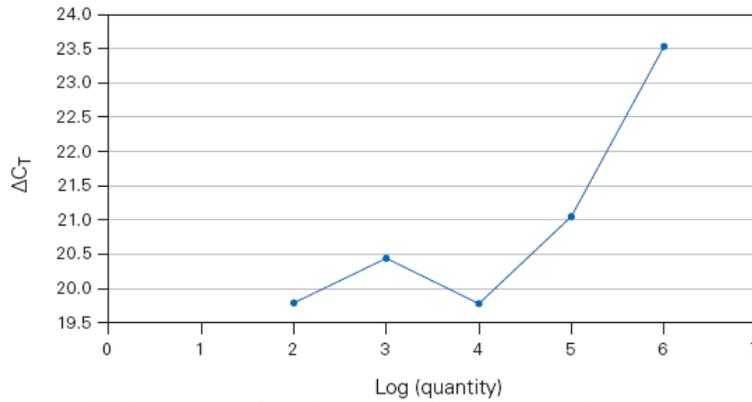
Figure 2 shows that the RNA standards from the above Figure 1 were then normalized to each other and the ΔCt vales plotted.

Linearity

Linearity is the maintenance of constant reverse transcription efficiency with increasing RNA mass. From the Efficiency formula:

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

one can extrapolate that an assay operating at 100% efficiency will double once every cycle, or 10-fold every 3.32 cycles.

RNA standard curves that have slopes less than -3.3 (more negative) indicate a loss of efficiency with increasing RNA mass.

The shallow non-parallel RNA standard curves in Figure 3 show that the reverse transcription chemistry of Kit A is neither 100% efficient (due to the poor slopes) nor will it maintain the quantitative relationship between different transcripts. The High Capacity cDNA Reverse Transcription Kit shows good linearity across many different targets with increasing templates inputs.
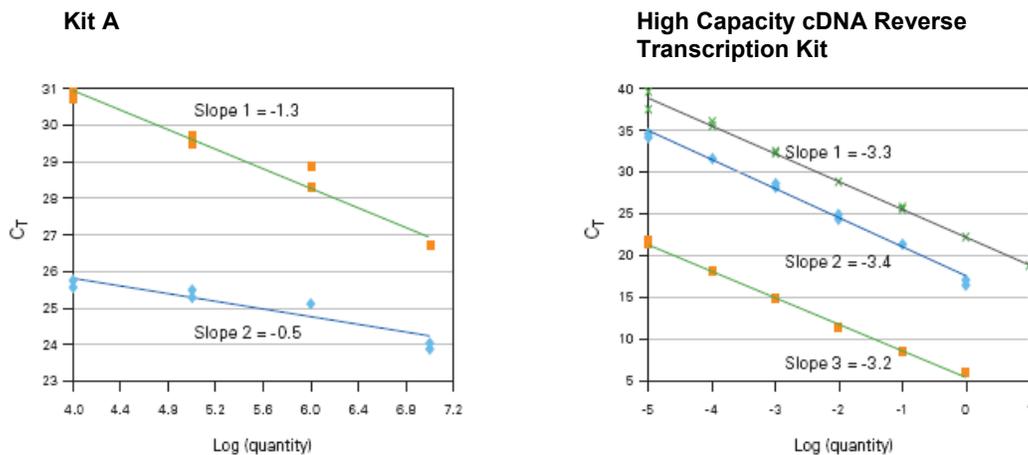
Figure 3

Efficiency

Efficiency is the percentage of target RNA molecules that are converted into cDNA along the entire transcript length. A higher RT efficiency allows the use of less RNA and the detection of lower expressed transcripts.

In Figure 4, the High Capacity cDNA Reverse Transcription Kit shows about a 5 cycle decrease in the target gene (T) C_t. This 5 cycle decrease corresponds to a 32-fold higher RT efficiency (2^5=32) than Kit A.
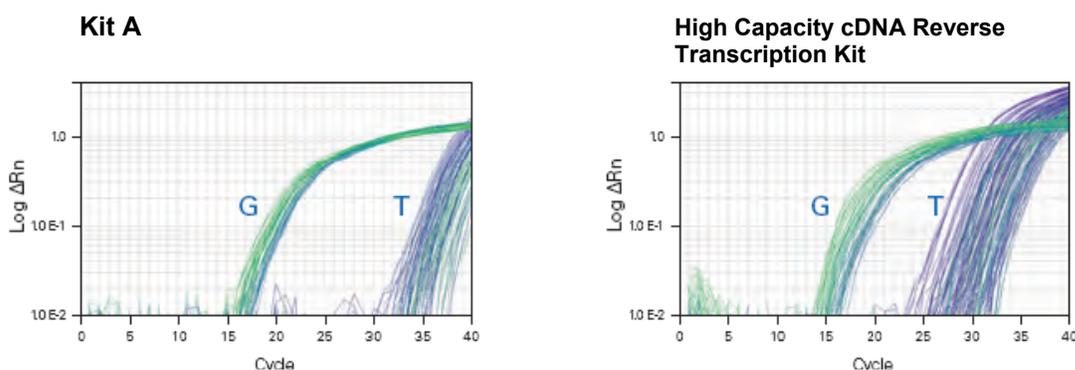
Figure 4

Conclusion

Reverse transcription kits can vary in their performance. Many kits are not formulated for use in real-time PCR applications and kit performance can also be affected by lab dependent factors such as the RT reaction volume and quality of the RNA samples. By using above demonstrated dynamic range test one can evaluate the linearity, capacity and efficiency of their chosen RT chemistry and determine if it is appropriate for their real-time PCR experiment.

II. 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. You can visit the Applied Biosystems store to review reverse transcription and real-time PCR chemistries and use the Real-Time PCR Decision Tree to assist you in selecting the necessary reagents for your gene expression study.

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.

A common approach for two-step RT-PCR is by using 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

[Reverse Transcription kit](#) and the TaqMan® Universal PCR Master Mix ([with](#) or [without](#) [AmpErase® UNG](#)). Additionally, you can use the [TaqMan®Gene Expression Master Mix](#). Refer to Appendix A of the [High-Capacity cDNA Reverse Transcription Kits 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.

III. 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 Reverse Transcription Kits 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 Poisson 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. To overcome this Poisson distribution of high variance in Ct values is to run more replicates or to run your samples at a higher dilution point.

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.

IV. 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 are referred to as inhibition plots.

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

Inhibition Plot Sample #1

$y = -3.33x + 20$

$R^2 = 1$

Y-axis: C_T (values 19, 24, 29, 34)
X-axis: Log Input (values -4, -3, -2, -1, 0)

Legend:
- ◆ Y
- ■ Predicted Y
- — Linear (Predicted Y)
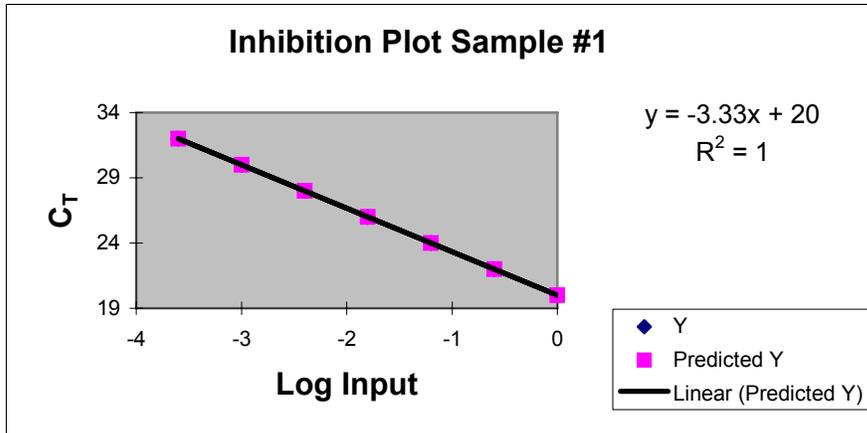
$E = (10^{-1/-slope} - 1) \times 100$
$E = (10^{-1/-3.33} - 1) \times 100$
$E = 9975\%$

Figure 2: Inhibition Plot Sample #1 demonstrating no inhibition

Inhibition Plot Sample #2

$y = -1.1905x + 26$

$R^2 = 0.495$

Y-axis: C_T (values 19, 24, 29, 34)
X-axis: Log Input (values -4, -3, -2, -1, 0)

Legend:
- ◆ Y
- ■ Predicted Y
- — Linear (Predicted Y)
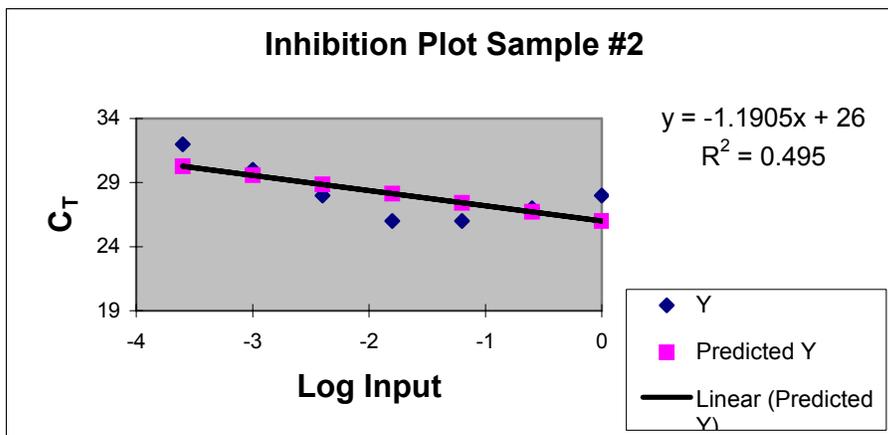
$E = (10^{-1/-slope} - 1) \times 100$
$E = (10^{-1/-1.1905} - 1) \times 100$
$E = 592\%$

Figure 3: Inhibition Plot Sample #2 demonstrating presence of inhibitor

The data in Inhibition Plot #1 show a real-time PCR assay amplifying ~ 100% efficiency (slope = –3.3), without inhibition. Inhibition Plot #2 has a slope that is shallower, and the PCR efficiency is calculated to be greater than 100% (592%). The slope is suggesting a PCR efficiency greater than 100%, and 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.

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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.

V. 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 performed without reverse transcriptase (No RT control or negative RT control) 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.

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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 with the same conditions. This universality of design, which is central to all the Applied Biosystems assays, as well as the Primer Express® Software, requires little, if any optimization, provided the assays are run using the TaqMan® Universal Master Mix or TaqMan® Gene Expression 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® Endogenous Controls
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® 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 Reverse Transcription Kit for reverse transcription and the TaqMan® Universal PCR Master Mix or the TaqMan® Gene Expression 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 or White Paper: The Design Process for a New Generation of Quantitative Gene Expression Analysis Tools: TaqMan® Probe-based Assays for Human and Other Model Organism Genes.   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 a unique **Assay ID**.

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

Hs = *Homo sapiens*   **At** = *Arabidopsis thaliana*

Mm = *Mus musculus*   **Dm** = *Drosophila melanogaster*

Rn = *Rattus norvegicus.*   **Ce** = *Caenorhabditis elegans*

Rh = *Macaca mulata* (Rhesus)   **Cf** = *Canis familiaris*

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.
_u1	An assay whose primers are each within separate exons. The probe resides within one of the two exons, or potentially within an exon between the upstream forward primer exon location, and the downstream reverse primer location. These assays may detect gDNA, unspliced transcripts, or partially spliced transcripts.
_ft	An assay that is designed to detect a fusion transcript. The primers and probe are located on either side of the fusion breakpoint, not directly on the breakpoint.

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

For example, the TaqMan® Gene Expression Assay for human GAPDH, **Hs99999905_m1**, indicates that this is an assay whose probe spans an exon junction and it does not detect genomic DNA.

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

The Online Guide for TaqMan® Gene Expression Assays describes how to search and order assays through the Applied Biosystems website. Additional information about the ordering process and appropriate documentation can be found in the Appendix section entitled "Ordering TaqMan® Gene Expression Assays".

2. Custom TaqMan® Gene Expression Assays

Custom TaqMan® Gene Expression Assays are custom-designed assays for expression studies. Custom TaqMan® Genotyping Assays are used for end-point qualitative 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® Gene Expression Assays offerings. Applications that involve viral detection, species other than those currently available in the TaqMan® Gene Expression Assays product line, detection of specific pathogens, and splice junction variants are some examples of applications that would benefit from 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 Assay service requires that before you submit your target sequence you verify its integrity by using several bioinformatics 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 assay that is ready to use with TaqMan® Universal PCR Master Mix or TaqMan® Gene Expression Master Mix and is ready to load on your Applied Biosystems real-time PCR instrument. 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 Custom TaqMan® Gene Expression Assays Part Numbers.

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

a. Verify the Quality of your Sequence (important for all custom designs 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 the RepeatMasker Home Page. Repeat Masker screens DNA sequences for interspersed repeats (ex. ALU sequences) and DNA sequences of low-complexity. The

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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, high homology within a gene family, or location of SNPs and other sequence ambiguities. 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.

Refer to the tutorial Bioinformatic Evaluation of a Sequence for Custom TaqMan Gene Expression Assays for more detailed instructions on assessing sequence quality.

b. Submit the sequence.

After you verify the sequence integrity, your sequence is entered into the File Builder software and submitted for ordering. Download the File Builder software and view the demonstration of its use before submitting your sequence. We recommend using the "Build a new file using the Wizard" feature.
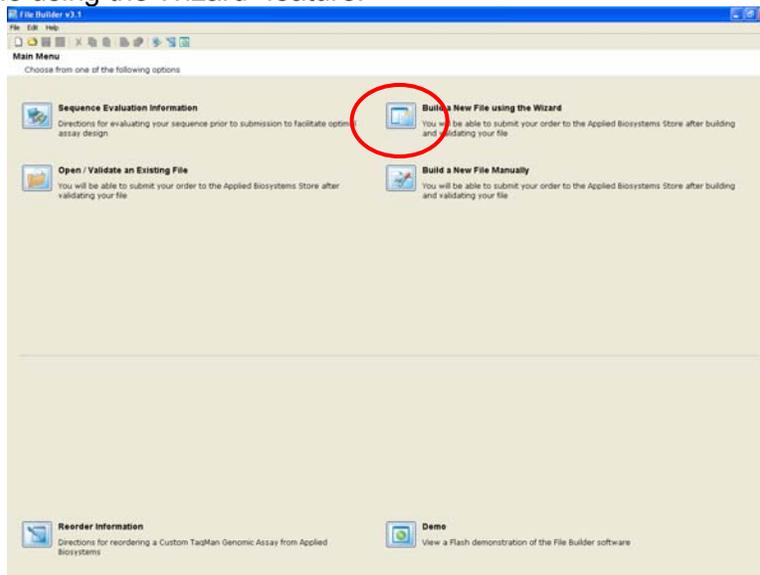
Sequence records require at least one target coordinate site. For gene expression assay submissions, the coordinate identifies a target site for the location of the probe. Having a TaqMan® MGB probe designed over an exon-exon junction should exclude detection of genomic DNA in a real-time PCR reaction. However, if the gene of interest is a single exon gene, you can use the Wizard function to assign the target method as "Automatic."

Note: At least one coordinate is required for each sequence record. The Custom TaqMan® Genomic Assays design software evaluates the target sites *in the order they are listed in the sequence record.* The first target site that meets the design criteria is used for the assay. If you prefer one target site to another, place that

![Applied Biosystems logo]

target site first in the sequence record. 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 a 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 Custom 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 "Custom TaqMan® Gene Expression Assays Part Numbers".

3. TaqMan® MicroRNA Assays

MicroRNAs (miRNAs) are naturally occurring highly conserved small RNAs (18-25 nucleotides in length) that are found in both plant and animal genomes. MicroRNAs are target specific and utilize the RNA interference pathway to regulate gene expression. They modulate gene expression one of two ways:

a) Translational suppression: When there is an imperfect sequence match of miRNA and target mRNA.
b) mRNA degradation: When there is an exact sequence match between miRNA and target mRNA, the target mRNA is directed towards destruction.

TaqMan® MicroRNA Assays include a miRNA specific stem-loop RT primer and a gold standard TaqMan® primer and probe set. The stem-loop RT primer accomplishes two particular tasks. One, it is specific for only the mature miRNA target and two, it extends the 5' end of the RT amplicon and thus making it amenable to downstream PCR amplification.
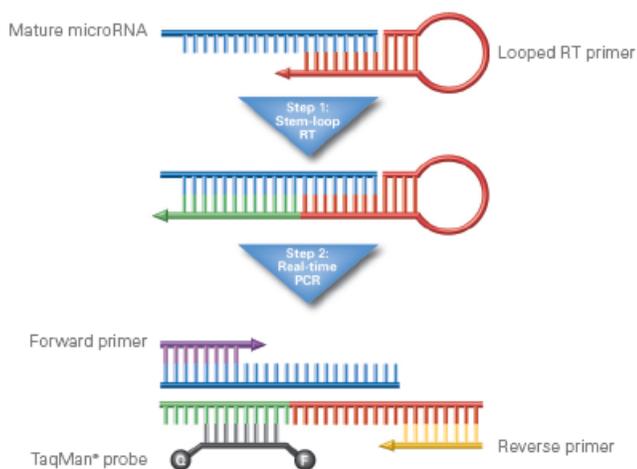
TaqMan® MicroRNA Assays are extremely sensitive and require as little as 1-10 ng of total RNA input into the TaqMan® MicroRNA Reverse Transcription Kit. Assays are

available to detect the following model organisms: *Homo sapiens* (human), *Mus musculus* (mouse), *Rattus novergicus* (rat), *Arabidopsis thaliana*, *Caenorhabditis elegans*, and *Drosophila melanogaster*.

4. TaqMan® Endogenous Controls

TaqMan®—Endogenous Controls are primer and probe sets designed to amplify commonly used endogenous control sequences in cDNA samples using the 5' nuclease assay. TaqMan® Endogenous Controls are available for human, mouse, rat and eukaryotic gene targets. In addition, they have been optimized for use with the TaqMan® Universal PCR Master Mix with or without AmpErase® UNG or TaqMan Gene Expression Master Mix and use identical thermal cycling conditions as all other AB assays. Certain TaqMan® Endogenous Control formulations are available in a primer limited formulation and contain probes labeled with the VIC® reporter dye. This allows multiplexing of TaqMan® Endogenous controls with TaqMan® Gene Expression Assays primer and probe sets, provided that the control gene is more abundantly expressed than the target gene.

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 or target, you can choose to have a custom TaqMan® Gene Expression assay generated. When the 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 TaqMan® MGB probes 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 dsDNA and is 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.

SYBR® Green reagent however, 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 sequences for synthesis. Then, should a TaqMan® probe-based assay be needed 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:

- [Power SYBR® Green PCR Master Mix and RT-PCR](#)

- [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 ofEndogenous 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 the same 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 or using 2100 Agilent Bioanalyzer) 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*.

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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](.).

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 $\Delta\Delta CT$ 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® Endogenous Control](.) formulations are available as primer-limited assays that contain probes labeled with the VIC® reporter dye. This allows multiplexing of TaqMan® Endogenous Controls with TaqMan® Gene Expression Assays, 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 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](.)".

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 (Custom)
- TaqMan® Low Density Arrays Gene Signature Arrays
- TaqMan® Cytokine Gene Expression Plate 1
- TaqMan® Human Endogenous Control Plate

1. TaqMan® Low Density Arrays (Custom)

TaqMan® Low Density Arrays are an efficient, flexible technology that enables real-time PCR in a low-density array format and streamlines gene expression studies using the Applied Biosystems 7900HT Real-Time PCR 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 and sample usage. 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 array.

After you receive your custom configured TaqMan® Low Density Arrays, combine your cDNA sample(s) with TaqMan® Universal PCR Master Mix or TaqMan® Gene Expression 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 the need for 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. TaqMan® Low Density Array Gene Signature Arrays

The TaqMan® Low Density Array Gene Signature Arrays are a collection of preconfigured with TaqMan® Gene Expression Assays and they query genes involved in common biological pathways. All panels are available for human samples, and some panels are also available for mouse and rat. Gene Signature Panels are currently available for the following pathways:

- Alzheimer's Array (Human and mouse)
- Endogenous Control Array (Human, mouse, and rat)
- GPCR Array (Human, mouse, and rat)
- Immune Array (Human and mouse)
- Inflammation Array (Human and rat)
- Phosphodiesterase Array (Human and rat)
- Stem Cell Pluripotency Array (Human and mouse)
- Human ABC Transporter Array
- Human Angiogenesis Array
- Human Apoptosis Array
- Human Nuclear Receptor Array
- Human Protein Kinase Array

Note: Check the TaqMan® Low Density Array Gene Signature Arrays site frequently since there will periodic updates.

3. TaqMan® Cytokine Gene Expression Plate 1

The TaqMan® Cytokine Gene Expression Plate 1 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 an 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 1 Product Information and Protocols".

4. TaqMan® Human Endogenous Control Plate

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 individual TaqMan® Gene Expression Assays (see Appendix B for more information about which assays are on the control plate.

Refer to "Identification and Selection of Endogenous Controls for Relative Quantitation".

Section VI
Ordering Real-Time PCR Reagents

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. Log in to the online store.
2. Select Real Time PCR in the Store Catalog (under Products and Serivces), then 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. 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 a custom synthesis of Sequence Detection Primers and TaqMan® probes:

1. Select Real Time PCR in the Store Catalog (under Products and Serivces
2. Select Custom Primers/Probes .
3. Select TaqMan® MGB Probes, TaqMan® TAMRA™ Probes, or Sequence Detection Primers and locate the items you want to purchase.
4. Click on the Configure button to enter the sequences you desire synthesized.
5. After you enter the sequences, select Add to Basket.
6. To process the order, your Shopping Cart, then fill out the requested information to complete the order.

To order TaqMan Gene Expression Assays please refer to our Online Ordering Guide for TaqMan® Gene Expression Assays.

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 ($\Delta\Delta$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 ($\Delta\Delta$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 ($\Delta\Delta C_T$)

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 the White Paper: 5' Nuclease Assays for Validating Hits from Fluorescent Microarrays. Applied Biosystems offers real-time PCR software products that perform the $\Delta\Delta C_T$ 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).
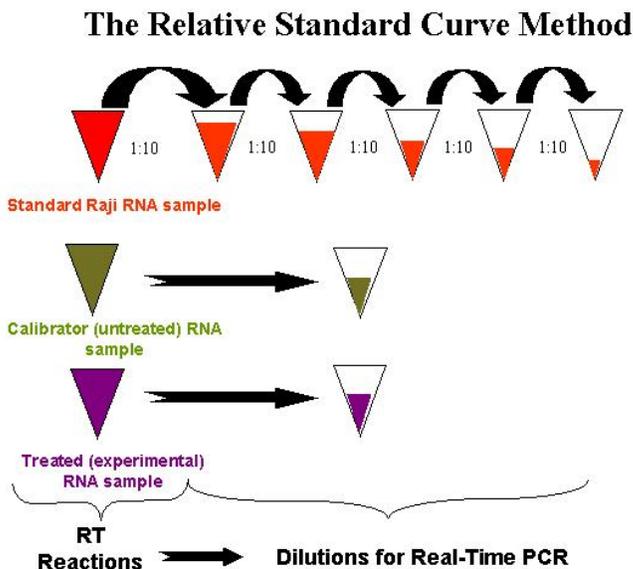

The Relative Standard Curve Method

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.
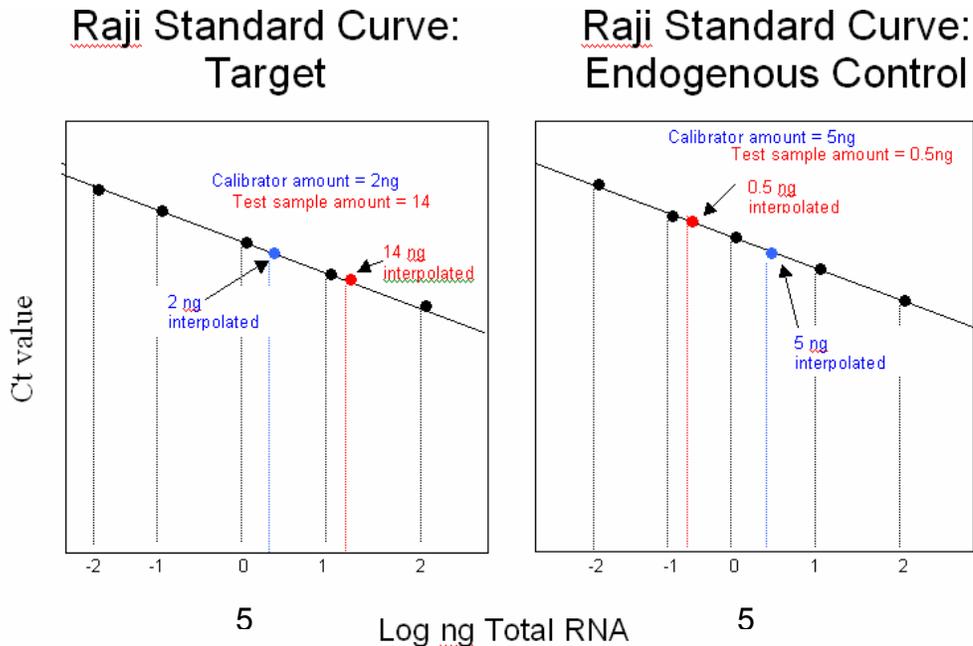

Figure 5: Raji Standard Curve Plots: log of input versus C_T value

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).

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

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

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).

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

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

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.

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.

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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.

Normalized target value = $\dfrac{\text{kidney c-}\textit{myc}\text{ quantity mean}}{\text{kidney GAPDH quantity mean}}$ = $\dfrac{0.41}{1.02}$ = 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.

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

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

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

$$cv = \frac{s}{x} = \frac{\text{standard deviation}}{\text{mean value}}$$

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.

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

b) Calculate the standard deviation

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

$$cv = \frac{s}{x} = \frac{\text{standard deviation}}{\text{mean value}}$$

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.

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3. The Comparative C_T Method (ΔΔ C_T Method)

The Comparative C_T Method, also referred to as the $\Delta\Delta C_T$ 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 $\Delta\Delta C_T$ 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 $\Delta\Delta C_T$ Calculation Is Valid.

Note: TaqMan® Gene Expression Assays have amplification efficiencies of 100%, in high quality samples. 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 are free contaminants (carryover phenol, proteins, inhibitors etc).
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.

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-*my*c – 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.
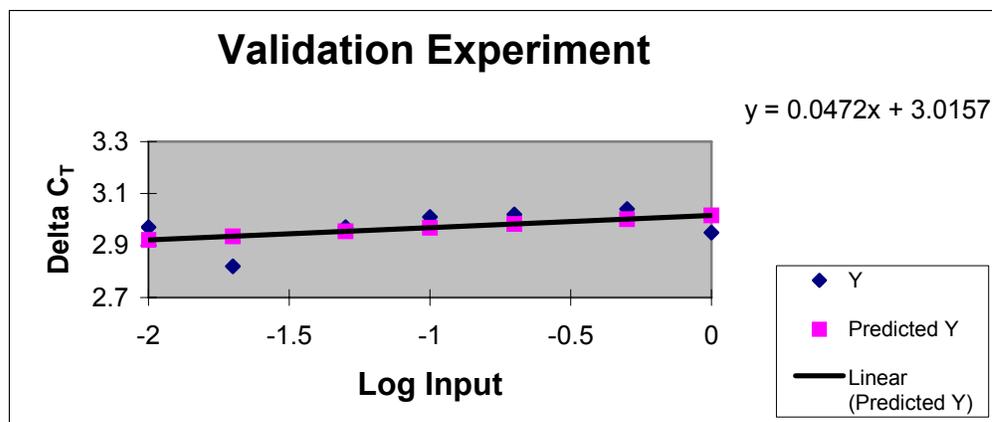

Figure 6: Validation plot of ΔC_T vs. log input amount of RNA

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 redefining 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.

    3. 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)

Step 1: 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.

Step 2: Calculate the ΔC_T value.

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

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 ΔΔC$_T$ 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 ΔΔC$_T$ 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 $\Delta\Delta C_T$ method are usually expressed as a range, which is a result of incorporating the standard deviation of the $\Delta\Delta C_T$ value into the fold-difference calculation.

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

$\Delta\Delta C_T + s = –2.5 + 0.1 = –2.4$

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

and

$$\Delta\Delta C_T + s = -2.5 - 0.1 = -2.6$$

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

e. What If a $\Delta\Delta C_T$ Value Is Positive?

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

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

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.

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 manually 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 ($<5\mu l$).

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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.

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.

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 Reverse Transcription Kit	4368814	4375575
TaqMan® Gene Expression Master Mix	4369016	4371135
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
TaqMan® PreAmp Master Mix Kit	4364130	4366127
Real-Time PCR Systems Chemistry Guide	N/A	4348358
TaqMan® Gene Expression Assays: Protocol	N/A	4333458
RNase Inhibitor	N808-0119	N/A

a. Ordering TaqMan® Gene Expression Assays:

1. Searching for Assays:

The Online Guide for TaqMan® Gene Expression Assays describes how to search for and order assays. The following highlights some of the search steps covered in the guide:

1. Login to the Applied Biosystems Online Store.
2. On the main page under Assays and Arrays, click TaqMan® Gene Expression Assays.
3. Click on TaqMan® Gene Expression Assays under Individual Assays.
4. Click on the Assay Search tab to find an assay of interest.

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 Search
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 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 My Basket to order the Assays, then, click the add to 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® Gene Expression Products: Protocol	4333458
TaqMan® Gene Expression Master Mix Protocol	4375575
TaqMan® Universal PCR Master Mix Protocol	4304449
TaqMan® Fast Universal PCR Master Mix, No AmpErase® UNG: Protocol	4351891
High-Capacity cDNA Reverse Transcription Kit Protocol	4322169
TaqMan® PreAmp Master Mix Kit Protocol	4366127

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c. Custom TaqMan® Gene Expression Assays Part Numbers

Part Description	Part Number	Number of reactions at 50μL reaction volume	Number of reactions at 20μL reaction volume
Small Scale, 20X	4331348	140	360
Medium Scale, 20X	4332078	300	750
Large Scale, 60X	4332079	1,160	2,900

d. Custom TaqMan® Gene Expression Documentation

Document Title	Document Part Number
Custom TaqMan® Gene Expression Assays Protocol	4334429
TaqMan® Gene Expression Master Mix Protocol	4375575
TaqMan® Universal PCR Master Mix Protocol	4304449
TaqMan® Fast Universal PCR Master Mix, No AmpErase® UNG: Protocol	4351891
High-Capacity cDNA Reverse Transcription Kit	4322169
TaqMan® PreAmp Master Mix Kit Protocol	4366127

II. 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 individual TaqMan® Gene Expression Assays.

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

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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

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* The human endogenous control gene targets from the TaqMan® Human Endogenous Control Plate are also available as individual TaqMan® Gene Expression Assays. Click on the assays IDs (ex: Hs99999910_m1) in the above table in part "a" to order the assays individually.

c. TaqMan® Human Endogenous Control Plate Product Documentation

Document Title	Document Part Number
TaqMan® Human Endogenous Control Plate Protocol	4308134
User Bulletin #5 Multiplex PCR with TaqMan® VIC Probes	4306236

III. TaqMan® Endogenous Controls

Document Title	Document Part Number
User Bulletin #5 Multiplex PCR with TaqMan® VIC Probes	4306236

IV. TaqMan® Low Density Arrays (Custom)

a. Documentation

Document Title	Document Part Number
Applied Biosystems 7900HT Micro Fluidic Card: Getting Started Guide	4319399
TaqMan® Low Density Array Formats	127MI57-02

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V. TaqMan® Low Density Arrays Gene Signature Panels

a. Documentation

Document Title
Mouse Alzheimer's Panel Map
Human Alzheimer's Panel Map
Human Endogenous Control Panel Map
Mouse Endogenous Control Panel Map
Rat Endogenous Control Panel Map
Human GPCR Panel Map
Mouse GPCR Panel Map
Rat GPCR Panel Map
Human Immune Panel Map
Mouse Immune Panel Map
Human Inflammation Panel Map
Rat Inflammation Panel Map
Human Phosphodieterase Panel Map
Rat Phosphodieterase Panel Map
Human ABC Transporter Panel Map
Human Angiogenesis Panel Map
Human Apotosis Panel Map
Human Nuclear Receptor Panel Map
Human Protein Kinase Panel Map
Human Stem Cell Pluripotency Panel Map
Mouse Stem Cell Pluripotency Panel Map

Note: *Check the* TaqMan® Low Density Array Gene Signature Arrays *site frequently since there will be periodic updates.*

VI. TaqMan® Cytokine Gene Expression Plate 1

a. TaqMan® Cytokine Gene Expression Plate 1 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 (2 x 96-well plates)	4307265

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b. TaqMan® Cytokine Gene Expression Plate Product Documentation

Document Title	Document Part Number
TaqMan® Cytokine Gene Expression Plate 1 Protocol	4306744
TaqMan® Gene Expression Master Mix Protocol	4375575
TaqMan® Universal PCR Master Mix Protocol	4304449
TaqMan® Fast Universal PCR Master Mix, No AmpErase® UNG: Protocol	4351891
High-Capacity cDNA Reverse Transcription Kit	4322169
TaqMan® PreAmp Master Mix Kit Protocol	4366127

VII. TaqMan® MicroRNA Assays

a. TaqMan® microRNA Assays Reagents

Item	Reagent Part Number
TaqMan® MicroRNA Reverse Transcription Kit	4366596
TaqMan® Gene Expression Master Mix	4369016
TaqMan® Universal PCR Master Mix (with AmpErase UNG)	4304437
TaqMan® Universal PCR Master Mix (no AmpErase® UNG)	4324018
TaqMan® Fast Universal PCR Master Mix, No AmpErase® UNG	4352042

b. TaqMan® microRNA Assays Documentation

Document Title	Document Part Number
TaqMan® MicroRNA Assays Protocol	4364031
TaqMan® Gene Expression Master Mix Protocol	4375575
TaqMan® Universal PCR Master Mix Protocol	4304449
TaqMan® Fast Universal PCR Master Mix, No AmpErase® UNG: Protocol	4351891

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