Methods and Application Guide

Introduction to Quantitative PCR

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Introduction to Quantitative PCR
Methods and Application Guide

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Introduction

You have made an excellent decision in purchasing the Mx3000P™ Real-Time PCR System†. You will find that at Stratagene, we are committed to providing the most comprehensive and easy-to-use support programs to our customers. To that end, *Introduction to Quantitative PCR* was written as a methods and application guide by our Field Applications Scientists and Technical Services Department in order to ensure that you are provided with the start-up support necessary to begin using this instrument, as well as an explanation of the theoretical basis for the materials used in quantitative PCR techniques. This guide is also designed for more experienced scientists, who will find clear guidelines for data analysis and interpretation of results to ensure better quality of experimental results.

After completing the installation of your Mx3000P Real-Time PCR System and performing the recommended validation protocol, the instrument is ready to start running real-time experiments. The next considerations for starting to use real-time methods in your research are:

1. choosing the most appropriate QPCR chemistry, and
2. learning assay design, assay optimization and data analysis methods.

You will find that *Introduction to Quantitative PCR* provides clear steps for learning the details of QPCR methods, how to use these methods effectively, and the most appropriate analysis techniques to provide reliable and reproducible results.

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Real-Time vs. Endpoint Quantitative PCR

PCR technology is widely used to aid in quantifying DNA because the amplification of the target sequence allows for greater sensitivity of detection than could otherwise be achieved. In an optimized reaction, the target quantity will approximately double during each amplification cycle. In quantitative PCR (QPCR), the amount of amplified product is linked to fluorescence intensity using a fluorescent reporter molecule. The point at which the fluorescent signal is measured in order to calculate the initial template quantity can either be at the end of the reaction (endpoint QPCR) or while the amplification is still progressing (real-time QPCR).

In endpoint QPCR, fluorescence data are collected after the amplification reaction has been completed, usually after 30–40 cycles, and this final fluorescence is used to back-calculate the amount of template present prior to PCR. This method of quantification can give somewhat inconsistent results, however, because the PCR reaction efficiency can decrease during later amplification cycles as reagents are consumed and inhibitors to the reaction accumulate. These effects can vary from sample to sample, which will result in differences in final fluorescence values that are not related to the starting template concentrations. As shown in Figure 1, the data collected at the reaction endpoint are not uniform even when identical samples are being amplified.

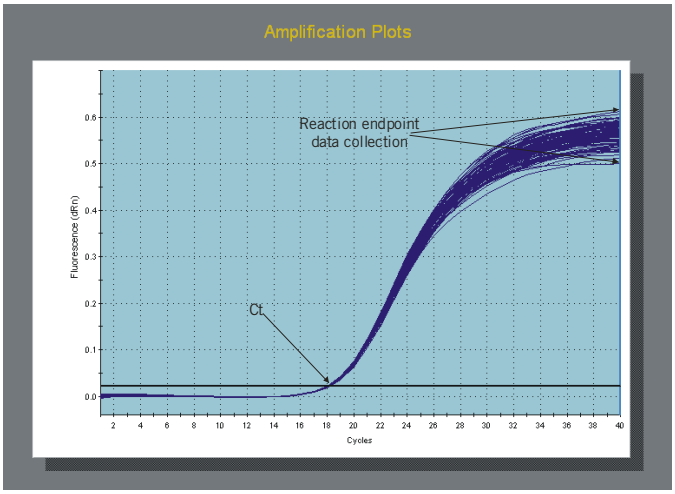

Figure 1
QPCR run with 96 identical reactions. Note that the PCR reaction endpoint variation (i.e. 40 cycles) is much greater as the reaction progresses.

The data spread of endpoint values demonstrates that data measured following amplification are not uniform or reproducible enough to be useful for the precise measurements required for gene expression analysis.

The more sensitive and reproducible method of real-time QPCR measures the fluorescence at each cycle as the amplification progresses. This allows quantification of the template to be based on the fluorescent signal during the exponential phase of amplification, before limiting reagents, accumulation of inhibitors, or inactivation of the polymerase have started to have an effect on the efficiency of amplification. Fluorescent readings at these earlier cycles of the reaction will measure the amplified template quantity where the reaction is much more efficient and, hence, more reproducible than at the endpoint. A fluorescent reporter molecule (such as a double stranded DNA binding dye, or a dye labeled probe) is used to monitor the progress of the amplification reaction. The fluorescence intensity increases proportionally with each amplification cycle in response to the increased amplicon concentration, with QPCR instrument systems collecting data for each sample during each PCR cycle. The first cycle at which the instrument can distinguish the amplification generated fluorescence as being above the ambient background signal is called the "Ct" or threshold cycle. This Ct value can be directly correlated to the starting target concentration for the sample. The greater the amount of initial DNA template in the sample, the earlier the Ct value for that sample (Figure 2). The Mx3000P analysis software determines the Ct value for each sample, based on certain user-defined parameters. If a standard curve dilution series has been run on the same plate as the unknown samples, the software will compare the Ct values of the unknown samples to the standard curve to determine the starting concentration of each unknown. Alternatively, the software can use the Ct values to generate relative comparisons of the change in template concentration among different samples.

Real time quantitative PCR is being used in a growing number of research applications including gene expression quantification, expression profiling, SNP analysis and allele discrimination, validation of microarray data, GMO (genetically modified organisms) testing, monitoring of viral load and other pathogen-detection applications.

The reporter molecule used in real-time reactions can be (1) a sequence-specific probe composed of

an oligonucleotide labeled with a fluorescent dye plus a quencher (e.g. TaqMan® probes [hydrolysis probes], Molecular Beacons and Scorpions) or (2) a non-specific DNA binding dye such as SYBR® Green I that fluoresces when bound to double-stranded DNA. The criteria that should be used to select the chemistry for your QPCR experiment are based on the following considerations:

1. The level of sensitivity and accuracy required for the data analysis.
2. The budget available to support the project.
3. The skill and experience of the researcher in designing and optimizing QPCR assays.

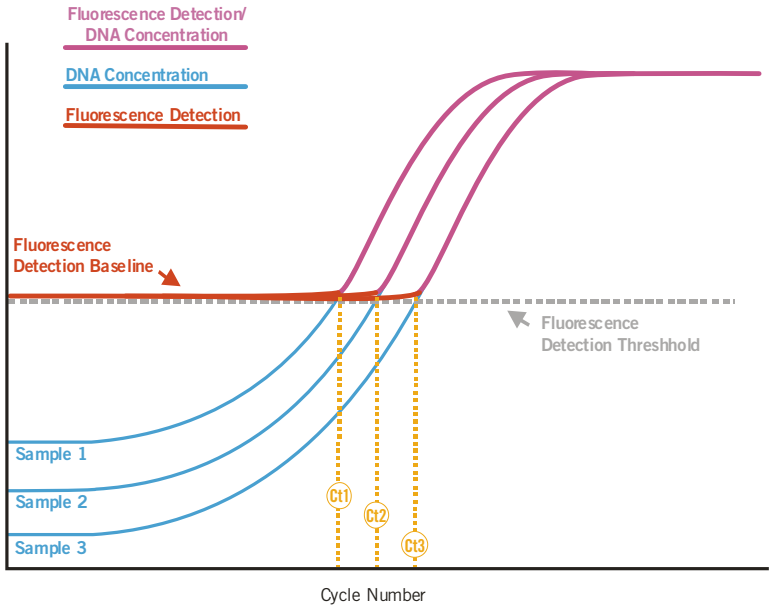

Figure 2

Principles of real-time fluorescence detection and QPCR target concentration measurements using threshold cycle (Ct). The Ct is inversely proportional to the initial copy number. Only when the DNA concentration has reached the fluorescence detection threshold can the concentration be reliably inferred from the fluorescence intensity. A higher initial copy number will correlate to a threshold cycle.

Experimental Design

The core idea that will guide the development of your experimental design is: "What is the fundamental scientific question that you are trying to answer?". For each project there are a number of considerations that need to be addressed:

- What is the goal of the experiment?
- What question is to be answered?
- What is the system being studied?
- What is the total number of genes to be analyzed?
- What control samples (calibrators) and genes (normalizers) will be used to measure the changes in expression levels?
- What is the source of the target sequence?
- Are there any limitations to the amount of target material available?
- What is the sensitivity required to obtain the data necessary to answer the experiment's fundamental question?

The answers to these questions will determine which QPCR approach is best suited to the requirements and objectives of the experiment (Figure 3).

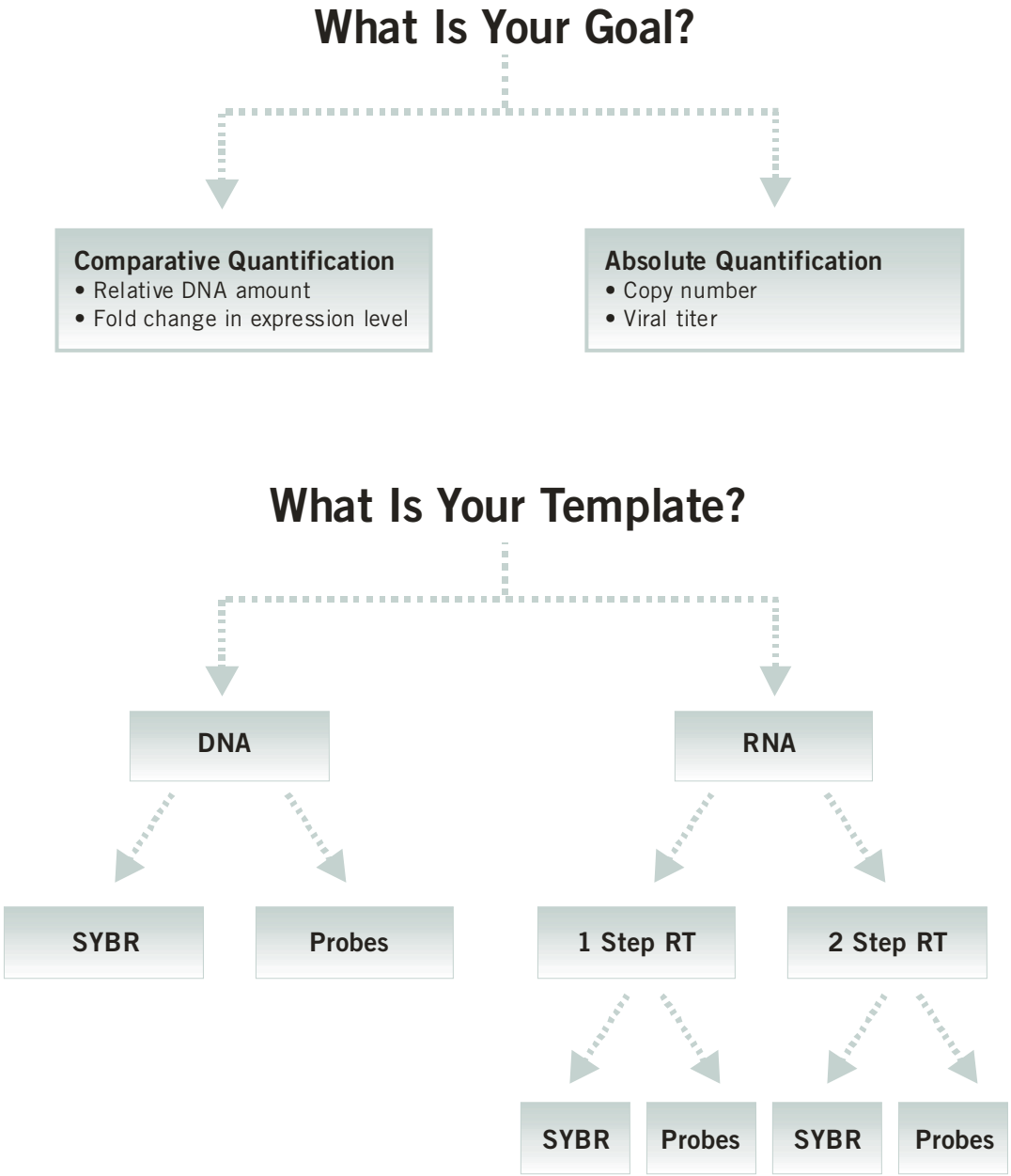

Figure 3
Flowchart showing a typical experimental design process based on the goals and requirements of the assay

QPCR Chemistry Options

One of the first things to consider in experimental design is which QPCR chemistry to use in the real-time PCR experiment. Each has its own advantages and drawbacks. The overall objective and requirements of the research project must be considered in deciding on the most appropriate detection chemistry. This guide will cover the design of experiments using the most commonly utilized chemistries: DNA Binding Dyes (SYBR Green I), TaqMan and Molecular Beacons. Chemistries such as Scorpions, Amplifluor® Primers, LUX Primers, FRET probe pairs (also known as Hybridization Probes), or Invader® Probes will work in the Mx3000P system, but they are beyond the scope of this document.

DNA Binding Dyes

DNA binding dyes such as SYBR Green I are cost effective and easy to use, especially for researchers who are new to using QPCR techniques. These same factors make SYBR Green I a common choice for optimizing QPCR reactions.

When free in solution, SYBR Green I displays relatively low fluorescence, but when bound to double-stranded DNA its fluorescence increases by over 1000-fold. The more double-stranded DNA that is present, the more binding sites there are for the dye, so fluorescence increases proportionately to DNA concentration. This property of the dye provides the mechanism that allows it to be used to track the accumulation of PCR product. As the target is amplified, the increasing concentration of double stranded DNA in the solution can be directly measured by the increase in fluorescence signal (Figure 4). Compared to probe-based methods, SYBR Green I assays are relatively easy to design and optimize. All that is necessary is to design a set of primers, optimize the amplification efficiency and specificity, and then run the PCR reaction in the presence of the dye.

One limitation of assays based on DNA-binding dye chemistry is the inherent non-specificity of this method. SYBR Green I will increase in fluorescence when bound to any double-stranded DNA (dsDNA). Therefore, the reaction specificity is determined solely by the primers. Consequently, the primers should be designed to avoid non-specific binding (e.g. primer dimer formation). Otherwise it is possible that the measured fluorescence may include signal contamination resulting in artificially early Ct values, giving an inaccurate representation of the true target concentration. A non-specific signal cannot always be prevented, but its presence can be easily and reliably detected by performing melting curve analysis on the PCR products from every run.

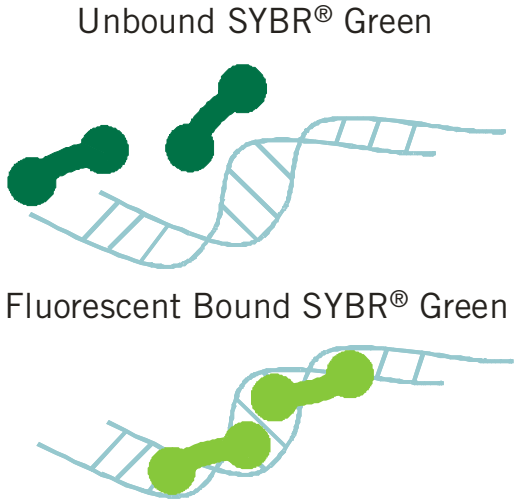

Figure 4
SYBR® Green I detection mechanism; double-stranded DNA in the reaction is bound by the dye. In the bound state, SYBR Green I is 1000 fold more fluorescent than in the unbound state. As PCR amplification increases the amount of dsDNA present, the fluorescence signal increases proportionately.

Following the amplification reaction, the PCR products can be melted and the SYBR Green I fluorescence detected. As the temperature increases, the DNA melts and the fluorescence intensity decreases. The temperature at which a DNA molecule melts depends in part on its length; therefore, if the PCR products consist of molecules of homogeneous length, a single thermal transition will be detected. On the other hand, the presence of more than one population of PCR products will be reflected as multiple thermal transitions in the fluorescence intensity. In this way, the fluorescence versus temperature curve (also known as the dissociation curve) is used to differentiate between specific and non-specific amplicons based on the Tm (melting temperature) of the reaction end-products.

DNA binding dyes are often used for initial expression validation screening of microarray samples as well as for other gene expression applications not requiring exceptional sensitivity and specificity. Optimization of primers to use with SYBR Green I chemistry is straightforward and provides a high level of QPCR experimental design success.

Probe-Based Chemistries

AS compared to non-specific chemistries such as SYBR Green I dye, a higher level of detection specificity is provided by using an internal probe with primers to detect the QPCR product of interest. In the absence of a specific target sequence in the reaction, the fluorescent probe is not hybridized, remains quenched, and does not fluoresce. When the probe hybridizes to the target sequence of interest, the reporter dye is no longer quenched, and fluorescence will be detected. The level of fluorescence detected is directly related to the amount of amplified target in each PCR cycle.

A significant advantage of using probe chemistry compared to using DNA binding dyes is that multiple probes can be labeled with different reporter dyes and combined to allow detection of more than one target in a single reaction (multiplex QPCR).

Linear Probes

Linear probes (hydrolysis or TaqMan probes) are the most widely used and published detection chemistry for QPCR applications. In addition to the PCR primers, this chemistry includes a third oligonucleotide in the reaction known as the probe. A fluorescent dye, typically FAM, is attached to the 5′ end of the probe and a quencher, historically TAMRA, is attached at the 3′ end. Increasingly, dark quenchers such as the Black Hole Quenchers (BHQ) are replacing the use of TAMRA because they provide lower background. As long as the two molecules (reporter and quencher) are maintained in close proximity, the fluorescence from the reporter is quenched and no fluorescence is detected at the reporter dye's emission wavelength. TaqMan probes use a FRET (Fluorescence Resonance Energy Transfer) quenching mechanism where quenching can occur over a fairly long distance (100Å or more, depending on the fluorophore and quencher used), so that as long as the quencher is on the same oligonucleotide as the fluorophore, quenching will occur.

The probe is designed to anneal to one strand of the target sequence just slightly downstream of one of the primers. As the polymerase extends that primer, it will encounter the 5′ end of the probe. *Taq* DNA polymerase has 5′–3′ nuclease activity, so when *Taq* DNA polymerase encounters the probe it displaces and degrades the 5′ end, releasing free reporter dye into solution. Following the separation of reporter dye and quencher, fluorescence can be detected from the reporter dye (Figure 5).

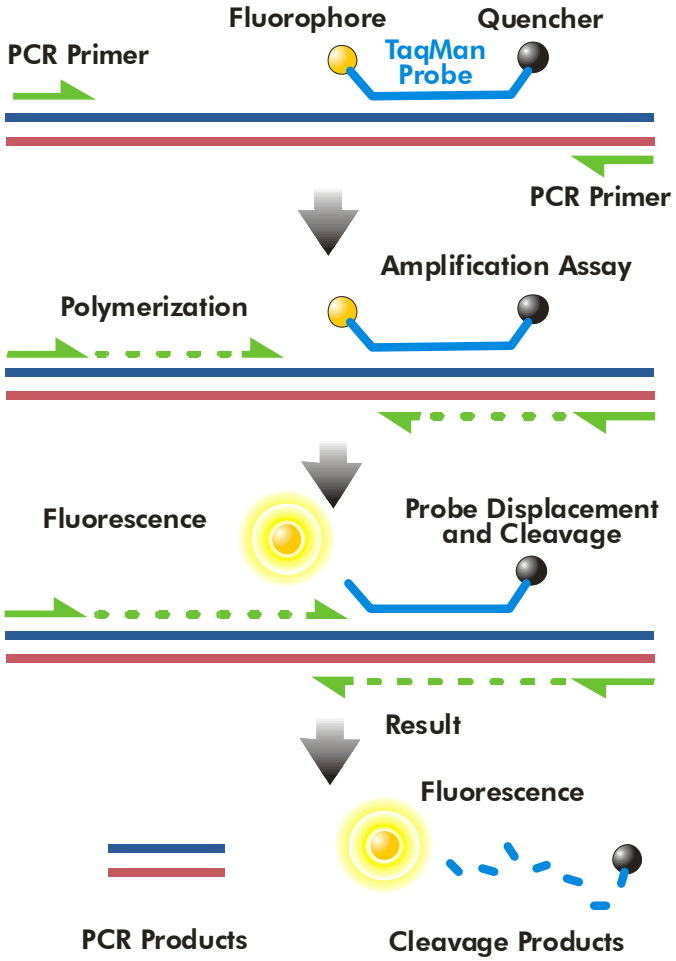

Figure 5
TaqMan® probe chemistry mechanism. These probes rely on the 5′–3′ nuclease activity of *Taq* DNA polymerase to cleave a dual-labeled probe during hybridization to the complementary target sequence.

In order to optimize probe binding and subsequent cleavage, it is critical to adjust the thermal profile to facilitate both the hybridization of probe and primers, and the cleavage of the probe. To meet both of these requirements, linear probes will generally use a two-step thermal profile with a denaturing step (usually at 95°C) and a combination annealing/extension step at 60°C, 7–10°C below the Tm of the probe. If the temperature in the reaction is too high when *Taq* DNA polymerase extends through the primer (such as at a standard extension temperature of 72°C) the probe will be strand-displaced rather than cleaved and no increase in fluorescence will be seen.

TaqMan chemistry can be used for single nucleotide polymorphism (SNP) detection or mutation analysis in a multiplex reaction where a separate probe is

designed for each allele and each probe is labeled with a different fluorophore (e.g. with FAM and HEX). Each probe is designed so that it is complementary to one allele sequence and not the other. However, in these assays, it can be challenging to optimize conditions to prevent the probes from annealing nonspecifically to the wrong allele. In general, enhanced specificity for SNP and allele discrimination analysis is achieved by using either one of the structured probe chemistries described in the "Structured Probes" section (below) or with a new type of TaqMan probe known as a MGB TaqMan (Minor Groove Binder) probe. The MGB probes are similar to the standard TaqMan probes, but they include the addition of a minor groove-binding moiety on the 3′ end that acts to stabilize annealing to the template. The stabilizing effect that the MGB group has on the Tm of the probe allows for the use a much shorter probe (down to ~13 bp). The shorter probe sequence is more susceptible to the destabilizing effects of single bp mismatches, which makes these probes better than standard TaqMan probes for applications that require discrimination of targets with high sequence homology.

Structured Probes

Structured probes contain stem-loop structure regions that confer enhanced target specificity when compared to traditional linear probes. This characteristic enables a higher level of discrimination between similar sequences and makes these chemistries well suited for SNP and allele discrimination applications.

Molecular Beacons include a hairpin loop structure, where the central loop sequence is complementary to the target of interest and the stem arms are complementary to each other (Figure 6). One end (typically 5′) of the stem is modified with a reporter fluorophore and the other end carries a quencher. Rather than using a FRET-quenching mechanism similar to TaqMan probes, Molecular Beacons rely on ground-state or static quenching, which requires the fluorophore and quencher to be in very close proximity for quenching to occur. Historically, DABCYL or Methyl Red have been used for this application. In the absence of target sequence, the stem loop structure is energetically favored and this places the fluorophore and quencher immediately adjacent to one another so that quenching will occur. In the presence of the target sequence, the annealing of the loop sequence to the target is the preferred conformation. When annealed to the target, the fluorophore and quencher are separated, and the reporter fluorescence can be detected.

Figure 6
Molecular Beacon chemistry mechanism. The Molecular Beacon includes a hairpin loop structure, with the loop complementary to a target sequence and the stem formed by the addition of internal complementary sequences. When hybridized to the target, the fluorophore and quencher are far enough apart to allow fluorescence to be detected.

In the absence of the specific target, the Molecular Beacon's thermodynamic properties favor the formation of the hairpin over mismatched binding. This property gives Molecular Beacons the increased mismatch discrimination that makes them well suited for applications such as SNP detection and allele discrimination.

Since the Molecular Beacon chemistry does not rely on the 5′ to 3′ exonuclease activity of *Taq* DNA polymerase, it can be used in a traditional three-step thermal profile. When the thermal cycling ramps up to 72°C and the *Taq* DNA polymerase extends to where the Molecular Beacon probe is annealed, the probe will simply be displaced and it will assume the hairpin loop conformation again. Because formation of the Molecular Beacon hairpin loop is a reversible process, the probe will be recycled with each PCR cycle.

Careful design of the Molecular Beacon stem is critical to ensure optimized performance of the reaction. If the stem structure is too stable, target hybridization can be inhibited. In addition, if the Molecular Beacon probe does not fold in the expected stem loop conformation, it will not quench properly. Any Molecular Beacon probe should be tested after synthesis to verify that it is behaving as expected before it is used in any QPCR assays. Melt curves can be used to make this determination (Figure 7). By melting the Molecular Beacon alone, in the presence of its perfect complement, or of a mismatched sequence, the dynamics of the reaction can be easily compared and used to determine the optimal temperature for fluorescence measurement and mismatch discrimination.

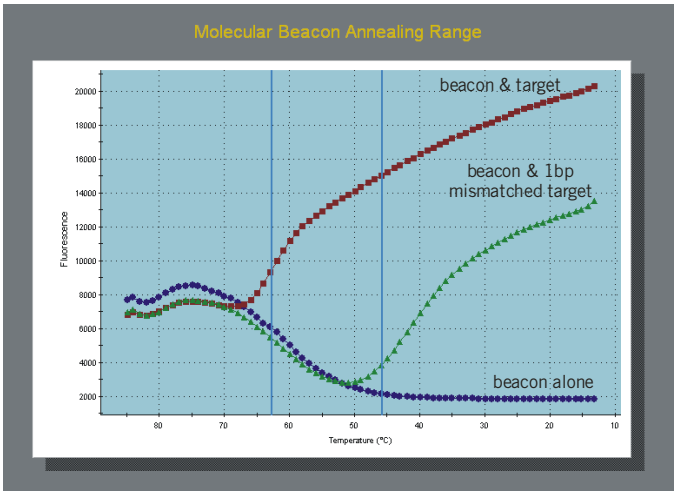

Figure 7
Example of a Molecular Beacon melting curve. Temperature is decreasing left to right on the X axis, and fluorescence is plotted on the Y axis. The window between the two vertical blue lines represents a suitable annealing temperature range to discriminate two alleles with a 1 bp difference.

Scorpions probe chemistry functions in a manner somewhat similar to Molecular Beacons, but rather than having a separate probe, the hairpin structure is incorporated onto one of the primers. The fluorophore is attached to the 5′ end of the primer and the 3′ end is complementary to the target and serves as a site for extension initiation. A quencher is located between the primer and probe region of the oligo, so that when the probe is in the hairpin configuration the reporter dye is located adjacent to the quencher. Following amplification and incorporation of the hairpin probe, the newly created strand is able to adopt a new structure. The loop sequence in the hairpin is complementary to the extension product of the probe/primer. During the subsequent round of denaturation and annealing, the loop sequence will anneal to the newly formed complement within the same strand of DNA. In this conformation, the fluorophore is separated from the quencher so fluorescence is produced. The primer also contains a "PCR blocker" in the hairpin which prevents the stem-loop structure from being copied during PCR by extension from the other primer.

Since the annealing of the loop sequence with the downstream PCR product is an intramolecular interaction, it is kinetically more favorable than probe systems which require two separate molecules to interact (the probe and template). For this reason, Scorpions typically result in higher fluorescence signal compared to TaqMan and Molecular Beacons. As with Molecular Beacons, Scorpions also do not rely on the 5′–3′ exonuclease activity of *Taq* DNA polymerase, so the reaction can be performed using a three-step thermal profile with the optimal extension temperature for the polymerase (72°C).

One disadvantage of the Scorpions chemistry is that the design and optimization of the probe structure is often much more challenging than with either Molecular Beacons or TaqMan probes, and as a result Scorpions are not generally suggested for those who are new to QPCR.

Methods of Quantification

There are two basic quantification methods, and each is suitable for different applications: absolute quantification and relative quantification.

Absolute quantification: The most direct and precise approach for analyzing quantitative data is to use a standard curve that is prepared from a dilution series of control template of known concentration. This is known as "standard curve" or "absolute" quantification. The absolute quantification approach is used when it is important to the experimental design and objective of the project to measure the exact level of template in the samples (e.g. monitoring the viral load in a sample).

A variety of sources can be used as standard templates. Examples include a plasmid containing a cloned gene of interest (GOI), genomic DNA, cDNA, synthetic oligos, in vitro transcripts, or total RNA such as Stratagene's QPCR Human Reference RNA.

Figure 8 describes a basic setup for standard curve quantification. Keep in mind that selection of template is dependent upon the application being pursued. The most critical consideration is that the primer set be optimized to work efficiently with the standards and the experimental source material or tissue.

Following amplification of the standard dilution series, the standard curve is generated by plotting the log of the initial template copy number against the Ct generated for each dilution. If the aliquoting was accurate and the efficiency of the amplification does not change over the range of template concentrations being used, the plot of these points should generate a straight line. This line is the standard curve. Comparing the Ct values of the unknown samples to this standard curve allows the quantification of initial copy numbers (Figure 9).

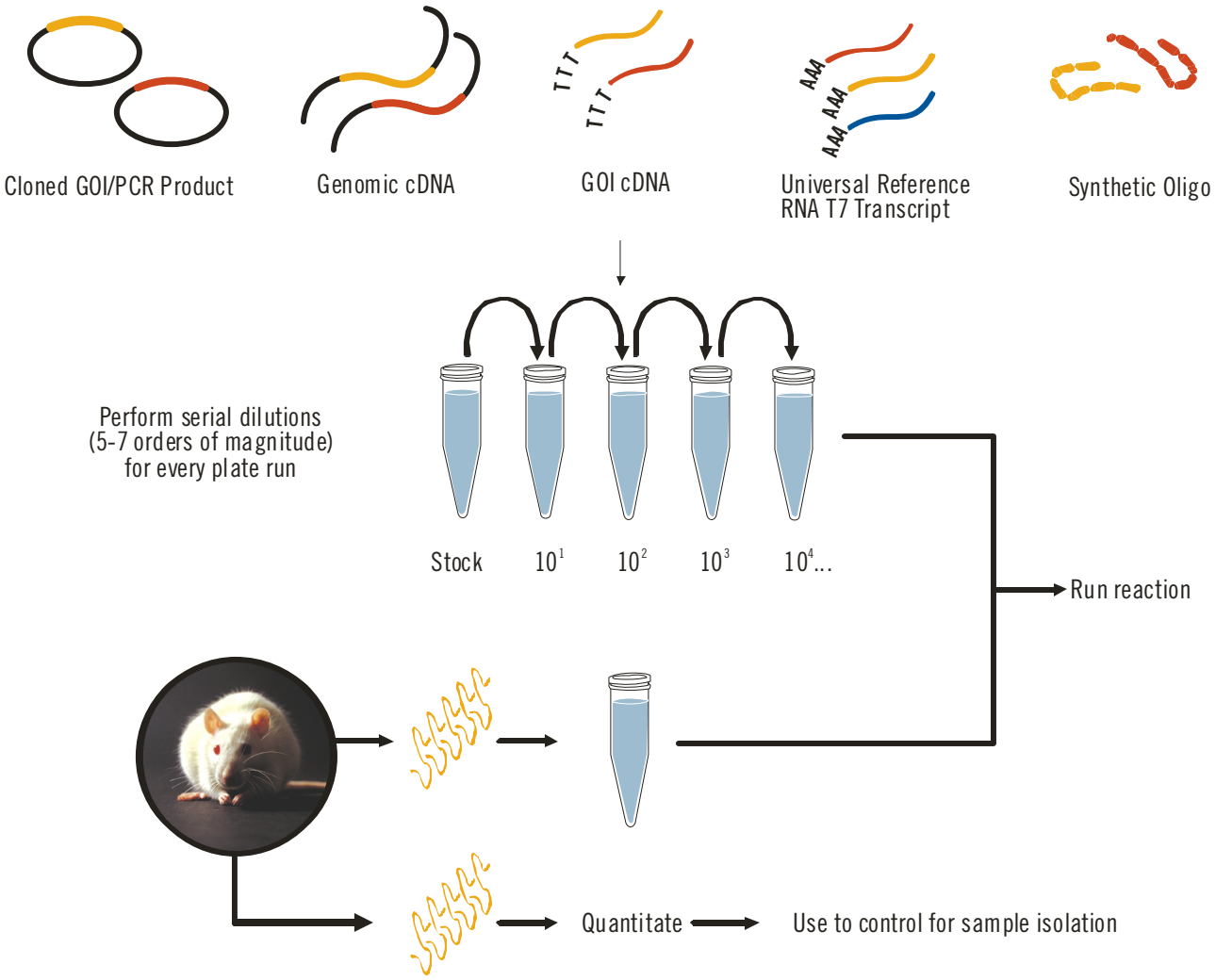

Figure 8

Experimental setup for standard curve quantification. Using a known starting concentration of template from one of a variety of sources, a dilution series is performed. These samples are run under the standard well type on the same plate as your unknowns. By comparing the Ct values of the unknowns to the Ct values of the standards, the starting template quantities for the unknown samples can be calculated.

Ideally, a standard curve will consist of at least 4 points, and each concentration should be run at least in duplicate (the more points the better). The range of concentrations in the standard curve must cover the entire range of concentrations that will be measured in the assay (this may be several orders of magnitude). Conclusions cannot be drawn from samples whose calculated initial quantity exceeds the range of the curve.

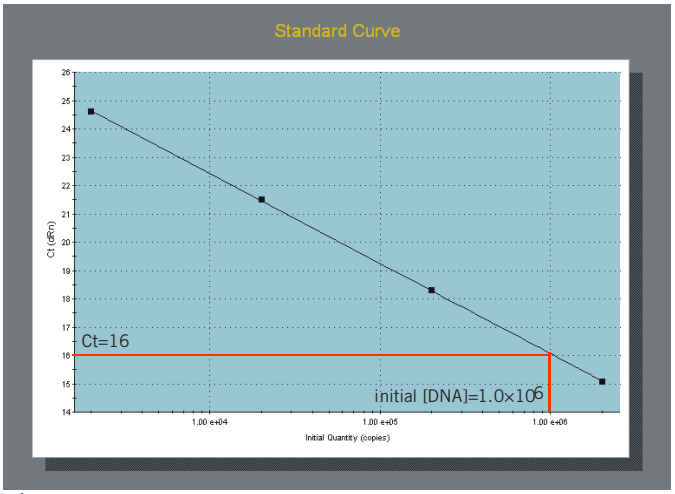

Figure 9
Illustration of the theory behind standard curve quantitation. The log of the initial template quantity is plotted against the Ct values for the standards. By comparing the Ct values of the unknowns to this Standard Curve plot, the initial template quantities for the unknown samples can be determined.

In addition, the curve must be linear over the whole concentration range. The linearity is denoted by the R squared (Rsq) value (R^2 or Pearson Correlation Coefficient) and should be very close to 1 ($\geq$ 0.985). A linear standard curve also implies that the efficiency of amplification is consistent at varying template concentrations. If the standard curve becomes non-linear at very low template concentration, it is probably approaching the limit of detection for that assay. Unknown samples that have Ct values that fall within a non-linear section of the standard curve cannot be accurately quantified. Ideally, the efficiency of both the standard curve and sample reactions should be between 90 and 110%. One hundred percent efficiency implies perfect doubling of amplicon each cycle. If the efficiency is significantly less, this implies the reaction is being slowed in some way, either from inhibitors present in the reaction mix or suboptimal primer sets or reaction conditions. Efficiencies significantly above 100% typically indicate experimenter error (e.g. miscalibrated pipettors, PCR inhibitors, probe degradation, formation of nonspecific products, and formation of primer dimers). Primer dimer formation is typically of greatest concern with SYBR green I assays where any double-stranded product will be detected. Deviations in efficiency can also be due to poor serial dilution preparation as well as extreme ranges of concentrations that either inhibit PCR (high template amounts) or exceed the sensitivity of that particular assay (very low amounts). The most important aspect is to have the efficiencies of standards and targets within about 5% of each other if possible, with both near 100%.

Once the reactions for the standard curve and the samples have been optimized, Ct values can be compared to each other and an initial template quantity can be estimated. It is important to remember that for this type of quantification a standard curve must be run on the same plate as the unknown samples. Replicates can vary in Ct when run at different times or on different plates, and thus are not directly comparable to other runs. Also keep in mind that the "absolute" quantity obtained from the standard curve is only as good as the DNA/RNA quantification methods used to measure the standards, so you must take care to use very clean template and to perform replicate measurements (whether using UV spectrophotometry or nucleic acid binding dyes such as RiboGreen® and PicoGreen®). There should also be at least 2–3 no template control (NTC) wells and for QRT-PCR runs at least 2–3 no reverse transcriptase control wells. A more detailed description of standard curve analysis will follow in the Data Analysis section.

Relative Quantification: Although standard curve (or Absolute) quantification can be useful in determining absolute quantities of target, the majority of scientific questions regarding gene expression can be accurately and reproducibly answered by measuring the relative concentration of the gene of interest (GOI) in unknown samples compared to a calibrator, or control sample. Here, the calibrator is a baseline for the expression of a given target gene. This can be a zero time point in a time-course experiment or an untreated sample that will serve as a benchmark to which the other samples can be compared. Using this approach, differences in Ct value between an unknown sample and calibrator are expressed as fold-changes (i.e. up or down regulated) relative to the calibrator sample. In addition to comparing the expression of the target gene alone in a control versus experimental sample, it is always a good idea to normalize the

results with a normalizing target, typically a gene whose expression is constant in both the control (calibrator) and experimental samples. This normalization controls for differences in RNA isolation and in the efficiency of the reverse transcription reaction arising from sample to sample and experiment to experiment. Normalizers are explained in more detail in the following section.

When designing a comparative quantification experiment, it is not necessary to run a standard curve on every plate as you would for absolute quantification. Rather the results are expressed as the fold difference between the target and normalizer in experimental versus calibrator samples. However, it is usually not accurate to assume that the amplification efficiency in any reaction is going to be 100%, or that the same concentrations of template molecules will be detected at a given Ct value each time the assay is run. Actual amplification efficiency values for a particular reaction can be established via a standard curve measurement during assay design, and multiple standard curves should be run to verify that this efficiency measurement is reproducible (typical run-to-run variability is in the 5% range).

Controls

One of the most important considerations in a QPCR experiment is appropriate controls. The specific controls that are needed will vary somewhat according to the experiment type, but there are certain controls, such as No Template Controls (NTC), that should be included in every run.

In QRT-PCR experiments, especially those based on comparative quantification, it is important to include a normalizer gene (also called a reference gene). In order to generate meaningful data that can be compared from run to run, sample to sample, and lab to lab, it is essential to quantify the normalizer gene side by side with the Gene of Interest (GOI). The normalizing gene is typically a "housekeeping" gene (HKG) whose expression should be constant under the experimental conditions of the assay. This constant level of expression must be verified experimentally, as the expression of housekeeping genes can vary under certain conditions. The most common housekeeping genes used are GAPDH and β-actin, which are ubiquitously expressed, but there is evidence that their level of expression can vary considerably (Radonic, Thulke *et al*. 2004) (Bustin 2002). Alternative references like 18S or 28S rRNA have also been shown to be up and down regulated under different conditions (Radonic, Thulke *et al*. 2004) and may not be applicable when poly A(+) RNA is used as the template source. When working with a whole animal, it may be useful to normalize to total cell number as well. In any case, it is crucial to select a reference or even multiple references that have been empirically tested to be consistent across all experimental conditions in your assay. You can find initial data in the literature or from microarray data (genome-www5.stanford.edu/).

Because the expression level of the normalizer is constant, any variation in the Ct of the normalizer can be attributed to other sources of variation, such as efficiency of the reverse transcription reaction, yield of the RNA purification, or variations in the number of cells from which the RNA was isolated. These sources of variation will affect the normalizer and the GOI equally, so differences in the Ct of the normalizer from sample to sample can be used to correct for any variation in the Ct of the GOI that is not due to changes in expression level. The most essential characteristic for successful normalizer genes is that they are not affected (induced or suppressed) by the changing experimental conditions. It is also important to choose a normalizer that has an expression level and an amplification efficiency that is similar to that of the GOI. During assay design, it should also be confirmed that these amplification efficiency values are reproducible. If they are not, the normalization results cannot be considered reliable.

Any of the references above (housekeeping genes or rRNA) are also known as endogenous references because they are part of the RNA pool. Because it can be difficult to find a truly constant reference, an alternative is to use an external or exogenous reference. An exogenous reference would be an RNA spike (in vitro transcript for example), that can be added in a defined amount to the extracted RNA. This has the advantage that reference gene expression levels are no longer a concern, but RNA isolation variances must still be controlled for. For greatest control, endogenous and exogenous references can be combined in a single assay. Figure 10 describes the advantages of each type of normalizer for comparative quantification.

Positive controls can be used to provide consistent positive reference data points in a given experiment. The positive control material can also be used to create a standard curve with dilutions that span the

expected unknown sample concentrations. If a standard curve is performed in a given run, theMx3000P software can use this to perform absolute quantification of unknown samples by plotting the Ct values of the unknowns on the standard curve. Some examples of appropriate positive control templates include plasmids containing the target sequence, purified PCR products, synthetic oligonucleotides, and Stratagene's QPCR human reference RNA.

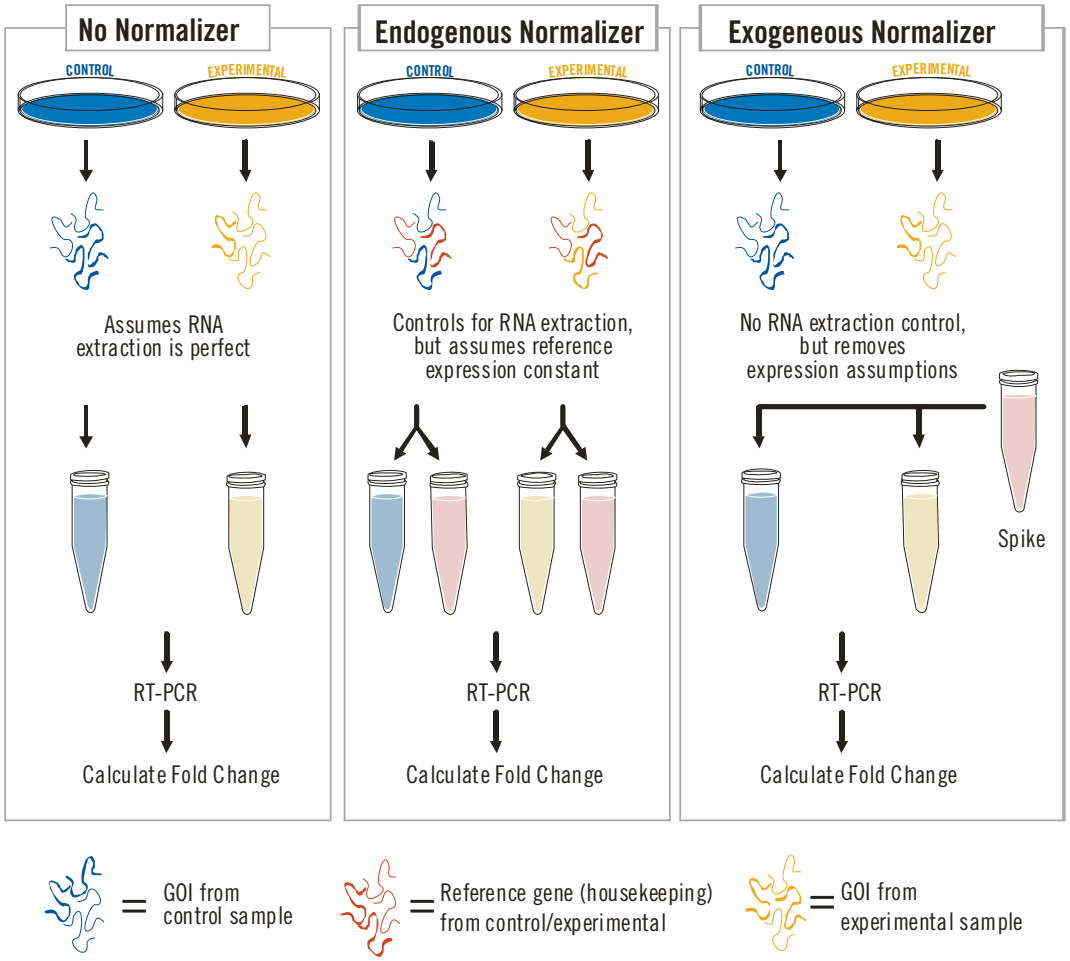

Figure 10
The use of exogenous and endogenous normalizers in QRT–PCR.

Positive controls are especially important to avoid false negatives due to DNA quality, PCR inhibitors, etc., typically associated with heterogeneous samples (e.g. applications such as pathogen detection, GMO testing, or mutation detection). In these experiments, inclusion of a positive control in all the amplification reactions validates the absence of detection of the target of interest as a true negative sample.

Negative controls are often overlooked in experimental design but they are one of the most important components of any QPCR assay. "No Template Controls" (NTC), and "No Reverse Transcription Controls" (No RT) provide a mechanism to control for external contamination or other factors that can result in a non-specific increase in the fluorescence signal. Ideally, signal amplification should not be observed in the NTC sample wells. If the NTCs do cross the threshold, their Cts should be at least five and preferably more than ten cycles from the Cts of your least concentrated samples. If the Cts of the NTCs are less than five Cts above the samples containing template, the Cts should not be considered accurate. Another common negative control is a NAC, or "No Amplification Control", which includes all the reaction components except for the polymerase. This is useful if you suspect that you may be seeing an increase in fluorescence in your reaction that is not due to actual amplification (e.g. your probe is degrading).

When performing qualitative PCR (generating a positive or negative determination on whether or not a given sequence is present) it is necessary to include at least three NTC wells, or three dye-specific negative control wells, in order to determine statistically whether or not real amplification has occurred.

Passive Reference Dye: Although it is not an amplification control, it is common practice when performing QPCR to include a reference dye in the reaction mixture. The reference dye is not linked to any amplification effect. Therefore, the fluorescence from this dye should be constant throughout the amplification reaction. Provided concentration and volume are equal in every well of the reaction, theoretically the fluorescence intensity for the reference dye should be the same in every sample. The fluorescence signal for the fluorophores in the reaction can be normalized to the reference dye by dividing the raw fluorescence intensity at each cycle for the dye of interest by the fluorescence intensity from the reference dye at the same cycle in the same tube. This will act to correct or "normalize" any signal level differences (e.g. those caused by differences in plasticware transparency and reflectivity, or volume differences due to aliquoting errors). This correction is not required, but if a reference dye is designated, it is performed automatically by the Mx software. Corrected data are designated as Rn or dRn in the amplification plots and Text Report. The most commonly used reference dye is ROX. Pure ROX dye should be used at a final concentration of 30 nM in the Mx3000P system or the Mx4000® multiplex quantitative PCR system.*,††

Ultimately, the objective of using real-time quantitative PCR experiments is to determine the absolute quantity of the target sequence present in the sample or to monitor the fold changes of genes in response to experimental conditions. For accurate data analysis and meaningful statistics using either of these approaches, the appropriate positive and negative controls must be included with each real-time assay.

Primer and Probe Design

Primer and probe design is viewed as the most challenging step of setting up a new QPCR experiment. However, the availability of numerous primer and probe design software programs coupled with a set of easy to follow design rules makes the process relatively simple and reliable.

The first step in primer/probe design is to acquire the sequence of your gene of interest. Numerous publicly available sequences can be found in open access databases such as NCBI (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide). After the sequence is obtained, a primer/probe design software program should be used in order to simplify and maximize success for the design process. (If you are requested to enter buffer conditions by the design software, use 100 mM monovalent cation and 5 mM Mg^{++}.)

Designer software packages are available both as freeware on the internet and through most oligonucleotide vendors. A representative list of primer design software can be found in the "Useful Websites" section. In addition, this list can be accessed from the Mx3000P software (under the Tools menu, select "QPCR Internet Links").

The sequence region to be used as the detection template must be considered carefully. Regions with obvious secondary structure or long runs of the same nucleotide (e.g. non-coding regions) should be avoided. For detection of coding sequence specific to RNA targets it is advisable, where possible, to design the probe to span exon-exon boundaries. This prevents the detection of sequences from residual genomic DNA in the RNA prep. In circumstances where this is not an option, the RNA sample should be treated with DNase prior to the RT step. This is an efficient approach and results in minimal loss of sample when carried out on a column-based purification system.

In QRT-PCR, the method of cDNA synthesis is also relevant to primer design if oligo dT priming is used. It is generally safe to assume that the Reverse Transcriptase (RT) reaction has transcribed between 500–1000 bases from the polyA site with quantitative linearity, so it is best to design the assay towards the 3´ of the sequence. The presence of SNPs and splice variants within a sequence should also be considered, as these must either be avoided or targeted as required for the goal of the experiment.

For optimal performance, the region spanned by the primers (measured from the 5´ end of each primer) should be between 60–120 bp in length for probe-based chemistries, and between 100–400 bp in length if SYBR Green I will be used. In order to maximize the efficiency of the PCR amplification, it is best to keep the target length relatively short. With SYBR Green I it is advantageous to use a slightly longer target so more of the dye molecules can bind to the amplified product and produce higher fluorescent signal.

General rules for primers used in all chemistries are that they will be between 15–30 bp in length, and the theoretical Tm of the two primers should be within 2°C of each other. It is best to try to avoid G/C clamps at the 3´ ends of the primers to prevent these oligos from folding on themselves or annealing non-specifically. The five bases at the 5´ terminal end generally should contain no more than two guanines and cytosines, although it is acceptable to have three in the final 5 bases if no two pyrimidines (i.e. thymine, cytosine) are adjacent. Since thymidine tends to mis-prime more readily than the other bases, a 3´ terminal T should be avoided if possible. The 5´ end of the primers also should not contain an inverted repeat sequence that would allow it to fold on itself. It is also very important to run a BLAST search on all primer and probe sequences to make sure they will not potentially anneal to other targets. (www.ncbi.nlm.nih.gov/blast/Blast.cgi)

Probes should not contain runs of the same base (avoid more than three of the same base), and optimally should contain more “C” than “G” nucleotides. Guanine is an effective fluorescent quencher and should not be adjacent to the reporter dye.

For TaqMan probes, where possible, the probe is best placed towards the 5´ end of the amplicon. Historically the probe has usually been situated 3–12 bp downstream of the primer on the same strand, but recent evidence seems to indicate that the distance from the upstream primer to the probe is less important than previously thought. TaqMan probes are generally between 20–30 bp in length. Ideally they should have balanced GC content, although probes with varying content (20–80% GC) can still be effective. The Tm requirements of the probe will most often dictate the specific %GC.

TaqMan assays are conventionally performed as a two-step PCR reaction consisting of a product melt at 95°C, followed by primer annealing and *Taq* DNA polymerase elongation at 60°C (Figure 11). For these assays the probe is designed with a Tm 8–10°C higher than the primer Tms. Using the higher Tm for the probe ensures hybridization to the target before extension can occur from the primer, so there will always be a corresponding increase in fluorescent signal for every amplified copy that is produced.

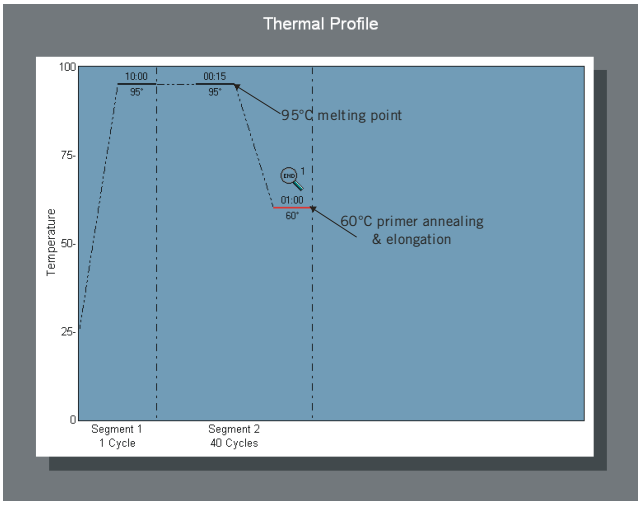

Figure 11
Typical two-step PCR thermal profile used for TaqMan® QPCR reactions.

Since TaqMan chemistry requires using the same thermal profile for each reaction, primers should always be designed with a Tm of approximately 60°C, and the hydrolysis probe with a Tm around 70°C. Optimization of the assay is accomplished by adjusting primer concentration rather than optimizing according to annealing temperatures (this is detailed under the section entitled "Primer Optimization Guidelines").

Molecular Beacons should be design to anneal at 7–10°C higher than the primers, to allow hybridization before primer extension. Scorpion probe regions anneal at the same temperature as the primers or even at 1°C lower since these bind to the newly synthesized DNA strand.

For Molecular Beacons, the stem sequence should be designed to be about 5–7 bp in length and it should have a similar Tm to the melting temperature of the probe region in the loop. As a general rule, stem sequences that are 5 bp long will have a Tm of 55–60°C, stems that are 6 bp long with have a Tm of 60–65°C, and stems that are 7 bp long will have a Tm of 65–70°C. Before having the Molecular Beacon probe synthesized, it is also useful to use an oligo folding program like mfold (www.bioinfo.rpi.edu/applications/mfold/) to verify that the sequence will form the desired stem-loop structure. For the folding temperature use your annealing temperature, and for the ionic conditions enter the [Na^+] as 100 mM and the [Mg^{++}] as 5 mM. Unlike TaqMan probes, Molecular Beacons are usually designed so that the probe is annealed closer to the midpoint between the two primers, rather than adjacent to the upstream primer. This will ensure that any low-activity extension by the polymerase at the annealing temperature will not displace the probe before the fluorescence reading is taken.

A Scorpions probe sequence should be approximately 17–30 bp in length. It is best to place the probe no more than 11 bp upstream of the complementary target sequence. The farther downstream this complementary sequence is, the lower the probe efficiency will be. The stem sequence should be about 6–7 bp in length, and contain sufficient pyrimidines so that the Tm of the stem loop structure is 5–10°C higher than the Tm of the primer sequence to the target, and the ΔG (Gibb's free energy) value for the stem loop confirmation is negative. The more negative the ΔG value, the more likely the folding will occur. You can calculate ΔG values for oligo pairs at www.bioinfo.rpi.edu/applications/mfold/ using the 2-state hybridization server, with parameters of 25°C, 100 mM [Na^+] and 5 mM [Mg^{++}]. In general, you should try to design your primers and probes so all the oligo pairs have ΔG values of –2 or greater (more positive). Similar to Molecular Beacons probes, it should be verified using the mfold site listed above, that the Scorpion probe will form the proper folding confirmation.

For all QPCR reactions, it is a good idea to verify that all of the oligos (primers and probe) that will be used together in the same reaction will not form dimers, particularly at the 3´ ends. The 3´ complementarity can be checked by scanning the sequences manually. If you are using primer design software, the program itself may run a check to make sure the sequence choices it picks are not complementary to each other.

Dye and Quencher Choice

When designing a fluorescent probe, it is necessary to ensure that the fluorophore and quencher pair is compatible, given the type of detection chemistry. In addition, when designing multiplexed reactions the fluorophores and quenchers chosen for the different targets should minimize the spectral overlap between them, to avoid possible crosstalk issues (Table 1).

Filter Set	Ex Wavelength	Em Wavelength
Alexa 350	350	440
FAM/SYBR Green	492	516
TET	517	538
HEX/JOE/VIC	535	555
CY3	545	568
TAMRA	556	580
ROX/Texas Red	585	610
CY5	635	665
FR 640	492	635
FR ROX	492	610
FR CY5	492	665

Table 1
Parameters of the Mx3000P system filter sets. FR 640, FR ROX and FR Cy5 are available only as a custom set.

For TaqMan probes, the most historically common dye/quencher combination is FAM with a TAMRA quencher. This combination will certainly work well, but in recent years dark quenchers have become more popular. Dark quenchers emit the energy they absorb from the fluorophore as heat rather than light of a different wavelength. They tend to give results with lower background, and are especially useful in a multiplex reaction where avoidance of light from the quencher giving cross-talk signal with one of the dyes is important. The most commonly used dark quenchers and the range of emission wavelengths at which they are efficient are:

- **Black Hole Quenchers (Biosearch Technologies):**
  BHQ-1 480–580 nm
  BHQ-2 550–650 nm
  BHQ-3 620–730 nm

- **Iowa Black (Integrated DNA Technologies)**
  Iowa Black-FQ 420–620 nm
  Iowa Black-RQ 500–700 nm

A good listing of TaqMan fluorophore and quencher combinations can be found at the Integrated DNA Technologies website at http://www.idtdna.com/Catalog/DualLabeledFluorescentProbes/Page1.aspx. Molecular Beacons have historically used DABCYL quenchers, which work with a wide range of fluorophores. Molecular Beacons can also be used with the BHQs. A good listing of the different fluorophore and quencher choices can also be found at Integrated DNA Technologies website at http://www.idtdna.com/Catalog/MolecularBeacon/Page1.aspx. Scorpions probes can use fluorophore and quencher choices similar to Molecular Beacons, but in addition the probe must include a chemical blocker such as hexaethylene glycol to prevent the polymerase from extending through the hairpin loop region.

In an Mx3000P system, the choice of dyes will be limited by the four filters you chose to have installed in the instrument. When multiplexing, you should choose dyes that are as spectrally distinct from each other as possible. In general, for duplex reactions the most popular combination is FAM and HEX (JOE/VIC) with ROX as the reference dye. For triplex, FAM, HEX (JOE/VIC), and CY5 with ROX as the reference dye are suggested. For quadriplex, we suggest FAM, HEX (JOE/VIC), Texas Red®, and CY5. Any fluorophores that have little to no spectral overlap are best suited for this type of application.

When multiplexing, Dark Quenchers can be especially useful (as mentioned above). TAMRA can be an effective quencher, but the emission spectra for TAMRA do have some overlap with other dyes such as ROX, HEX, and CY3. With Dark Quenchers, background from the quenchers will not be an issue.

Probe and Primer Synthesis

Primers used for QPCR can be synthesized with any oligo synthesizer or purchased from a commercial oligo house. Probes will also require the addition of dye and quencher molecules. Additionally, Molecular Beacon and TaqMan probes will also need to be blocked at the 3´ end to prevent them from acting as primers and producing extension products. An important consideration to take into account in the preparation of the primers is how they are purified. You can use desalted primers with probe-based detection chemistry. If the primers are to be used with SYBR Green I detection, however, it is preferable that they be HPLC purified. With the SYBR Green I method, any double stranded DNA will give the dye a place to bind and produce fluorescent signal. Since any truncated primers that are present have the potential to anneal nonspecifically, the presence of these in a SYBR Green I reaction may produce artificially high signal and non-specific amplification.

Sequence specific probes should be double HPLC purified or PAGE purified to prevent any truncated sequences from annealing non-specifically and to remove any free dye that may be present in order to reduce background.

Once the oligonucleotides are received, it is best to aliquot them into smaller volumes before storing them at –20°C. Multiple freeze/thaw cycles can damage oligos, and probes with fluorescent tags are especially susceptible to this sort of degradation. Primers should be aliquoted into volumes that will not require that they be thawed more than 20 times, and probes should be stored in volumes that will result in no more than five freeze/thaw cycles.

After the probe has been ordered, an easy test that can be performed to ensure that the probe is quenching properly is to read the fluorescence from an aliquot of a probe, then perform a nuclease digestion of that aliquot and take a second fluorescence reading. Digesting the probe will free the fluorophore from quenching and you should see an increase of $\geq$5000 counts in the raw fluorescence signal. This digestion can be performed using 100 nM of probe in 25µl 1× buffer with 10U DNase or S1 Nuclease, incubated at room temperature for 30 minutes.

QRT-PCR Reactions: One-step vs. Two-step

Prior to performing QRT-PCR reactions (quantitative RT-PCR), a decision must be made whether to use single-step or two-step QRT-PCR. This decision should be based on whether or not the sample source is limiting.

If a single target gene is to be analyzed from each sample and the sample material is not limiting, a single step procedure is likely the most time-efficient and economical approach to use. In this case, the target RNA is isolated, and the target-specific reverse transcription (RT) reaction is performed in the same tube as the subsequent QPCR reaction, with a target specific primer. This single-tube protocol has the advantage that additional manipulation of the generated cDNA is not required, and so the potential for contamination is greatly reduced.

If the source of the sample is limited, difficult to isolate, or if multiple targets are to be quantified from each sample, the two-step QRT-PCR method is generally preferred. After the total cDNA is generated using random primers, oligo dT primers, or a combination of both, the cDNA can be quantified if desired and then archived for subsequent experiments. A portion of this cDNA is then used for in QPCR reactions. With this method, cDNA from a single RT reaction can be used to analyze several genes.

Using the two-step approach allows for the use of optimal buffer conditions for the reverse transcriptase and for the DNA polymerase individually. Using a one-step reaction requires the same conditions for both enzymes, which may not be optimal. Note that if you wish to carry out a dUTP/UNG decontamination strategy to prevent carry-over contamination between experiments, a two-step RT-PCR is required so that the Uracil N-Glycosylase treatment can be performed prior to PCR amplification.

The disadvantage of the two-step approach is the additional sample handling that is required, with the possibility of contamination and sample loss. However, most RT reactions will yield sufficient cDNA to perform 20 QPCR reactions, and the additional step can be worth the time and hands-on attention required for the process. The clear advantage of two-step QRT-PCR is that a maximum amount of information can be determined from minimal RNA. Additionally, because reverse transcription of all targets occurs simultaneously, the variation in RT from reaction to reaction is reduced for more uniform and reproducible data.

Reagent Choice

The Mx3000P system was specifically designed as an open format system that will work with reagents from most manufacturers. Some considerations should be taken into account when choosing which chemistry to use, both from an instrument standpoint and from the standpoint of the specific experimental requirements, as follows:

Reference Dye Considerations

From an instrument standpoint, there is a difference in the concentration of passive reference dye (usually ROX) that should be used in the Mx3000P vs. other systems. The white light excitation system in the Mx3000P system and the system's dye-specific filters will excite and measure the fluorescence for ROX very efficiently. In systems that do not allow excitation at ~584 nm (including laser-based systems), ROX is excited very inefficiently, so a higher concentration of the reference dye is used to compensate for the low ROX signal. If a kit that is designed for one of these systems is used in the Mx3000P system, the high concentration of ROX will create oversaturated signal on the ROX channel and result in normalization problems. Because of this, some master mixes containing ROX could cause problems with normalization if its reference dye concentration is too high. In the Mx3000P system, ROX should be used at a final concentration of approximately 30nM of free dye. Some master mixes contain a short oligonucleotide labeled with FAM and ROX that causes emission from ROX by energy transfer or FRET. The presence of these oligonucleotides is compatible with fluorescence detection in the Mx3000P system.

QPCR Reagents

Other than the requirement imposed by the reference dye concentration, the Mx3000P system should work with any of the commercially available QPCR master mixes, or PCR reagents purchased and optimized separately. Polymerase and buffer from any source can be used in the Mx3000P system. Normally, however, *Taq* DNA polymerase is the polymerase of choice for QPCR. If TaqMan chemistry is used, it requires the 5´ to 3´ exonuclease activity of *Taq* DNA polymerase. Since the products of a QPCR reaction are usually not used for any downstream applications such as cloning or sequencing, high fidelity proofreading enzymes are generally not required. More importantly proofreading enzymes should not be used with any probe-based QPCR method. These enzymes typically utilize a 3´ to 5´ exonuclease activity in order to remove any mis-incorporated bases, and this exonuclease activity could act to digest your probe. The most important criterion for any QPCR polymerase chosen is the efficiency and speed of amplification.

A complete list of our QPCR and QRT-PCR reagent products is located on pages 47–49. All of these kits utilize our SureStart® *Taq* DNA polymerase, which is a hot-start polymerase designed to ensure high specificity. All of the QRT-PCR kits utilize StrataScript™ RNase H(–) reverse transcriptase to reduce RNA degradation and increase cDNA yield. Figure 12 specifically highlights the line of reagent kits for QPCR. For standard QPCR reactions using probe chemistries, the Brilliant® QPCR master mix[†††, ††††] provides all the buffer components in a pre-optimized solution. The Brilliant® QPCR core reagent kit[†††, ††††] offers the reagents in separate tubes so you can perform your own buffer optimization. If you wish to carry out a dUTP/UNG decontamination procedure, the Brilliant® *Plus* QPCR core reagent kit[†††, ††††] includes a dNTP mix containing dUTP and a stock of the Uracil-N-Glycosylase enzyme. For SYBR Green I detection, the Brilliant® SYBR® Green QPCR core reagent kit[†††, ††††] and Brilliant® SYBR® Green QPCR master mix[†††, ††††] both come with the SYBR Green I dye included.

All Brilliant kits are also available for use in both one-step and two-step QPCR reactions (Figure 13). Both the one and two-step kits are available as either standard kits or Plus format kits containing the reagents necessary for dUTP/UNG decontamination. The one-step kit is available as a core reagent kit or master mix, however the two-step kit is only available as a core reagent kit.

The FullVelocity™ technology[†††, †††††, ††††††, **] is a new high-speed reagent system for both QPCR and QRT-PCR that delivers sensitive, specific and reproducible results with significantly shorter overall run times than other QPCR reagents. This technology uses a novel multi-enzyme formulation which has been extensively performance-tested for fast cycling conditions. The enzyme formulation consists of a unique non-*Taq* DNA polymerase that has been engineered for high speed QPCR, together with an additional thermostable protein that provides highly specific cleavage of hydrolysis probes during each cycle. FullVelocity technology enables you to reduce your overall cycling times by

greater than 40% while using any primers and probes designed for use in a TaqMan assay. The FullVelocity technology is also available as a one-step QRT-PCR Master Mix.

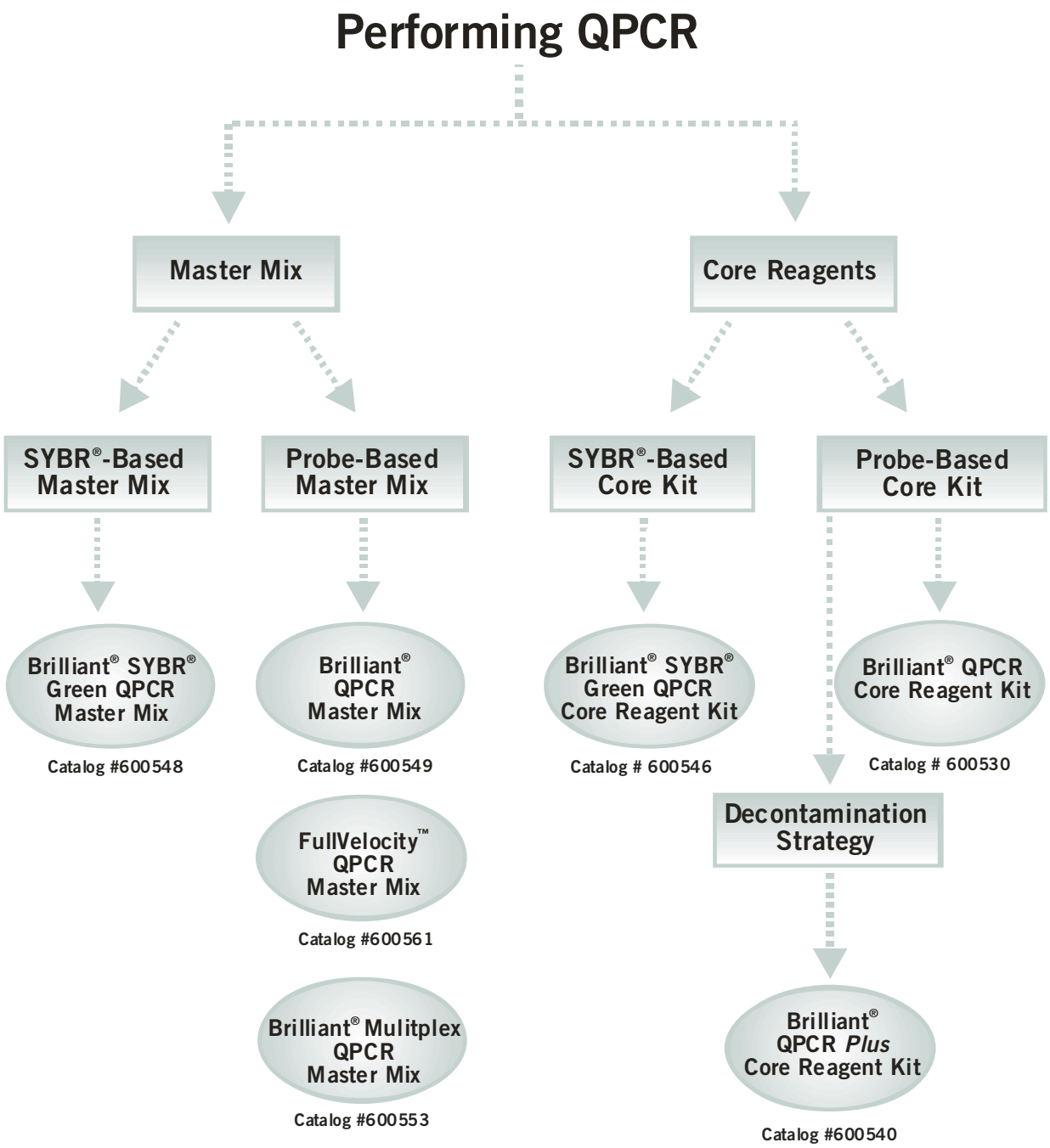

Figure 12
Flowchart showing the Stratagene QPCR reagent kit best suited to each experimental design. This flowchart is not meant to be all-inclusive; for a detailed explanation of Stratagene's products related to QPCR, see the Reagent & Ordering Information.

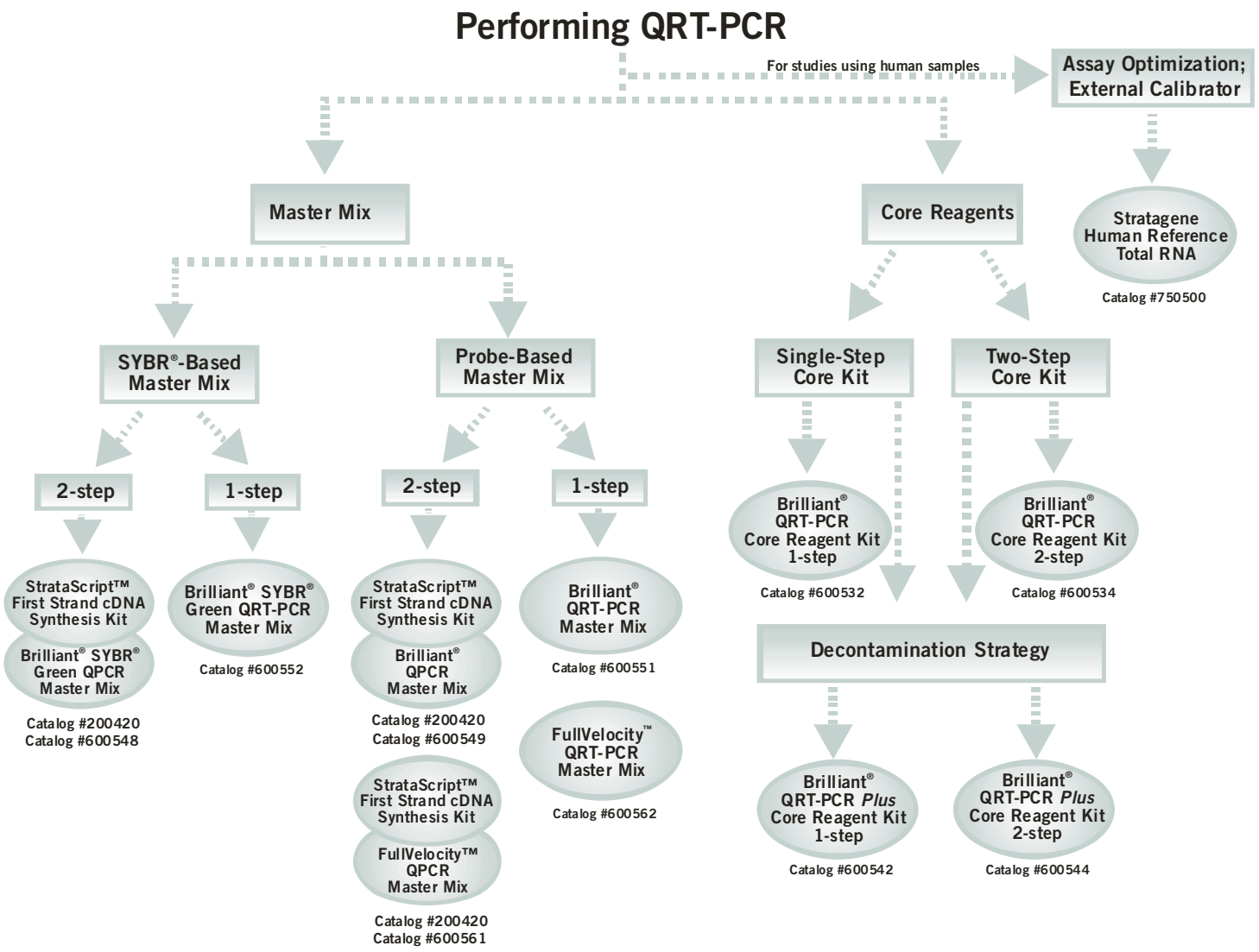

Figure 13
Flowchart showing the Stratagene QRT-PCR reagent kit best suited to each experimental design. This flowchart is not meant to be all-inclusive; for a detailed explanation of Stratagene's products related to QRT-PCR, see the Reagent & Ordering Information.

Assay Optimization

To ensure efficient and accurate quantification of the target template, QPCR assays should be optimized and validated. This process requires the use of an abundant and quantifiable control template. The most common source of control templates is target that has been cloned into a plasmid (must be linearized and purified), a purified PCR fragment, genomic DNA, a synthetic oligonucleotide, or a stock of cDNA from cell culture. Stratagene offers a QPCR-specific human Universal Reference RNA, made from multiple cell lines that provide coverage of 85% of all genes, which can also be used as a template for optimization reactions.

Assays are most efficiently optimized by first evaluating the primer concentrations (forward and reverse) for a given template, then testing different probe concentrations, and finally (if required) optimizing the Mg^{++} concentration.

In most experiments it is sufficient to use a standard concentration of $MgCl_2$ depending on the type of QPCR chemistry employed in the assay. For linear hydrolysis probes (TaqMan), begin with a final concentration of 5.0 mM $MgCl_2$. Molecular Beacons use a lower concentration of 3.5 mM. Scorpions use a lower concentration of 1.5 mM to 2.5 mM $MgCl_2$. Therefore a single concentration must be determined that will work for all the assays. Higher Mg^{++} concentration tends to favor hybridization, and therefore excessive $MgCl_2$ can promote the formation of primer-dimers. While primer-dimers are not detected by sequence specific probe chemistries, they can cause the reactions to be inefficient and therefore less sensitive. When multiplexing, a standard Mg^{++} concentration must also be used.

In SYBR Green I assays, primer-dimers generate contaminating signal making it difficult to get reliable quantitative data from the experiment. Therefore, for SYBR Green I assays it is best to use 1.5 mM to 2.5 mM $MgCl_2$ concentration.

Primer Optimization Guidelines

Depending on the QPCR chemistry being utilized for the assay, different ranges of primer concentrations should be tested. For SYBR Green I, relatively low primer concentrations are used to avoid primer-dimer formation. For most SYBR Green I applications, primer concentrations ranging from 50–300 nM should be tested.

For sequence specific probe chemistries like TaqMan and Molecular Beacons, a wider range of primer concentrations needs to be considered. Typically, primer concentrations ranging from 50–900 nM should be tested. However, not all assays require the testing of all these primer concentrations. In many cases testing a range from 50–600 nM will suffice. To demonstrate the more complex of these two primer optimization strategies, this guide will illustrate the optimization matrix for primer concentrations from 50–900 nM. These reactions will be run in duplicate with the appropriate controls.

Primer Optimization with SYBR Green I

SYBR Green I is inexpensive and easy to use, making it ideal for use in primer optimization. Since SYBR Green I dye is a DNA binding dye, it will generate signal from both specific and non-specific products. The generation of all products can be easily visualized on a melt curve following the amplification reaction. Therefore, SYBR Green I dye can be used to determine both primer performance and primer specificity at different concentrations. As a result, the entire primer optimization process can be completed before ordering the sequence-specific probe. This is desirable because if the primers are not working it may be necessary to redesign them. Since this may also involve redesigning the probe, it is worthwhile to run this test prior to ordering the probe. For an example of how to set up your plates for primer optimization, refer to page 40.

Primer Optimization Data Analysis

Using the Mx3000P software, you can analyze dR or dRn. Analysis of dRn is only applied if a passive reference dye (e.g. ROX) is used in the experiment. If no reference dye is used, then analyze dR. In the following examples dRn is used. Once the run is completed, examine the Ct and dRn Last values for each primer combination. Select the primer combination that results in the lowest Ct value and the highest dRn Last value. When optimizing primers using SYBR Green I, it is also crucial to analyze the melt curve data for each primer concentration pair to ensure a single homogenous product is being generated. If several primer combinations give very similar results, pick the primer combination with the lowest overall concentration.

Dissociation (Melting Curve) Analysis

All products generated during the PCR amplification reaction are melted at 95°C, then annealed at 55°C and subjected to gradual increases in temperature. During the incremental temperature changes fluorescence data are collected until the reaction reaches 95°C. The result is a plot of raw fluorescence data units versus temperature (Figure 14). This view of the data may appear difficult to interpret at first, but the rapid linear decrease in fluorescence between 82°C and 84°C where the major product melts is obvious in Figure 15.

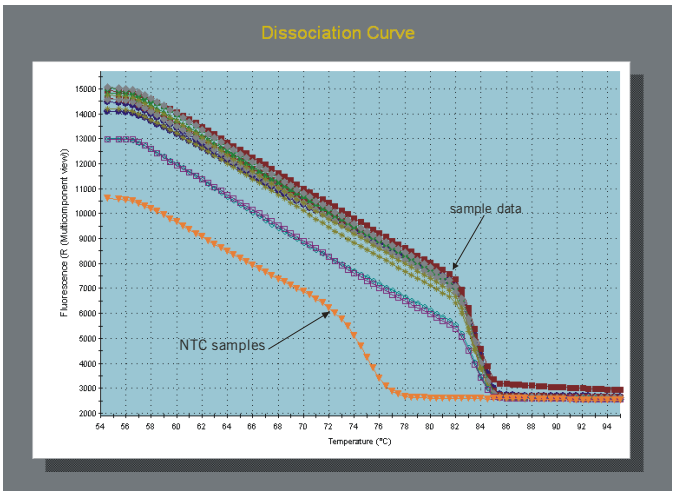

Figure 14

Raw fluorescence signal change plotted as a function of increasing temperature. The higher traces show a rapid melt between 82°C and 84°C. The NTC samples show a change in plot shape around 72°C.

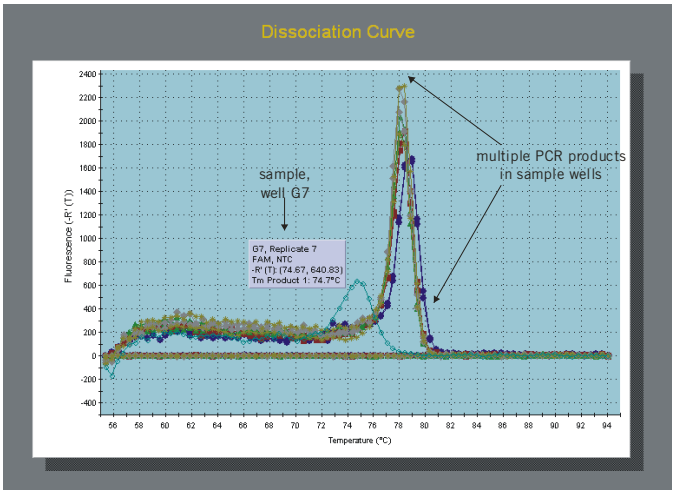

Figure 15

The negative first derivative of raw fluorescence plotted against increasing temperature during the melt curve. Sample well G7 is highlighted to indicate a small amount of primer-dimer product melting in the NTC sample. The other plots indicate melts at 78°C and 79°C, indicating two PCR products in some of the sample wells.

In analyzing the various combinations of primer in Figure 15, you can see one major peak at 78°C for the majority of the samples, but a few samples have peaks at 79°C. The distinct melting peaks indicate multiple PCR products in this assay. A fully optimized assay will contain only a single melt product. The Cts from these wells should not be trusted as accurate, and no meaningful quantification can be based on these data.

Two steps are required to interpret results from a SYBR Green I melt curve analysis. The first step is to review the PCR products produced by the samples in the reaction. In the example shown in Figure 16, the presence of a single homogeneous melt peak for all sample reactions confirms specific amplification. The data from this reaction are reliable and meaningful for analysis and interpretation. The second step is to evaluate the NTC sample well for the presence of primer-dimer formation. It is acceptable to observe a small amount of primer-dimer formation in the NTC wells, but if there is a corresponding peak in the sample amplification plots the Cts from these wells cannot be trusted as accurate.

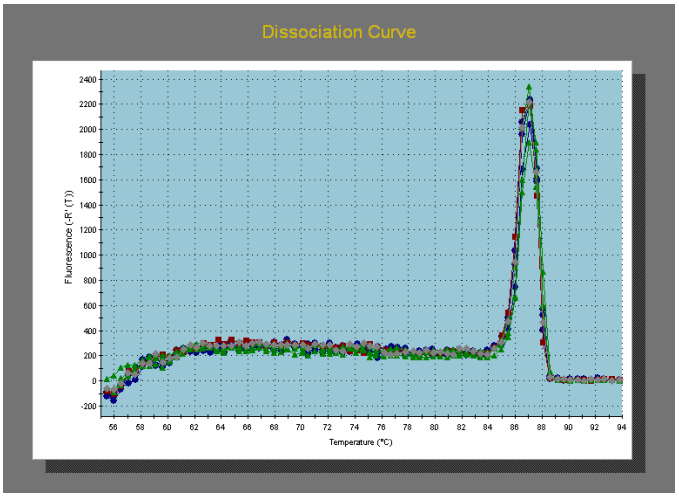

Figure 16

The first derivative of raw fluorescence plotted against an increase in temperature. The single melt peak at 86.5°C indicates a single PCR product is being amplified in these samples.

Choosing the Correct Primer Concentration

Figure 17 and Figure 18 show an example of a 100 nM–900 nM primer matrix in the presence of a linear hydrolysis probe. Based on Ct only, all primer concentration combinations give comparable results. It is best to get the lowest Ct values possible, but it is often more important to reduce the overall primer concentration if you are planning to use this as part of a multiplex assay. When multiplexing, the lower the overall concentration, the less chance that the reactions will interfere with one another. After analyzing the amplification plots, 300 nM forward primer concentration and 900 nM

reverse primer concentration was chosen because of its lowest Ct and highest dRn.

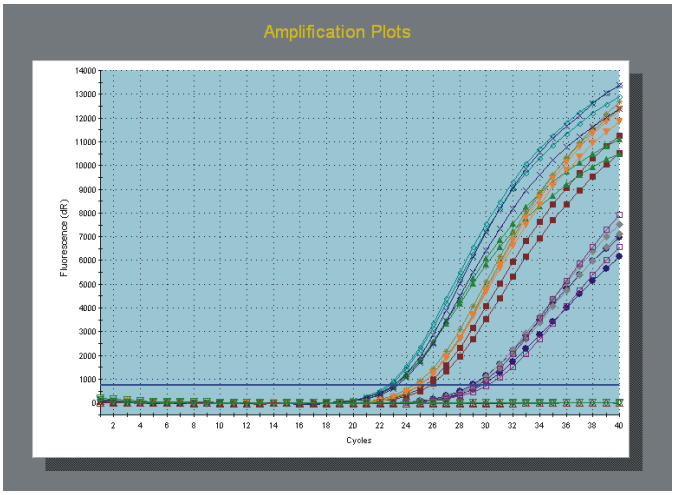

Figure 17
Amplification plots of the primer matrix optimization with each cycle number plotted vs. dRn fluorescence. Different primer concentration combinations generated Ct values between 22.5 and no Ct measured.

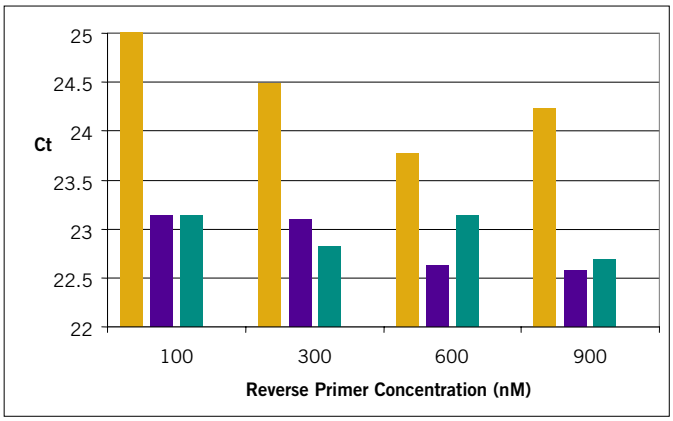

Figure 18
Primer optimization matrix data. Forward primer concentrations tested against reverse primer concentrations with average Ct values listed. Forward primer concentrations (in nM) include: 100, yellow; 300, purple; 600, blue. Forward primer concentrations of 900 nM resulted in no Ct, and are not shown here. Based on the "No Ct" for 900nM of forward primer, this concentration is too high and likely inhibitory in the reaction.

Primer Optimization with Fluorescent Probes

If you prefer to optimize the assay with a fluorescent probe in each phase of the process, the first step is still to determine the optimal primer concentrations. A good starting concentration for linear hydrolysis probes is 200 nM although lower concentrations of 100 nM can be used if probe quantity is a concern.

The procedure for performing the primer optimization matrix experiment is nearly identical to that listed for the SYBR Green I procedure on page 40. The one major exception in this approach is the thermal profile to be used for the linear hydrolysis probe experiment. Linear hydrolysis probes (TaqMan probes) use a two-step thermal profile, and Scorpions or Molecular Beacon probes use a three-step thermal profile with pre-determined optimal annealing temperatures. Fluorescent probe thermal profiles do not employ a melt curve.

Primer Optimization Data Analysis

Analysis of probe-based primer optimization is similar to SYBR Green I primer optimization analysis, but does not include the melt curve component. Optimal primer combinations are still determined by the lowest Ct value and highest dRn Last value.

Probe Concentration Optimization Guidelines

After the optimal primer concentrations have been determined, it is necessary to determine the optimal probe concentration for the assay. Fluorescent probe concentrations typically range from 50nM to 300nM for linear hydrolysis probes and Molecular Beacons, while other QPCR chemistries, like Scorpions, might require concentrations as high as 500nM. For an example of how to set up a probe optimization reaction, refer to page 41.

Probe Optimization Data Analysis

Analysis of fluorescent probe optimization is similar to primer optimization analysis. Select the probe combination that results in the lowest Ct value and the highest dRn Last value. If several probe combinations give very similar results, pick the lowest probe concentration (Figure 19).

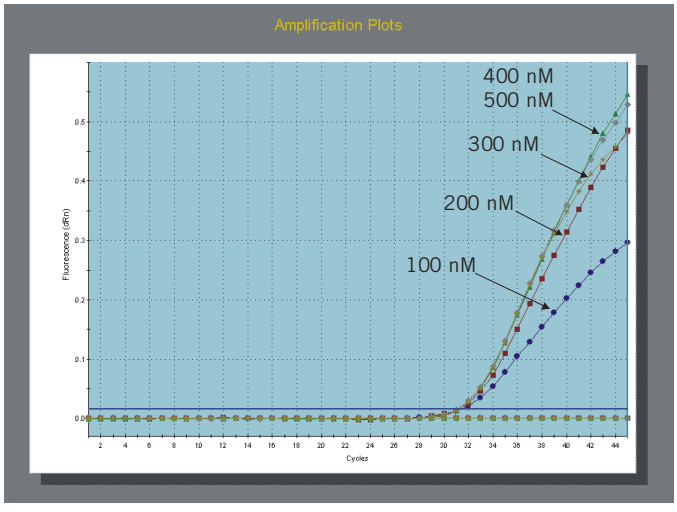

Figure 19
Probe optimization data plotted as cycle number vs. dRn fluorescence. All probe concentrations generate the same Ct value. However, the dRn Last values are decreased with 100 nM 200 nM and 300 nM of probe. Based on these data 200 nM of probe would be optimal.

Standard Curves

After determining optimal primer and probe concentrations for the assay, we recommend testing the overall performance of the QPCR reaction in terms of efficiency, precision, and sensitivity. Data generated from a serial dilution of a positive control template (standard curve) are an excellent means of determining the overall performance of a QPCR assay. The dilution series should encompass a large range of concentrations to ensure the reaction performs at equal efficiency for high and low concentrations of starting template, ideally encompassing the expected levels of target to be encountered with the experimental samples. To accomplish this objective, a three-fold to ten-fold dilution series over several orders of magnitude should be generated in triplicate. For example, for gene expression experiments, a typical serial dilution would consist of five points of a five-fold serial dilution, starting with 100 ng of total RNA per reaction (or the cDNA equivalent amount).

PCR Reaction Efficiency

The slope of the standard curve is used to determine reaction efficiency. Since the PCR reaction is based on exponential amplification, if the efficiency of PCR amplification is 100% the amount of template will double with each cycle, and the standard curve plot of the log of starting template vs. PCR cycles which generate a linear fit with a slope between approximately –3.1 and –3.6 are typically acceptable for most applications requiring accurate quantification (90–110% reaction efficiency). If the amplification reaction is not efficient at the point being used to extrapolate back to the amount of starting material (usually the Ct is used for this purpose), then the calculated quantities may not be accurate.

Precision

The standard curve should be run in triplicate (or at least duplicate) so that it is possible to determine the precision of pipetting, the reproducibility, and the overall sensitivity of an assay. Rsq is the fit of all data to the standard curve plot and can be influenced by accuracy of the dilution series, and overall assay sensitivity. If all the data lie perfectly on the line, the Rsq will be 1.00. As the data fall further from the line, the Rsq decreases. As the Rsq decreases it is more difficult to determine the exact location of the standard curve plot thus decreasing the accuracy of quantification. An Rsq value $\geq$0.985 is acceptable for most assays.

Sensitivity

The slope and Rsq values of the standard curve help determine the sensitivity of a given assay. If the slope of the standard curve is lower than –3.322, the Rsq is below 0.985, and the data points indicate an upward trend in the standard curve plot at the lower starting template concentrations, this may indicate the reaction is reaching the threshold of sensitivity. In this case, further assay optimization or even redesign of the primers and probe may be necessary to extend the linear range. Alternatively, the points outside the linear range can be culled from the standard curve. However, unknown samples in that concentration range may not be trusted to give quantitative results.

Standard Curve Examples

Figure 19 and Figure 20 illustrate a four-fold dilution series standard curve over three orders of magnitude. In this example the data generate a linear standard curve with a slope of –3.401 which is well within the acceptable range of –3.1 to –3.6 and an amplification efficiency value (Rsq) of 98.6%, again, within the acceptable parameters described above.

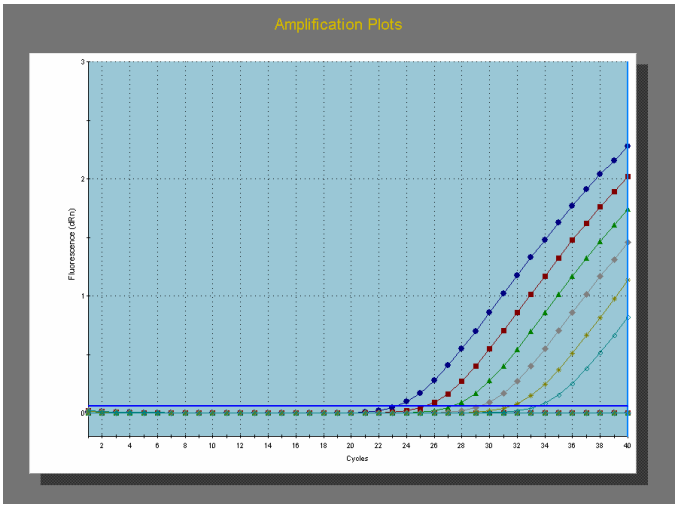

Figure 20
Amplification plots of standards in a four-fold dilution series over three orders of magnitude.

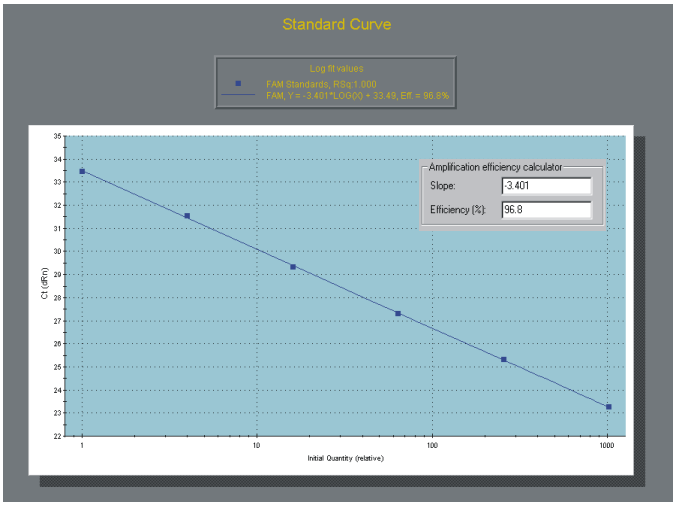

Figure 21
Standard curve generated with data from Figure 20, with slope and Rsq indicated.

Further Optimization

If the assay is still not performing well after the probe and primer concentrations are optimized, you can try altering the Mg^{++} concentration within the range of 3.5–5.5 mM for TaqMan or Molecular Beacons reactions or in the range of 1.5–3.5 mM for Scorpions or SYBR Green I reactions. If the reaction still does not work well after complete optimization is performed, it may be necessary to redesign the primers and/or the probe.

Multiplex Assay Considerations

If the experimental project requires many runs over time on the same set of genes (e.g. time course studies, metastasis progression research), it may be more cost effective and provide a higher level of statistical correlation to design a multiplex reaction to use for the duration of the study. The multiplex approach is particularly important when the template material is limited because this approach allows the maximum amount of data to be generated from each assay. It does require more time to design and optimize reactions that will all work together in the same tube, but the long term savings in reagent cost. The time from the reduced number of experimental runs can be substantial.

The primers and probes for multiplex QPCR reactions are designed the same as they would be for singleplex reactions, with a few extra considerations. All of the primers and probes that will be used in the same reaction should be of similar length, Tm, and GC content. Also, special care should be given to ensure that none of the oligos will interact with one another. In a singleplex reaction, the ΔG value for any two of the oligos in the solution should be –2 or greater (more positive). This may not always be possible for the large number of oligos in a multiplex assay, but minimally you should try to achieve ΔG values in the following ranges:

Singleplex: Greater than –2
Duplex: Greater than –4
Triplex: Greater than –6
Quadriplex: Greater than –8

When optimizing the relative primer concentrations, it is especially important with a multiplex assay to use the lowest primer and probe concentrations possible. The higher the oligo concentrations, the greater the chance the reactions will interfere with one another. Standard curves should be run during the assay optimization for all the reactions both singleplex and multiplex to ensure that the reactions do work together (Figure 22 and Figure 23). The efficiency in the singleplex reactions and the multiplex reactions should not differ by more than 5%, and the Ct values should not change by more than approximately 1 Ct.

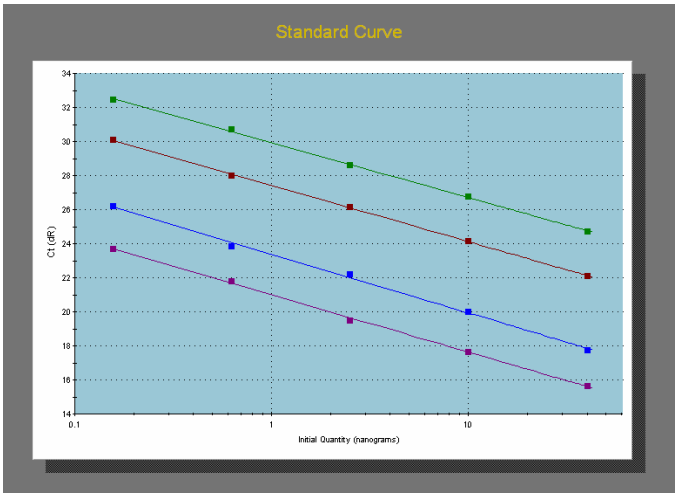

Figure 22
QPCR standard curve from FAM, HEX, CY5, and Texas Red® Singleplex Assays.

If the multiplex assays do not appear to be working well together, may be necessary to add additional reaction components to ensure that reagents are not limiting to the multiple reactions. In these cases, the *Taq* DNA polymerase and dNTP concentrations can both be increased by between 50–100%, and the buffer concentration can be increased from a 1× solution to a 1.5× solution. For this purpose, Stratagene offers a Brilliant® Multiplex QPCR

Master Mix[†††] preoptimized for the simultaneous amplification of multiple targets.

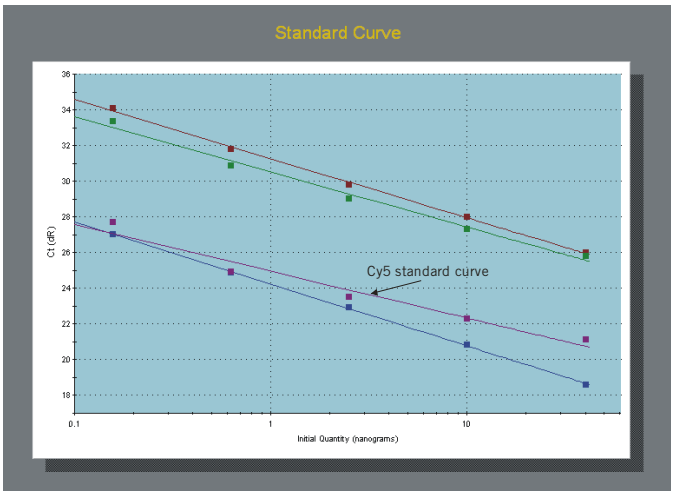

Figure 23
QPCR standard curves from a FAM, HEX, CY5, Texas Red® multiplexed assay. The CY5 primer/probe set slope shows ~140% efficiency, indicating some sort of interaction with other reactions to generate additional signal.

If one reaction in particular has a very early Ct or a very large dR compared to the other reactions, you can decrease the primer concentration in that reaction to prevent this from affecting the other reactions (this is often referred to as "primer limitation"). An effective way to do this is to try several reactions with a fixed RNA or cDNA concentration similar to that in the experimental samples, but different total primer concentrations.

The best primer concentration is that which has the lowest final fluorescence level, with an unchanged Ct value. If this still does not work, you can try removing the oligos one at a time to determine which oligos in particular are interfering with one another, and then redesign them.

Regardless of which approach is planned initially (individual target or multiplex), it is worthwhile to design all assays to be compatible for running in a standard format (e.g. standard thermal profile, $MgCl_2$ concentration, reagent concentration) to save time if multiplexing is required at some later point.

The Ideal Assay

Using the techniques described above you can be confident in your QPCR assay design. The ideal assay requires optimized primer sets, probe concentration, magnesium concentration, assay efficiency, and assay precision. Following the steps outlined above to optimization is the quickest and easiest method to ensure all aspects of the QPCR reaction are performing optimally. Achieving optimized assay performance will allow accurate quantification of experimental samples and reliable data analysis and comparison of the experimental study.

QPCR Experiment Data Analysis
Ensuring Your Ct Values Are Accurate

After the data are collected, it is best to examine them carefully to ensure the run went well and the assigned Ct values are accurate before you start looking at the calculated absolute or relative quantities. When first analyzing your amplification plots, you should follow these steps:

1. Look at the raw fluorescence values.
2. Look at the dissociation curves (if SYBR Green I was used).
3. Check the baseline settings.
4. Check the threshold.

Raw Fluorescence Values

When first looking at the data, it is best to view the amplification plots with the replicates set to "Treat Individually" on the Analysis Selection/Setup page (Figure 24). All wells that you want to be included in the analysis should also be highlighted on this page. Upon unloading the samples, verify that the caps are properly in place and that no loss of liquid due to evaporation is noticeable. If evaporation has occurred any anomalous wells can be culled from the analysis by de-selecting them on the Analysis Selection/Setup page. On this same page in the upper right corner, you can select which collection point will be used for the results if more than one collection point was set on the thermal profile.

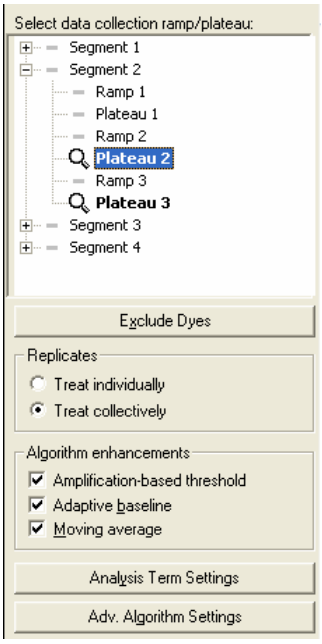

Figure 24
View of the control panel on the right side of the Analysis Selection/Setup screen. At the top of the panel, you can select the specific collection point that will be used for analysis. When first analyzing the data, the replicates should be set to "Treat individually".

You should next select the Results tab, and under Area to Analyze select the Amplification Plots page. When first viewing the data, it is good practice to view the raw data in order to ensure that the fluorescence intensity range is appropriate. To do this, set the Fluorescence to R (Multicomponent view). This will show the actual fluorescence values collected by the instrument for each well in every dye channel that was selected at every cycle. The minimum fluorescence values are usually greater than 3,000, and the maximum fluorescence values should be less than 45,000 fluorescence units. The fluorescence signal for the PMT detector approaches saturation at approximately 45,000 fluorescence units; if the signal exceeds this level the reliability of the data may be affected (Figure 25).

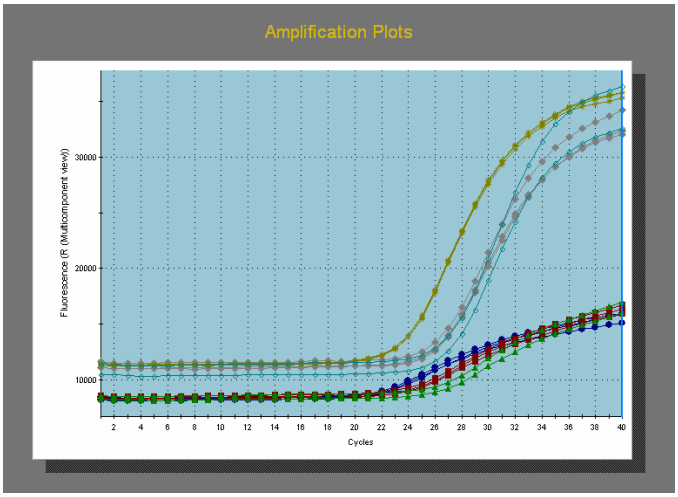

Figure 25
Example of raw amplification plots (Multicomponent view). The raw fluorescence counts are plotted on the Y axis. Fluorescence intensities should fall between 3,000–45,000.

Saturated signal of the reference dye may indicate that too high a concentration of the dye was used. If the signal level is very low in all of the channels, it may indicate the bulb in the instrument is failing and needs to be changed; however, if the signal is too low in any one channel, the Gain may be adjusted to increase the signal level. This can be done from the Instrument menu, under "Filter Set Gain Settings".

Dissociation Curves (Only for SYBR® Green I)

As mentioned previously, when the detection chemistry is based on dsDNA detection, such as SYBR Green I, you should run a melting (dissociation) curve at the end of your amplification reaction known as a dissociation curve. The purpose of the dissociation curve is to determine if anything

other than the gene of interest was amplified in the QPCR reaction. Because SYBR green I will bind any double stranded product, any non-specific amplification in your unknown wells will artificially increase fluorescence and make it impossible to accurately quantitate your sample.

To view the SYBR Green I dissociation curve, select the Results tab, and under Area to Analyze go to 'Dissociation curve' in the software. The best way to analyze the dissociation curve results is to set the fluorescence to –Rn´(T), although if you have not run a normalizing dye you should set this to –R´(T). In this view, every peak in the curve indicates a specific product is melting. Most QPCR products will melt somewhere in the range of 80–90°C, although this can vary given the size and sequence of your specific target. Ideally, you should see a single peak within this temperature range, and the melting temperature should be the same in all the reactions where you have amplified the same sample. If any secondary peaks or shoulders are seen on the peak of interest, it indicates that something other than your gene of interest is present among the reaction products. Since there is no accurate way to determine how much the amplified signal from each product is contributing to the Ct, if any secondary peaks are observed the Ct value from that well should not be considered accurate.

If secondary peaks are seen, other controls run in the reaction may give you an indication of what was causing this problem and how it can be prevented in the future. If these same secondary peaks are present in your NTC wells, it may indicate primer dimer formation or the presence of contamination by a sequence that was also amplified during the reaction. In the case of primer dimers, re-optimizing the reaction conditions may be necessary. On occasion, it may be necessary to re-design the primers. If the secondary peaks are not seen in the NTC wells, it could indicate non-specific primer binding or the presence of differentially spliced products. Performing a BLAST search following primer design may help decrease the incidence of this type of problems.

Setting the Baseline

Fluorescence intensity data (Amplification plots) can be described as a two-component function: a linear component or background and an exponential component that contains the relevant information. To isolate the exponential component, the linear contributions to fluorescence can be estimated and subtracted. This is what this chapter refers to as "baseline correction". It is a three-step process that is carried out for each amplification plot (i.e. each well and each dye):

1. Identify the range of cycles during which all contributions to fluorescence are strictly linear (no exponential increase in fluorescence).
2. Using the fluorescence intensity values during the cycles determined above, fit the data to a straight line (a function predicting the contribution of the linear components throughout the reaction)
3. Subtract the predicted background fluorescence from the measured fluorescence intensity during each cycle.

The resulting curve (dR or dRn in the amplification plots screen) corresponds to the change in fluorescence due to DNA amplification. The software always performs steps 2 and 3 above in the same fashion in order to isolate the exponential components of the curve. However, there are a few options for determining which cycles to use to estimate the contribution from the background fluorescence:

Adaptive baseline (default method): When this method of baseline correction is selected, the software will automatically select the appropriate cycles for each plot (each well and dye). The algorithm first looks for the beginning of the "baseline cycles" by comparing fluorescence intensity values between cycles. If these changes exceed a set amount (calculated by comparison to the overall cycle-to-cycle variability in the data), the next cycle is analyzed. This process is repeated until the changes in fluorescence are stable over a number of cycles. The first cycle to show steady fluorescence values is defined as the Baseline Start Cycle. The next step in the analysis is to define the end of the baseline. This is accomplished in a similar way, by looking at an increase in fluorescence that continues for multiple cycles and exceeds the changes in fluorescence up until that point. Once a significant increase in fluorescence is found, the cycle prior to the increase is defined as the Baseline End Cycle.

As can be seen in Figure 26, the starting and ending cycles which are used to generate the best fit baseline are different for the individual wells. As a rule, the higher concentration samples (e.g. wells C9 and C11) tend to have a shorter baseline range, and the lower concentration samples (e.g. wells C2

and C4) tend to have a longer baseline. Data analyzed in this way tend to provide more accurate estimates of the starting amount of a sample, so it is recommended that the Adaptive Baseline be used to assign the baselines unless the user chooses to adjust the baseline ranges manually.

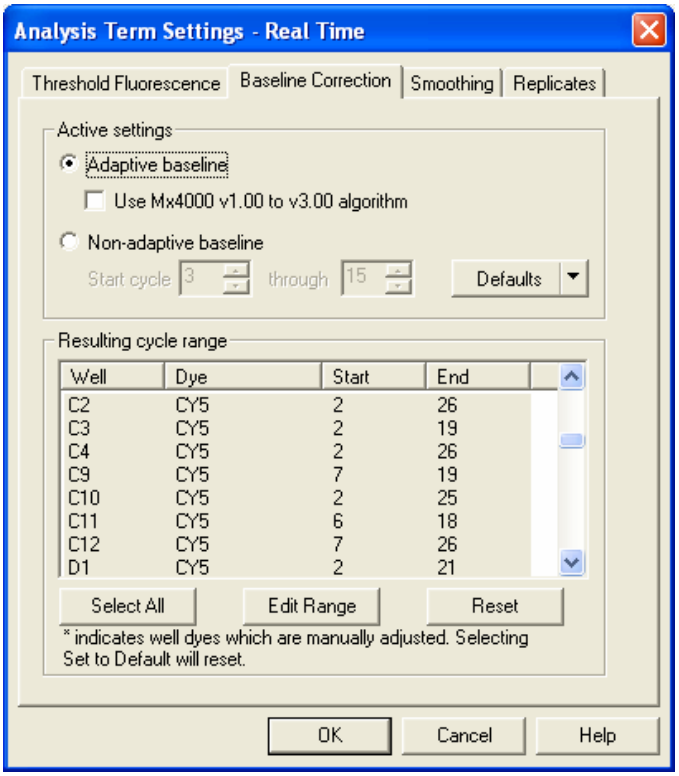

Figure 26
A view of the baseline correction screen in the Analysis Term Settings window. When the Adaptive Baseline is turned on, the software will set the baseline cycle range independently for each Amplification Plot.

Non-adaptive baseline: If the check-box for Adaptive baseline (in the Analysis Selection/Setup screen) is not selected, a common baseline range is set for all the amplification plots. Using this method, the software will set the baseline using the range of cycles specified in the Active Settings section of the Analysis Term Settings-Baseline Correction dialog box. The default baseline range is from cycle 3–15, but this range can be modified by the user in this same dialog box. The drawback for this method is that the range from cycles 3–15 is not necessarily the best baseline range to use for all curves. Samples that contain very high concentrations of template may have Cts earlier than cycle 15, in which case the software will be trying to fit a baseline through a region of the plot that is not flat. Samples that contain a low concentration of template may have later Cts, in which case the baseline would be more accurately set over a wider range of cycles. Consequently, this method of baseline correction will tend to be less accurate than the Adaptive Baseline.

Manually defined baseline range: With either the adaptive or non-adaptive baseline methods, it is possible that the baseline range selected by the software can be inaccurate (although this is less likely with the adaptive baseline). This would result in the amplification plots being skewed in such a way that they will cross the threshold at a different cycle than they would have if the baseline had been set more accurately (Figure 27). This will give inaccurate Ct values and thus will directly affect the calculation of the quantity of these samples. If this sort of inaccurate baseline setting occurs, it is most often the result of an amplification plot with a non-standard shape, which can happen when the starting sample concentration is quite high and the Cts very early (less than cycle 15). To correct for this, the starting and ending cycles can be set manually in the Analysis Term Settings Baseline Correction dialog box. When selecting the cycle range, it is best to view the amplification plots in the R (multicomponent) mode and search for flat segments in the early cycles, where there is little or no increase in the detected fluorescence. For the best results, the range selected should be as broad as possible, without including the first cycle in which there is a perceptible increase in fluorescence above background or any tailing in the early cycles (Figure 28).

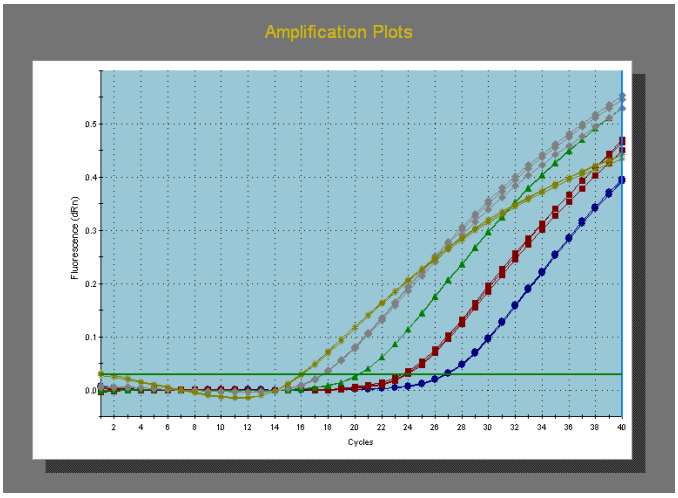

Figure 27
An example of an amplification plot where the baseline range is set incorrectly. In this case, the baseline range for the orange plot has been set to begin at cycle 3 and end at cycle 15, and this range includes the first part of the fluorescence shift.

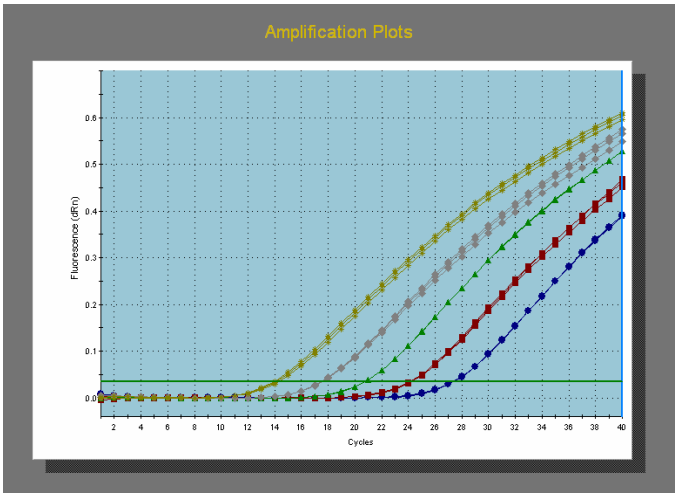

Figure 28
The Amplification plot from Figure 27 after the baseline range has been corrected to the cycle range 3–10.

Setting the Threshold

The basic principle used in the analysis of real-time PCR data is that the number of cycles necessary to reach a fixed concentration of amplicon in the reaction is an accurate estimator of the initial target concentration at the beginning of the reaction. Therefore, the number of cycles required to reach arbitrary fluorescence intensity should correlate well with initial target concentration, as fluorescence intensity values correlate with the concentration of the PCR products. This fluorescence value is referred to as the "threshold fluorescence", and the number of cycles required for any one reaction to reach it is the "threshold cycle" or "Ct".

Ct values correlate very well with initial target concentration as long as some assumptions are satisfied. Namely, that the kinetics of the reaction is approximately constant throughout the reaction and that they are also similar between any samples that are being compared to each other (e.g. standards and unknowns).

To satisfy these conditions, the threshold value has to be set at a point where all samples being analyzed display the same rate of increase in the fluorescence intensity, and ideally this increase responds to an exponential function. In addition, valid quantitative comparisons can only be done between PCR reactions that amplify the same target (i.e. use the same primer set).

There are different ways of setting the threshold value, two of which are software algorithms (amplification-based and background-based thresholds) and a manually set threshold.

Amplification-Based Threshold: (default method): This algorithm first determines the portion of the amplification plots where all of the data curves display an exponential increase in fluorescence. To do this, the software looks at the shift in fluorescence for each baseline-corrected curve and sets a point just above the baseline at 0% and the maximum of the first derivative as 100% amplification. As a default, the search range for the algorithm falls within 5–60% of this fluorescence shift for all the curves. This range can be manually adjusted based on personal preferences, by accessing the Analysis Terms Settings Screen (on the menu bar under Options). Under the Threshold Fluorescence tab select the Advanced Settings button to enter the new range.

Once the search range for the amplification-based threshold is established, the threshold value is set based on one of two different criteria. In experiments where there are at least two wells for each replicate, the algorithm calculates the threshold value that minimizes the standard deviation (σ) in Ct values for each replicate set. If there are no replicate wells, the algorithm will instead use a fixed amplification position. In such cases, the software sets the threshold at the midpoint of the Search Range. If the default search range of 5–60% is used, the threshold will be set at 32.5%.

Background-Based Threshold:
This method will be used by the software if the check-box for the amplification-based threshold is deselected in the Algorithm Enhancements box on the Analysis Selection/Setup screen. As the name implies, this method determines the threshold based on the background fluorescence in the experiment. The software determines the standard deviation for all selected wells based on a common set of cycles early during the reaction. This standard deviation (σ) value is multiplied by the background sigma multiplier (default 10), and the resulting quantity is set as the threshold. The cycle range used to calculate the standard deviation will default to cycles 5–9, but this range can be changed manually from the Analysis Terms Settings Screen (on the menu bar under Options) by selecting the Threshold Fluorescence tab. The sigma (σ) multiplier can also be changed in that window. Typically, only early cycles are selected for the

background cycle range to ensure the background is being calculated from a range where increasing signal from the PCR amplification has not begun to affect the fluorescence values. If amplification becomes noticeable during early cycles, it may be necessary to lower the background cycle range. If there are large tails on the amplification plots that extend beyond cycle 5, it may be necessary to raise the background cycle range.

It is typical for the background-based algorithm to set a lower threshold than the amplification-based algorithm. This is caused by the amplification-based method's requirement for the threshold to contact all the amplification plots within a range in which exponential increase in fluorescence is evident, and above a certain minimum percentage of this range. Thus, the amplification-based threshold is most likely to select a threshold which will generate Ct values in a range where all samples are amplifying exponentially.

Manually-Set Threshold: Normally the software-based methods will select a good threshold, but in cases where the curves do not conform to the assumptions made by the algorithm, an incorrect threshold may be calculated. Good indicators of improperly-set threshold values are false positives (Ct values obtained from negative control wells), known positive samples giving very late Cts or no Cts at all, or non-linear standard curves. There are other possible causes of all these results which will be discussed later, but manually adjusting the threshold is one way to correct these errors.

When manually adjusting the threshold, it is best to view the amplification plots in a semi-log scale. To do this, double click anywhere within the blue background area of the amplification plots. This will open the Graph Properties window, and under the section for Y-axis select the button for Log Scale and click on the OK button at the bottom of the Graph Properties window. In the log scale, the amplification plots will normally appear rather noisy during the baseline cycles, due to the log scale. Following the baseline cycles, relatively straight lines rise upward in the region where amplification begins. These plots will eventually reach a plateau (Figure 29).

To adjust the threshold for each dye collected, move the cursor over the threshold on the amplification plot. When it is over the threshold, the cursor will appear as a double-headed vertical arrow, allowing the threshold line to be moved up or down. Notice that if there are standard wells selected, the parameters of the best fit line and the measured efficiency of amplification are displayed at the bottom of the screen. Alternately, on the screen to the right of the amplification plots the threshold fluorescence values for each dye channel are listed on the screen. A numerical value for the threshold can be entered there.

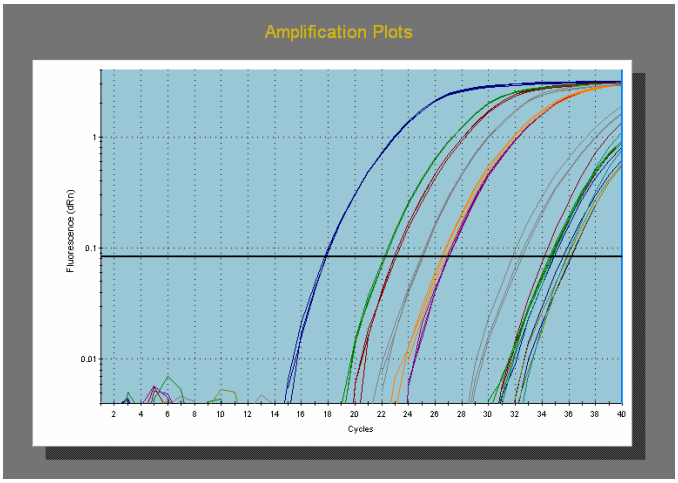

Figure 29
Amplification Plots viewed with the Y Axis set to a log scale. The optimal setting for the threshold is the point where all the log plots are linear and parallel, as shown in where the threshold is set here.

Ideally, the threshold should be set in the region where the plots are all linear and where they are all as close as possible to parallel to one another. The threshold should not be so high that it crosses any of the plots where they are starting to plateau and are no longer linear. If possible, the threshold line should be placed above the highest points of the fluorescent plots in the early (background fluorescence) cycles. Of the two methods the software has of automatically setting the threshold, the amplification-based threshold most closely resembles the way it is normally set manually.

Controls

Prior to moving on to analysis of the results, it is important to verify that the controls are behaving as expected. If this is not the case, the quantitative results may not be accurate, and further troubleshooting may be necessary.

Ideally, none of the negative control wells should cross the threshold, although it is not uncommon to see the negative controls drift across the threshold during late cycles. If the negative controls are displaying sigmoid-shaped amplification curves, the

fact that real amplification of the negative control is taking place would be indicated. This may be due to template contamination or excessive primer dimer formation.

Whether this will affect the Cts of the unknown samples will depend on the level of the signal in your negative controls. If the Cts of the negative control wells are ten cycles higher than the Cts of any of the unknown wells, it is safe to assume that these results are accurate. If the Cts in the negative control wells are within five cycles of any of the unknowns, this may call the validity of the results into question. Under these circumstances it may be necessary to troubleshoot the reaction to determine the source of signal in the negative control wells. The type of negative control well from which the signal was detected can provide an important indication of the source of the trouble. A shift in the No RT controls would indicate possible genomic DNA contamination. A shift in the NAC control wells could indicate probe degradation and a shift in the NTC wells may indicate primer dimer formation (when performing a SYBR Green I assay), or contamination. If the shift in the negative control wells is due to primer dimers, you can determine if the primer dimers are also forming in the unknown wells by looking at the dissociation curves.

If the positive control wells are not showing amplification, it will call into question whether any of the unknown wells that did not amplify are actually negative samples or whether this is due to non-specific failure of the PCR reaction (e.g. the presence of an amplification inhibitor). In this case, it may be necessary to troubleshoot the reaction conditions (e.g. different water and/or primer sources). The presence of PCR inhibitors in the template can also be identified by decreasing the amount of template used. If the Ct values tend to decrease or remain constant in the presence of lower amounts of template, this usually indicates the presence of an amplification inhibitor.

Standard Curve Quantification

After amplification, given that both the standards and experimental samples are amplifying efficiently, the Cts for each standard dilution can be determined and plotted against the initial template quantity. Sample Ct values can be used to estimate template quantity by comparing them to the standard curve. For this estimate to be accurate, the standard curve must be linear across the whole range of template concentrations in your assay and the measured efficiency of amplification near 100%.

A typical plate setup for a standard curve can be seen in Figure 30. The Ct values from each standard well will be used to create a standard curve. Figure 31 represents a typical standard curve constructed over three orders of magnitude (40 copies to 20,000 copies) on an Mx3000P instrument.

All	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard REF 2.00e+004	Standard REF 2.00e+004	Standard REF 2.00e+004	Standard REF 1.00e+004	Standard REF 1.00e+004	Standard REF 1.00e+004	Standard REF 5.00e+003	Standard REF 5.00e+003	Standard REF 5.00e+003	Standard REF 2.50e+003	Standard REF 2.50e+003	Standard REF 2.50e+003
B	Standard REF 1.25e+003	Standard REF 1.25e+003	Standard REF 1.25e+003	Standard REF 6.25e+002	Standard REF 6.25e+002	Standard REF 6.25e+002	Standard REF 3.13e+002	Standard REF 3.13e+002	Standard REF 3.13e+002	Standard REF 1.57e+002	Standard REF 1.57e+002	Standard REF 1.57e+002
C	Standard REF 7.85e+001	Standard REF 7.85e+001	Standard REF 7.85e+001	Standard REF 3.93e+001	Standard REF 3.93e+001	Standard REF 3.93e+001	NTC REF FAM	NTC REF FAM	NTC REF FAM	Unknown REF FAM	Unknown REF FAM	Unknown REF FAM

Figure 30
Example of a standard curve plate setup. This two-fold dilution series would generate a 10-point standard curve in triplicate, from 20,000 copies down to about 40 copies

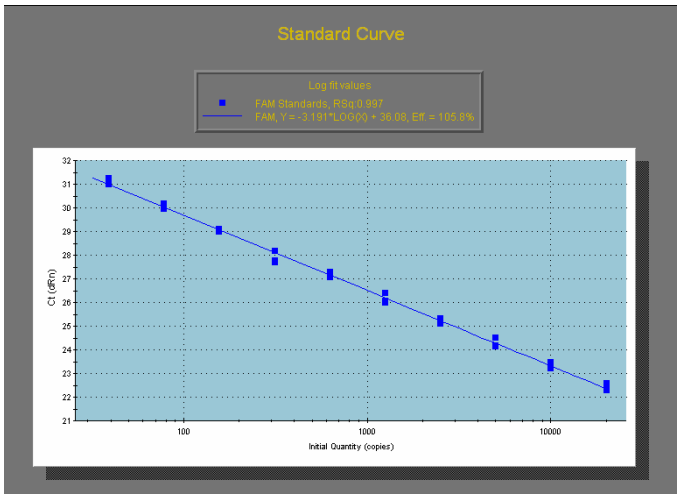

Figure 31
Standard curve demonstrating a two-fold dilution series, from 20,000 to 40 copies. At each standard dilution a one-cycle change in Ct value is observed. This direct correlation between fold-decrease in standard concentration and increased Ct value demonstrates that the doubling efficiency of this assay operates at approximately 100%.

Data from a standard curve run can be viewed in multiple formats including: standard curve, initial template quantity, and plate sample values. In the standard curve view, as seen in Figure 31, the efficiency and linearity will automatically be displayed by the software using the equation:

$$X_n = X_0(1+E)^n$$

Where X_n = amplified target amount (target quantity at cycle n); X_0 = starting quantity; E = efficiency of amplification; and n = number of cycles. When the efficiency is perfect (100% or 1), there is a perfect doubling of target amplicon every cycle; a 10–fold amplification should take 3.32 cycles ($2^{3.32} = 10$). In a plot of Ct versus the log of initial template, the slope should therefore be close to –3.32 (negative because a higher Ct means lower template amount). Because of this relationship, you can calculate the efficiency directly from the slope using the equation below:

$$\text{Efficiency} = [10(-1/\text{slope})]-1$$

Relative or Comparative Quantification

Figure 32 contains a typical plate setup for a comparative quantitation reaction. In this case, the normalizer and target genes were run in separate wells, although they can be multiplexed in the same well. There will be at least three different well types used for analysis: the unknowns (experimental samples), the calibrators (controls), and negative controls (e.g. NTC wells). If a normalizer gene is included in the experiment, then there will be unknown wells that have the target gene of interest and others that have the normalizer (these can be run in the same tube if you are multiplexing). The same is true of the calibrator wells. To compare the Ct differences between target and normalizer genes in the unknown versus calibrator samples, it is necessary to "associate" the wells to be compared. All associated wells will be represented by a letter in each well in the plate setup (Figure 32).

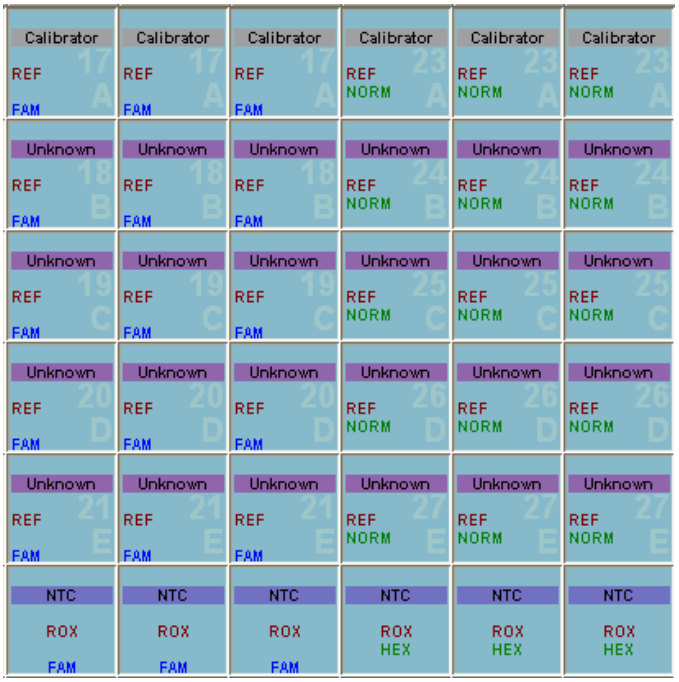

Figure 32
An example plate setup for comparative quantification where the gene of interest (GOI) and normalizer have been run in separate wells. The unknown wells containing the target gene (replicate 1) are associated with the unknown wells that contain the normalizer (replicate 2) by designating both of them as belonging to association group "A". Similarly, calibrators containing the target gene should be associated with calibrators containing the normalizer; here they are assigned to group "B". The dye channel where the normalizer is detected is designated "NORM" in the wells where the normalizer is amplified. Replicates are assigned the same number

When amplification is complete, the software will calculate the Ct differences between the target gene in the unknown and calibrator, and correct that by the difference in Cts for the normalizer gene in the unknown and calibrator samples. Given two samples (such as control and experimental samples), the ratio of starting amounts can be estimated according to the equation:

$$\frac{\text{Unknown}}{\text{Control}} = (1+E_{\text{target}})^{-\triangle \text{Ct}_{\text{target}}(\text{Control}-\text{Unknown})}$$

Where *E* corresponds to the efficiency of amplification of the target gene (a value from 0 to 1

that represents the number of amplification products generated during each cycle of the reaction per molecule of target sequence), Ct = threshold cycle (Ct), and "Control" represents the calibrator sample. Note that this equation does not include normalization to a reference gene.

For example, if there is difference of one cycle in the Ct values of a control and unknown samples, and the efficiency (E) is 100%, this would indicate a two-fold calculated difference in target gene expression. When incorporating a normalizer target for correction, the equation can be described as:

$$\text{Normalized } \frac{\text{Unknown}}{\text{Control}} = \frac{(1+E_{\text{target}})^{-\Delta Ct_{\text{target}}}}{(1+E_{\text{norm}})^{-\Delta Ct_{\text{norm}}}}$$

Here norm refers to the reference or normalizer gene. In the particular case where the efficiencies of amplification of both DNA sequences are equal to one (or 100%); the equation above is sometimes described as the "ΔΔCt" method because it can be reduced to the expression:

$$\text{Normalized } \frac{\text{Unknown}}{\text{Control}} = \frac{2^{-\Delta Ct_{\text{target}}}}{2^{-\Delta Ct_{\text{norm}}}} = 2^{-\Delta\Delta Ct}$$

In the Mx3000P software, the default value for amplification efficiency (E) is one. However, if preliminary experiments were done to establish the actual amplification efficiency for each target, these values can be specified. Under the "Analysis and Terms Settings", in the tab for "Efficiency settings" the new values can be entered in the form of efficiency value (as a percent from 0–100) or in terms of the slope of the standard curve for each target.

Initially, it may be beneficial to look at all the replicates individually to ensure there are no outliers that may skew the results. Once all outliers are eliminated, replicates can be treated collectively (under the "Analysis Selection/Setup" tab). The amplification plots for each replicate set then result from an average of the amplification plot of all replicate wells in a set, and a single Ct value is then calculated for each replicate set. The corresponding Ct values are applied to the equations above, resulting in a relative quantity value for the gene of interest corrected for the corresponding quantity of normalizer target in each sample or association group.

The Ct values of the target (GOI) and the normalizer should ideally be within about ten cycles of each other. This is important because it becomes more difficult to normalize accurately if, for example, the target is rare and the normalizer is abundant. Given that the normalizer gene expression was previously validated as relatively constant for all samples in the assay, any Ct differences in the normalizer between the control and sample pools should be due to differential sample loading or RNA extraction, and the normalized Ct differences should convey true expression level changes for the target gene.

The output for a comparative quantitation run is typically a relative quantity chart (Figure 33). A value of one indicates no change in target gene expression between that sample (replicate set) and the calibrator sample. Anything greater than one indicates up-regulation and anything lower than one indicates down-regulation.

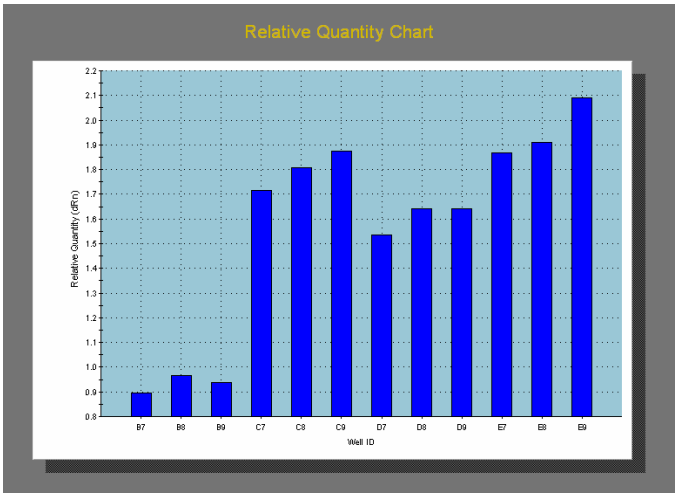

Figure 33
Relative quantification data where the expression levels of treated samples are compared to a control sample after gene normalization. These data are expressed in fold-differences of gene expression compared to a control sample. Samples B7–B9 are down-regulated while all others are up-regulated to varying degrees.

Qualitative PCR

In addition to Quantitative PCR, the Mx3000P system can be used in experiments where only a qualitative "Yes/No" answer is required regarding the presence or absence of a given template of interest. Because in experiments of this sort it is not critical to quantify the exact level of change in the signal, these assays can be performed either as real-time experiments or they can be based on the end-point fluorescence level.

For qualitative assays, it is not necessary to run a standard curve, but it is best to include both positive and negative controls. At the very

minimum, 1–3 NTC negative controls should be included on every plate to control for any contamination that might cause false positive results. In addition to these, it is best to include 1–3 known negative samples that closely mimic the sample preparation used with the unknown or experimental samples. It is also good practice to run at least one positive control sample that is known to contain your template of interest. When the positive control sample shows a significant fluorescence shift, it indicates that any negative results in the unknowns are real negatives, and are not just the result of a failure of the PCR reaction due to problems with the primers, probes, buffer, or polymerase.

With end point qualitative PCR, final calls can be based on the final fluorescence level or on the level of the fluorescence shift between a read taken prior to PCR amplification and a read taken at the endpoint. The unknowns are then compared to the negative control samples to determine if the unknowns are significantly greater than the negative control wells. If this is analyzed in the Quantitative Analysis Plate Read experiment type, the Mx3000P software will automatically make + or – calls for the unknowns as long as at least 3 NTC or dye-specific negative controls are run on the same plate. The software does this by performing a t-test based on either the raw final fluorescence values (Rpost), the normalized final fluorescence (Rn, post), the shift in raw fluorescence (Rpost–Rpre), the shift in normalized fluorescence (Rnpost–Rnpre), or the ratio of the raw fluorescence shift (Rpost/Rpre).

These values can be selected for the final call from the "p-values and final calls based on:" field on the Final Call Results page under the Results tab (Figure 34). The p-value is the probability that the mean of one set of sample data is different from the mean of another set of sample data. For example, if the user-defined confidence level for calls setting is 99%, a positive call (+) for an unknown well means that at most 1% of the time a measurement of sample identical to the control wells will produce a value as great as the actual measurement collected for the unknown well. The first set of sample data is always taken from the control wells in the analysis selection. When replicates are being treated individually, the second set of sample data consists of a single well (usually an unknown well). When replicates are being treated collectively, the second set of sample data consists of all of the replicates.

If the p-value exceeds the confidence level, the well/dye is called as detected and signified with a plus sign (+); otherwise it is called as not detected and is shown by a minus sign (–). The default confidence level setting is 99%, but this can be adjusted under the Analysis Terms settings.

For real-time qualitative PCR, the final call on whether amplification has taken place is generally based on the Ct values. Care should be taken when accepting these final calls from the software, because the only criterion used by the software in making the final call is whether or not the amplification plot for the unknown well crossed the threshold. If the Ct is within 5–10 cycles of any NTC or dye-specific negative controls, it may be better to assign that well a negative or indeterminate call. A p-value test or other statistical analysis can be applied to the Ct values of the unknowns and the negative controls to determine if they are significantly distinct. This sort of analysis is not currently available in the Mx3000P software, so the data would have to be exported and statistically evaluated elsewhere.

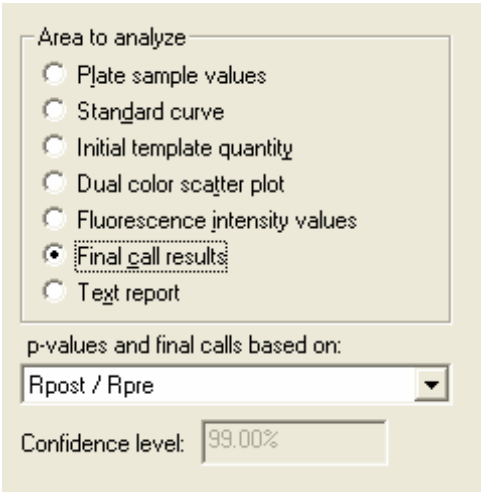

Figure 34
Control Panel to the right of the Final Call Results page in the analysis section of a Plate Read experiment. The drop down menu in the "p-values and final calls based on:" field can be used to control the data used to make the determination of whether amplification has taken place. In this example, the ratio between the post-amplified raw fluorescence and the pre-amplified raw fluorescence is used.

As with most other experimental techniques, careful planning during the assay design phase and consistent use of the proper controls are the key to success in QPCR. The guidelines described here suggest some principles and concepts of experimental design and analysis that will maximize the likelihood of reliable results.

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2. Radonic, A., S. Thulke, *et al*. (2004). "Guideline to reference gene selection for quantitative real-time PCR." Biochem Biophys Res Commun 313(4): 856-862.

Endnotes

† Practice of the patented polymerase chain reaction (PCR) process requires a license. The Mx3000P™ system real-time PCR system is an Authorized Thermal Cycler and may be used with PCR licenses available from Applied Biosystems. Its use with Authorized Reagents also provides a limited PCR license in accordance with the label rights accompanying such reagents.

†† Practice of the patented polymerase chain reaction (PCR) process requires a license. The Mx4000™ multiplex quantitative PCR system is an Authorized Thermal Cycler and may be used with PCR licenses available from Applied Biosystems. Its use with Authorized Reagents also provides a limited PCR license in accordance with the label rights accompanying such reagents.

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†††† Use of labeling reagents may require licenses from entities other than Stratagene. For example, use of fluorogenic probes in 5' nuclease assays may require licenses under U.S. Patent Nos. 6,214,979, 5,804,375, 5,210,015 and 5,487,972 owned by Roche Molecular Systems, Inc. and under U.S. Patent No. 5,538,848 owned by Applied Biosystems.

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* U.S. Patent No. 6.657,169.

** U.S. Patent Nos. 6,528,254, 6,548,250, and patents pending.

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Molecular Beacons are distinct from the Beacon® Fluorescence Polarization System, which is a registered trademark of PanVera Corporation, Madison, WI.

Appendix

Plate Set-up Example

1. Prepare master stock of primers at 100µM, aliquot and store at –70°C.
2. Dilute primers to 10 µM working solutions and store at –20°C.
3. Dilute forward and reverse primer concentrations according to the directions below (both 1µM and 5µM primer stocks are required to set up this set of primers), for a total of 52 reactions (25 primers in duplicate and 2 controls).

Set 1: reverse primer 50 nM

A. Add 3.0 µl [1 µM stock] reverse primer to each tube.

B. Add forward primer in the amount indicated in the table below:

	Amount [stock]	Resulting Concentration
tube 1	3.0 µl [1 µM stock]	(50 nM)
tube 2	1.2 µl [5 µM stock]	(100 nM)
tube 3	3.6 µl [5 µM stock]	(300 nM)
tube 4	7.2 µl [5 µM stock]	(600 nM)
tube 5	10.8 µl [5 µM stock]	(900 nM)

C. Add ddH_2O to a final volume of 21.6 µl

Set 2: reverse primer 100 nM

A. Add 1.2 µl [5 µM stock] reverse primer to each tube.

B. Add forward primer in the amount indicated in the table below:

	Amount [stock]	Resulting Concentration
tube 1	3.0 µl [1 µM stock]	(50 nM)
tube 2	1.2 µl [5 µM stock]	(100 nM)
tube 3	3.6 µl [5 µM stock]	(300 nM)
tube 4	7.2 µl [5 µM stock]	(600 nM)
tube 5	10.8 µl [5 µM stock]	(900 nM)

C. Add ddH_2O to a final volume of 21.6 µl

Set 3: reverse primer 300 nM

A. Add 3.6 µl [5 µM stock] reverse primer to each tube.

B. Add forward primer in the amount indicated in the table below:

	Amount [stock]	Resulting Concentration
tube 1	3.0 µl [1 µM stock]	(50 nM)
tube 2	1.2 µl [5 µM stock]	(100 nM)
tube 3	3.6 µl [5 µM stock]	(300 nM)
tube 4	7.2 µl [5 µM stock]	(600 nM)
tube 5	10.8 µl [5 µM stock]	(900 nM)

C. Add ddH_2O to a final volume of 21.6 µl

Set 4: Reverse Primer 600 nM

A. Add 7.8 µl [5 µM stock] reverse primer to each tube.

B. Add forward primer in the amount indicated in the table below:

	Amount [stock]	Resulting Concentration
tube 1	3.0 µl [1 µM stock]	(50 nM)
tube 2	1.2 µl [5 µM stock]	(100 nM)
tube 3	3.6 µl [5 µM stock]	(300 nM)
tube 4	7.2 µl [5 µM stock]	(600 nM)
tube 5	10.8 µl [5 µM stock]	(900 nM)

C. Add ddH_2O to a final volume of 21.6 µl

Set 5: Reverse Primer 900 nM

A. Add 10.8 µl [5 µM stock] reverse primer to each tube.

B. Add forward primer in the amount indicated in the table below:

	Amount [stock]	Resulting Concentration
tube 1	3.0 µl [1 µM stock]	(50 nM)
tube 2	1.2 µl [5 µM stock]	(100 nM)
tube 3	3.6 µl [5 µM stock]	(300 nM)
tube 4	7.2 µl [5 µM stock]	(600 nM)
tube 5	10.8 µl [5 µM stock]	(900 nM)

C. Add ddH_2O to a final volume of 21.6 µl

Set 6: Control Tubes

A. Tube 1 (NTC 1)
3.0 µl [1 µM stock] of forward primer
3.0 µl [1 µM stock] of reverse primer
15.6 µl ddH_2O

B. Tube 2 (NTC 2)
10.8 µl [5 µM stock] of forward primer
10.8 µl [5 µM stock] of reverse primer
0.0 µl ddH_2O

4. Prepare the final reactions using Stratagene's Brilliant® SYBR® Green QPCR master mix (supplied at 2× concentration with a separate tube of undiluted Passive Reference Dye) as indicated below. Note, do not add primers.

Reagent Master Mix	1 × 60 µl reaction	27 × 60 µl
2× SYBR Green I QPCR MM	30 µl	810 µl
Passive Reference Dye (1:200)	0.9 µl	24.3 µl
dH_2O	to be determined[a]	to be determined
Template (10^4 or 10^6 copies)	to be determined	to be determined
Final Volume (without primers)	38.4 µl	1036.8 µl

[a] must be adjusted depending on template concentration and amount to add.

5. Mix and pulse centrifuge to collect the master mix.
6. Add 38.4 µl of the reaction master mix to each primer condition tube. Mix gently and pipette 25 µl of each into two tubes or two wells of a 96 well plate.
7. Completely cap the tubes or plate wells, label and spin if necessary, place the tubes/plate in the thermal block of the Mx3000P™ system.
8. In the Mx3000P analysis software select the "SYBR Green (with Dissociation Curve)" module. This will load the correct thermal profile with melt curve. Enter the plate setup. It is optional to add an additional single endpoint data collection on the 72°C plateau. If necessary, change the annealing temperature to match the calculated Tm of the primers, in most cases this should be 55°–60°C.
9. Run the assay.

Probe Optimization Reaction Example

1. Prepare a master stock of probe at 100 µM, aliquot and store at –70°C.
2. Dilute probe to 5 µM working solution and store at –20°C.
3. Use 1µM probe stock to set up the probe matrix.
4. Set up the reaction master mix as indicated in step 3 of the Plate Set-Up Example above.
5. Pipette out the probe matrix as indicated below

	(50nM)	(100nM)	(200nM)	(300nM)	NTC 1	NTC 2
ddH_2O	17	14	8	2	17	2
Probe 1µM	3	6	12	18	3	18

6. Prepare the reagent master mix as indicated below.

Reagent Master mix	1 × 60µl reaction (µl)	12 × 60 µl
2x QPCR MM	30 µl	360 µl
Passive Reference dye (1/200)	0.9 µl	10.8 µl
dH_2O	to be determined[a]	to be determined
template (10^4 or 10^6 copies)	to be determined	to be determined
forward primer	to be determined	to be determined
reverse primer	to be determined	to be determined
[Probes in matrix)	20 in duplicate	20 in duplicate
Final volume reagent master mix (without probe)	40 µl	480 µl

[a] must be adjusted depending on template concentration and amount to add.

7. Add 40µl of the reaction master mix to each probe condition tube and mix gently
8. Pipette 25µl from each of the tubes in step 7 into two tubes or two wells of a 96 well plate.
9. Select "Quantitative PCR (Multiple Standards)" module in software, and enter plate set-up.
10. In the thermal profile delete the extension plateau and run only a two-step thermal cycle.
11. Run the assay.

QPCR Glossary

Experiment and Chemistry Terms

Allele Discrimination (Real-Time) Real-time measurements using Ct to determine the genotype of a DNA sample. To achieve discrimination, two probes labeled with two spectrally distinct dyes are used to identify the wild type and mutant alleles. A DNA sample genotype is determined by plotting the Ct value specific to the wild-type allele against Ct specific to the mutant allele.

Allele Discrimination (plate read) Plate read measurements of the fluorescence produced are used to determine the DNA sample genotype. To achieve discrimination, two probes labeled with two spectrally distinct dyes are used to identify the wild type and mutant alleles. Results are analyzed as follows: (1) if the fluorescent value of the unknown DNA sample is high for the wild type dye and low for the dye identifying the mutant, the sample is called wild type homozygote. (2) If the fluorescent value from the unknown DNA sample is high for the dye identifying the mutant and low for the wild type dye the sample is called mutant homozygote. (3) If the sample generates intermediate values for both dyes, it is called heterozygote.

Comparative Quantitation A QPCR analysis method that enables determination of relative gene expression compared to a calibrator (a single standard). This method is used to establish relative fold-increase in expression by assuming unchanging reaction efficiency. This method eliminates the requirement to include a standard curve with each reaction. Comparative quantitation can be applied to DNA and cDNA targets, and the most common application is the comparison of mRNA expression levels in treated versus untreated or normal versus diseased cells or tissue.

Dissociation Curve A melting curve protocol that reports the temperature on the X-axis, versus either fluorescence (R, Rn) or the first derivative of the fluorescence [–R'(T), –Rn'(T)] on the Y-axis. The analysis is used to verify the reliability of results from SYBR Green I quantitative experiments. SYBR Green I fluorescence exhibits a large increase upon binding to double-stranded DNA, and this can be used both to generate amplification plots real-time during the amplification and to obtain thermal denaturation profiles of the complex nucleic acid mixtures generated during PCR amplification. Typically, two semi-discrete populations with different transition temperatures can be identified in the first derivative plots. Populations with a Tm of 80°C or higher correspond to the larger PCR products, and are usually assigned to the specific DNA product. DNA products displaying melting temperatures less than 75 °C correspond to non-specific DNA products that are not necessarily homogeneous and may contain multiple PCR product species.

Dynamic Range The linear range of fluorescent signal (from the lowest to the highest in the experiment) that can be detected without saturating the system. A wide dynamic range in a real-time system confers the ability to detect samples with high and low copy number in the same run.

Molecular Beacon Melting Curve After the Molecular Beacon is manufactured, the melting characteristics should be verified using a melt curve analysis protocol to determine the Molecular Beacon's target specificity, melting temperature (Tm), and appropriate annealing temperature for subsequent PCR experiments. The melt curve displays the Molecular Beacon fluorescence at various temperatures in the presence or absence of single-stranded oligonucleotide target. For allele discrimination assays, the melting curve performed with the matched and the mismatched synthetic target defines the optimal temperature for assay discrimination performance.

Molecular Beacon Probes Hairpin-shaped fluorescence-labeled probes that can be used to monitor PCR product formation either during or after the amplification process. The free probe maintains the hairpin structure and causes quenching of the fluorophore. When the probe is annealed to target the fluorophore is separated from the quencher and releases fluorescence that can be detected.

Plate-Read (Endpoint) Experiments One measurement of the fluorescence is taken at the completion of the amplification reaction. Results are generally recorded as either a positive or negative call on whether amplification occurred, so this is not a real-time or quantitative PCR assay.

Qualitative Detection Allows you to determine the presence or absence of template of interest based on either Ct values or endpoint fluorescence.

Quantitative PCR Analysis Allows PCR product measurement and monitoring of the PCR reaction in a closed-tube system by measuring fluorescence intensity during each amplification cycle. Methods for both RNA and DNA are available to determine mRNA signal levels and/or DNA gene quantification. Quantitative PCR analysis software uses absolute standard curves, relative standard curves, or comparative methods for data analysis.

Quencher A compound used in QPCR experiments that absorbs the energy of the reporter dye in its excited state. The quencher can emit its own fluorescent signal (e.g. TAMRA) or emit no fluorescent signal (e.g. DABCYL, BHQ).

Real-Time Experiments Experiments that monitor and report the accumulation of PCR product by measuring fluorescence intensity at each cycle while the amplification reaction progresses. Data are collected at the end of each melt/elongation cycle of the thermal cycling, and is available for analysis by Mx3000P software while the run is in progress.

Reference Dye Dye used in real-time experiments for normalization of the fluorescence signal of the reporter fluorophore. The reference dye fluoresces at a constant level during the reaction. ROX is commonly used as a reference dye.

Reporter Dye The fluorescent dye used to monitor PCR product accumulation in a QPCR experiment. This can be attached to a probe (such as with TaqMan or Molecular Beacons) or free in solution (such as SYBR Green I). Also known as the fluorophore.

Sensitivity of Detection The level at which a given assay is able to detect low copy numbers. This is important when working with samples that have low expression levels.

TaqMan Probes Linear FRET fluorescence-labeled probes used to monitor PCR product formation either during or after the amplification process. As the DNA polymerase extends the upstream primers and encounters the downstream probe, the 5′ to 3′ nuclease activity of the polymerase cleaves the probe. Following cleavage, the reporter fluorophore is released into the reaction solution and fluorescence is detected.

Sample- and Well-Type Terms

Buffer A sample type containing only buffer, used to confirm the background fluorescence attributable to the buffer.

MB A sample type that corresponds to a well that contains only the Molecular Beacon in Molecular Beacon melting curve experiments.

MBMO A sample type that corresponds to a well containing the Molecular Beacon plus a single-nucleotide mismatched oligo in Molecular Beacon melting curve experiments.

MBO A sample type that corresponds to a well containing the Molecular Beacons plus the perfectly matched oligo in Molecular Beacon melting curve experiments.

NAC (No Amplification Control) In this sample type the polymerase/reverse transcriptase is omitted.

No RT Control (No Reverse Transcriptase Control) A sample type used in RT-PCR which contains all the reaction components except the reverse transcriptase enzyme.

NPC (No Probe Control) In this sample type all reaction components except the fluorescent labeled probe are present.

NTC (No Template Control) A sample type containing all the reaction components except the target(s).

Negative Dye Control Negative dye control wells contain all the PCR reagents and targets except the target (defined) dye. These wells, in combination with NTC wells, are used to calculate final +/– calls.

Positive Dye Control A sample type that corresponds to one of the dyes defined in optics configuration. Positive dye controls are used for decomposition analysis. In an experiment with more than one reporter, the positive controls are used to calculate the amount of cross talk between dyes caused from one dye crossing through to the filter of another dye and contributing to the total fluorescence recorded for that second dye. These controls contain all of the PCR reagents and the template for only one of the targets.

Standard A sample type containing reaction mixture with known concentration of the target nucleic acid. Using data from the standard wells and the threshold cycle (Ct) of the Unknown wells, the initial quantity of template in the Unknown wells can be calculated.

Unknown A sample type containing all reaction components plus the target nucleic acid. Fluorescence from the Unknown wells is measured and compared to NTC wells, Negative Control wells, and Standards to determine the initial template quantity and/or amplification of the target(s).

Analysis Terms

Amplification Plot The Amplification Plot view shows a plot of amplification cycles (on the X axis) versus fluorescence units (on the Y axis) for each ramp or plateau on which data are gathered.

Background An analysis setting that specifies the number of initial cycles of fluorescence data the software uses to calculate the background noise level. The region specified is typically in the cycle range before exponential amplification occurs. The Background Cycles are used for the default threshold fluorescence computation. The

standard deviation of the raw fluorescence for the specified cycles is calculated and multiplied by the constant Sigma multiplier (the default Sigma multiplier is 10) to yield the threshold fluorescence.

Baseline Correction For each well and each optical path the raw fluorescence data are fit over the specified range of cycles using a linear least mean squares algorithm to produce a baseline. The value of the baseline function is calculated for every cycle and subtracted from the raw fluorescence to produce the baseline corrected fluorescence (dR) and the normalized baseline corrected fluorescence (dRn).

Calibrator for Comparative Quantitation Fold-change expression measurements of sample compared to a reference sample (calibrator) is adequate for analysis of most expression data. This approach eliminates determination of absolute template quantity and running standard curves with each experiment. For example: the calibrator could be untreated HeLa cell culture in a study screening compounds that may induce apoptosis. In another example involving the expression of a cancer marker gene, the calibrator could be the normal, non-diseased part of the organ, and the sample (referred to in the Mx3000P software using the comparative quantitation experiment type as the Unknown) represents the diseased tissue of the same patient.

Collective Results Data analysis that averages all wells with the same replicate symbol, effectively treating the measurements as data coming from the same well.

Confidence Level The user-defined confidence level for calls is the statistical probability value required before the analysis algorithm will call amplification occurrence in a well. The default confidence level is 99%.

Dual Color Scatter Plot The Dual Color Scatter Plot software analysis view shows a plot of the dyes assigned to wells. Each plot point is the intersection of the either the fluorescence values or threshold cycle values for dyes assigned to a single well. For example, the X-axis may correspond to HEX while the Y-axis corresponds to FAM.

Initial Template Quantity The Initial Template Quantity software screen provides calculated quantities of template added to Unknown wells before thermal cycling. These quantities are interpolated from a standard curve constructed from the fluorescence recorded for the known quantities of template in the standard wells.

Multicomponent A term used to distinguish the contribution that each dye and the background makes to the total fluorescence detected in a sample.

p-value The probability that the mean of one set of sample data is different from the mean of another set of sample data. If the user-defined confidence level for calls setting is 99%, a positive call (+) for an Unknown well means that at most 1% of the time a measurement of sample identical to the control wells will produce a value as great as the actual measurement collected for the Unknown well.

R Squared The RSq value is a calculated assessment of the fit of the standard curve to the data points plotted. The RSq value will always be a value between 0 and 1. The closer the RSq value is to 1, the better the fit of the line to the data points.

Replicates Replicate/duplicate samples are used to increase the statistical significance and confidence of QPCR data. In plate set-up the Mx3000P software allows specifying certain wells for the program to average results. Selecting “Treat Individually” in the software analysis section directs the program to analyze each well independent of any replicate definitions. Selecting “Treat Collectively” directs the program to analyze all wells with the same replicate symbol as a group, effectively treating the measurements as all coming from the same well.

Sigma Measurement of the variability (standard deviation) of the fluorescence measured from all wells and multiple cycles. Typically, the sigma value is determined from the first few cycles, before the PCR reaction starts to affect the measurement. The Sigma multiplier is a user-defined number that is used in the analysis software program to multiply by sigma in the default method of calculating the threshold.

Standard Curve The QPCR Standard Curve is a correlation plot generated by running a series of standards of known template concentration and then plotting the known starting quantities against the measured Ct values. The range of concentrations run should span the expected unknown concentration range. On the X-axis, the concentration measured for each standard is plotted in log scale. On the Y-axis the Ct (threshold cycle) correlating to each standard is plotted. A best-fit curve is generated by the software, and the data are displayed for each individual dye or multiple dyes used in the experiment on the same graph. In the absolute quantitation method, Ct values for unknown samples are compared to the Standard Curve plot to determine the starting concentration of template in the unknown wells.

Threshold Cycle (Ct) The PCR cycle at which fluorescence measured by the instrument is determined to be at a statistically significant level above the background signal. The threshold cycle is inversely proportional to the log of the initial copy number.

Fluorescence Reading Terms

R: raw fluorescent reading in arbitrary units

dR baseline subtracted fluorescent reading

Rn fluorescent reading normalized to the reference dye

dRn baseline subtracted fluorescent reading normalized to the reference dye

Rpre the initial fluorescence reading

Rpost the final fluorescence reading

Rn, pre the fluorescence before thermal cycling normalized to the reference dye in a plate-read experiment

Rn, post the fluorescence after thermal cycling normalized to the reference dye in a plate-read experiment

Rpost–Rpre the total change in fluorescence in a plate-read experiment

Rn, post–Rn, pre the total change in normalized fluorescence in a plate-read experiment

Rpost/Rpre the final fluorescence reading divided by the initial fluorescence reading in a plate-read experiment

R Last the final fluorescence reading in a real-time experiment

dR Last the final fluorescence reading minus the initial fluorescence reading in a real-time experiment

Rn Last the final fluorescence in a real-time experiment normalized to a reference dye

dRn Last the normalized final fluorescence reading minus the normalized first fluorescence reading in a real-time experiment

R Last/R First the final fluorescence reading divided by the initial fluorescence reading in a real-time experiment

QPCR References & Useful Websites

QPCR References

For the most current list of QPCR references, please visit http://www.stratagene.com/citations

Useful Websites

Oligonucleotide and Assay Design Resources

http://www.stratagene.com/qpcr
Stratagene's QPCR homepage and link to application notes

http://www.mx3000p.com
Homepage for Stratagene's Mx3000P Real-time PCR System

http://www.mx4000.com
Homepage for Stratagene's Mx4000 Multiplex Quantitative PCR System

http://www.stratagene.com/faq/index.htm
Stratagene Frequently Asked Questions

http://www.gene-quantification.info/
Gene Quantification Homepage

http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
Primer 3 Homepage

http://biotools.idtdna.com/Primerquest/
Primerquest Homepage

http://164.164.144.45/OligoSysT/login.jsp
Oligosys Homepage

http://www.premierbiosoft.com/stratagene/stratagene
Beacon Designer (TaqMan, M. Beacons and multiplex assay design)

Oligonucleotide Properties Calculators

http://www.bioinfo.rpi.edu/applications/mfold/old/dna/form1.cgi
Zucker Mfold

http://biotools.idtdna.com/Analyzer/oligocalc.asp
IDT Oligoanalyzer

http://www.rnature.com/oligonucleotide.html
RNATure

Miscellaneous

http://www.ncbi.nih.gov/RefSeq/
RefSeq (Reference sequence database)

http://www.ncbi.nlm.nih.gov/spidey/
Spidey (cDNA to genomic alignment tool)

http://www.molecular-beacons.org/
All about Molecular Beacons

http://iubio.bio.indiana.edu/
General Molecular Biology Links and Tools

http://www.tataa.com/
TATAA Biocenter

http://medgen.ugent.be/rtprimerdb/index.php
real-time PCR Primer and Probe Database

http://www.abrf.org/ResearchGroups/NucleicAcids/Studies/Assaydesign-Primer3.pdf
Nucleic Acids Research Group study on Primer and Probe Design

Discussion Groups

http://groups.yahoo.com/group/qpcrlistserver/
QPCR Listserver

Reagent & Ordering Information

Sample Preparation Materials

Stratagene RNA and cDNA Sample Preparation for QPCR			
Absolutely RNA® Miniprep Kit		50 preps	catalog #400800
Absolutely RNA® Microprep Kit		50 preps	catalog #400805
Absolutely RNA® Microprep Kit, 96 well		2 plates	catalog #400793
		10 plates	catalog #400794
Absolutely RNA® Nanoprep Kit		50 preps	catalog #400753
StrataScript™ first-strand cDNA synthesis kit		50 reactions (5–10 µg each)	catalog #200420

Stratagene Real-Time QPCR Reagent Kits

FullVelocity™ QPCR & RT-QPCR Master Mix Kits for Probe-Based Detection			
FullVelocity™ QPCR Master Mix	• Sensitive, high-speed real-time quantification of DNA and cDNA using hydrolysis probes • Does not require re-optimization of primers or probes • Multiplex up to 2 DNA or cDNA targets • Master mix format • For two-step RT-PCR, combine with the StrataScript 1st strand cDNA synthesis kit	400 reactions	catalog #600561
		10 x 400 reactions	catalog #929561
FullVelocity™ QRT-PCR Master Mix	• High sensitivity QRT-PCR in less time • one-step master mix format	400 reactions	catalog #600562
		10 x 400 reactions	catalog #929562

Brilliant® Probe-Based Quantitative PCR Reagents			
Brilliant® QPCR Master Mix	• Compatible with any fluorescent chemistry including sequence-specific probes • Multiplex up to 2 DNA or cDNA targets • Master mix format • Contains dUTP • For two-step RT-PCR, combine with the StrataScript 1st strand cDNA synthesis kit	400 reactions	catalog #600549
		10 x 400 reactions	catalog #929549
Brilliant® Multiplex QPCR Master Mix	• Multiplex up to 4 targets • Master mix format • For two-step RT-PCR, combine with the StrataScript 1st strand cDNA synthesis kit	200 reactions	catalog #600553
Brilliant® QPCR Core Reagent Kit	• Core reagent format	400 reactions	catalog #600530
		10 x 400 reactions	catalog #929530
Brilliant® QPCR *Plus* Core Reagent Kit	• For decontamination using UNG • Core reagent format	400 reactions	catalog #600540
		10 x 400 reactions	catalog #929540
Brilliant® Passive Reference Dye	• To maximize performance on different instrument platforms • 1mM solution	10 x 100 µl	catalog #600536

Brilliant® Probe-Based Quantitative RT-PCR Reagents			
Brilliant® Passive Reference Dye	• To maximize performance on different instrument platforms	10 x 100 µl	catalog #600536
Brilliant® QRT-PCR Master Mix Kit, one-step	• Compatible with any fluorescent chemistry including sequence-specific probes • one-step QRT-PCR • Master mix format • Contains dUTP	400 reactions	catalog #600551
		10 x 400 reactions	catalog #929551

Brilliant® Probe-Based Quantitative RT-PCR Reagents

Brilliant® QRT-PCR Core Reagent Kit, one-step	• One-step QRT-PCR • Core kit format	400 reactions	catalog #600532
		10 x 400 reactions	catalog #929532
Brilliant® QRT-PCR *Plus* Core Reagent Kit, one-step	• One-step QRT-PCR • Core kit format • Contains dUTP	400 reactions	catalog #600542
		10 x 400 reactions	catalog #929542
Brilliant® QRT-PCR Core Reagent Kit, two-step	• Two-step QRT-PCR • Core kit format	400 reactions	catalog #600534
		10 x 400 reactions	catalog #929534
Brilliant® QRT-PCR *Plus* Core Reagent Kit	• Two-step QRT-PCR • Core kit format • Contains dUTP and UNG	400 reactions	catalog #600544
		10 x 400 reactions	catalog #929544

Brilliant® SYBR® Green QPCR and QRT-PCR Reagents

Brilliant® SYBR® Green QPCR Master Mix	• Superior sensitivity compared to other *Taq*-based QPCR kits • Multiplex up to 2 DNA or cDNA targets • Master mix format • Contains dUTP • For two-step RT-PCR, combine with the StrataScript 1st strand cDNA synthesis kit	400 reactions	catalog #600548
		10 x 400 reactions	catalog #929548
Brilliant® SYBR® Green QPCR Core Reagent Kit	• Core reagent format • For two-step RT-PCR, combine with the StrataScript 1st strand cDNA synthesis kit	400 reactions	catalog #600546
		10 x 400 reactions	catalog #929546
Brilliant® SYBR® Green QRT-PCR Master Mix Kit, one-step	• One-step QRT-PCR • Master mix format • Contains dUTP	400 reactions	catalog #600552
		10 x 400 reactions	catalog #929552

Brilliant® Probe-Based Quantitative RT-PCR Reagents

Brilliant® QRT-PCR Master Mix Kit, one-step	• Compatible with any fluorescent chemistry including sequence-specific probes • One-step QRT-PCR • Master mix format • Contains dUTP	400 reactions	catalog #600551
		10 x 400 reactions	catalog #929551
Brilliant® QRT-PCR Core Reagent Kit, one-step	• One-step QRT-PCR • Core kit format	400 reactions	catalog #600532
		10 x 400 reactions	catalog #929532
Brilliant® QRT-PCR *Plus* Core Reagent Kit, one-step	• One-step QRT-PCR • Core kit format • Contains dUTP	400 reactions	catalog #600542
		10 x 400 reactions	catalog #929542
Brilliant® QRT-PCR Core Reagent Kit, two-step	• Two-step QRT-PCR • Core kit format	400 reactions	catalog #600534
		10 x 400 reactions	catalog #929534
Brilliant® QRT-PCR *Plus* Core Reagent Kit	• Two-step QRT-PCR • Core kit format • Contains dUTP and UNG	400 reactions	catalog #600544
		10 x 400 reactions	catalog #929544
Brilliant® Passive Reference Dye	• To maximize performance on different instrument platforms	10 x 100 µl	catalog #600536

QPCR Reference RNA and Pre-made RNA, mRNA and cDNA			
Stratagene® QPCR Human Reference Total RNA	• QPCR Human Reference Total RNA • (Concentration 1 µg/µl)	25 µg	catalog #750500
Total RNA and Poly(A)+ RNA, Gold Standard	• Human, mouse and rat RNA, ready for QPCR	inquire	Many, please inquire
First-Strand cDNA, Human	• Human cDNA, ready for QPCR	25 ng	Many, please inquire