

Introduction to Quantitative PCR



Methods and Applications Guide



Agilent Technologies

Introduction to Quantitative PCR

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Introduction to Quantitative PCR

Whether you are a novice or experienced user, our goal is to ensure that you are running quantitative PCR (QPCR) experiments quickly, efficiently, and affordably. Our Mx family of QPCR Systems, MxPro QPCR Software, premiere QPCR Systems Service Program, complete line of QPCR and QRT-PCR reagents, and Fast Track QPCR Education Program is the total package for your QPCR research. At Agilent Technologies, we are committed to providing you with the most comprehensive and easy-to-use support programs. The *Introduction to Quantitative PCR Methods and Applications Guide* was written 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 your QPCR instrument, as well as an explanation of the theoretical basis for the materials used in QPCR 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 experimental results.

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. The guide starts with a brief introduction to QPCR and experimental design. This is perhaps the most crucial step in the QPCR process as it lays the groundwork for every other aspect of the assay. The guide then discusses important experimental design specifics such as primer and probe design, dye and reagent choice, assay optimization, and data analysis. Detailed discussions on Sample Preparation, Reverse Transcription Considerations, Gene Expression Normalization and Multiple Experiment Analysis are also included.

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 semi-quantitative PCR) or while the amplification is still progressing (real-time QPCR).

In endpoint semi-quantitative PCR, 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.

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 quantitative analysis. For applications that do not require a great deal of copy number discrimination, such as qualitative studies which just seek to determine whether or not a target sequence of interest is present or not, end-point measurements are generally sufficient.

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 fluorescence 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. Fluorescence readings at these earlier cycles of the reaction will measure the amplified template quantity where the reaction is much more reproducible from sample to sample than at the endpoint.

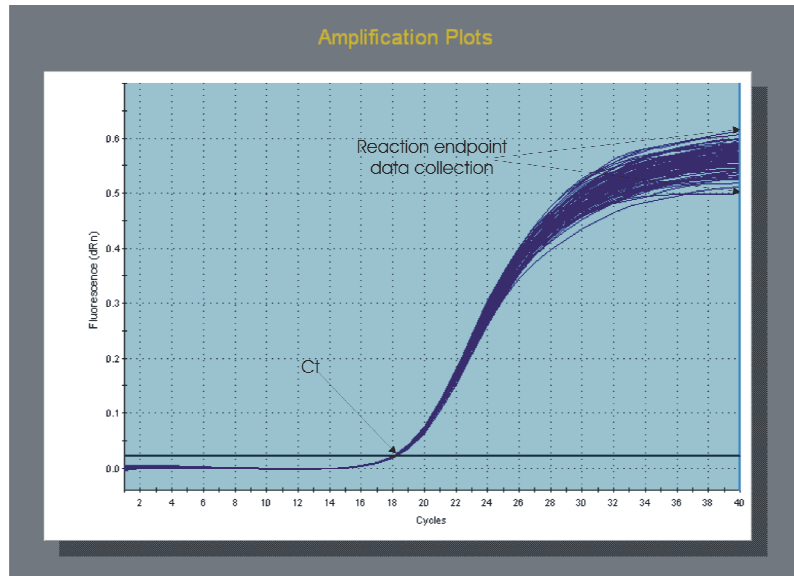


Figure 1
 QPCR run with 96 identical reactions. Note that the PCR reaction endpoint variation (i.e. 40 cycles) is much greater as the reaction progresses.

In real-time QPCR, 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. With each amplification cycle, the increase in fluorescence intensity is proportional to the increase in amplicon concentration, with the QPCR instrument system collecting data for each sample during each PCR cycle. The resulting plots of fluorescence vs. cycle number for all the samples are then set with their background fluorescence at a common starting point (a process known as baseline correction). Then, a threshold level of fluorescence is set above the background but still within the linear phase of amplification for all the plots. The cycle number at which an amplification plot crosses this threshold fluorescence level is called the “Ct” or threshold cycle. This Ct value can be directly correlated to the starting target concentration of the sample. The greater the amount of initial DNA template in the sample, the earlier the Ct value for that sample (Figure 2). The MxPro 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.

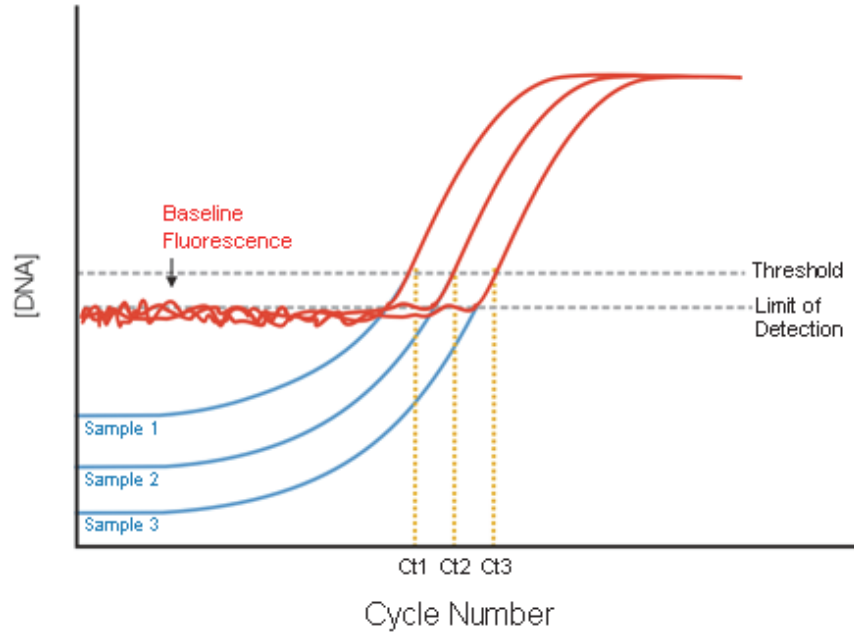


Figure 2

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 lower threshold cycle.

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

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 questions are 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 or reference genes) 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).

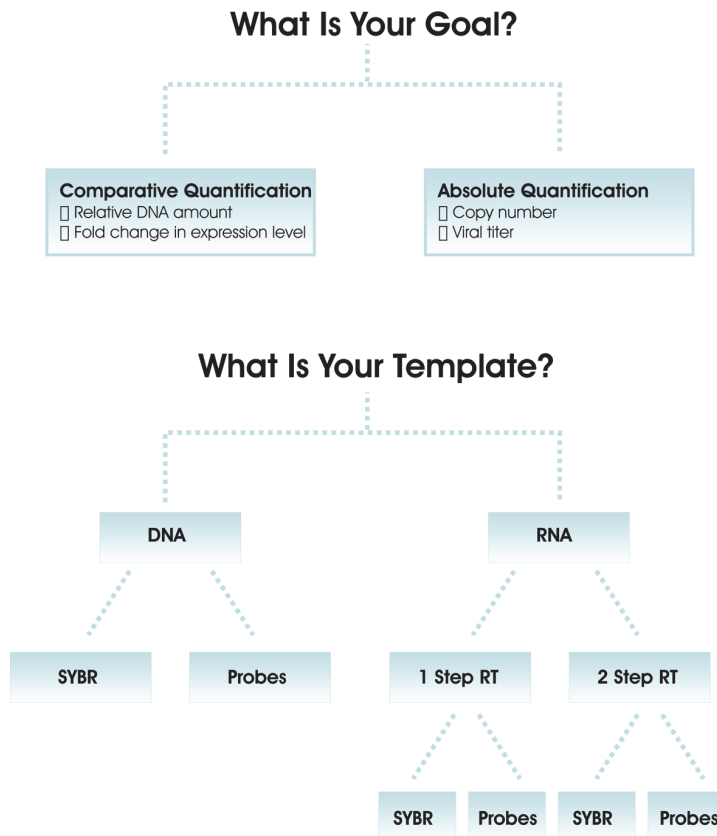


Figure 3
Flowchart showing a typical experimental design process based on the goals and requirements of the assay.

Methods of Quantification

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

Standard Curve

The most direct and precise approach for analyzing quantitative data is to use a standard curve that is prepared from a dilution series of template of known concentration. This is known as “standard curve” or “absolute” quantification. The standard curve 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 QPCR Human Reference Total RNA.

Figure 4 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.

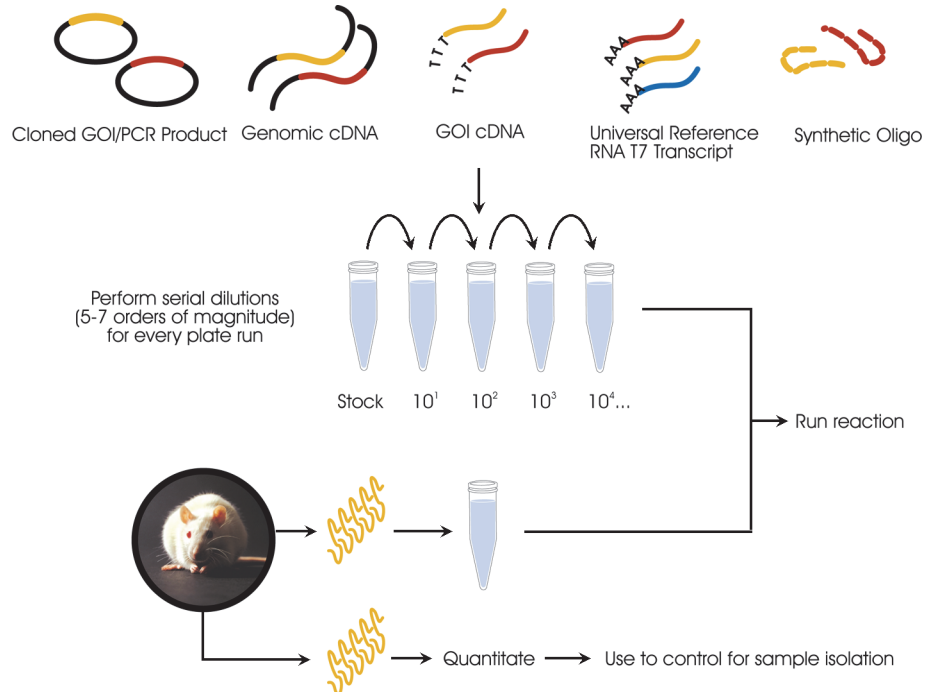


Figure 4
Experimental setup for standard curve quantification. Using a known starting concentration of template from one of a variety of sources, a dilution series is performed. These samples are run under the standard well type on the same plate as your unknowns. By comparing the Ct values of the unknowns to the Ct values of the standards, the starting template quantities for the unknown samples can be calculated.

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 aliquotting 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 linear regression line. This line represents the standard curve. Comparing the Ct values of the unknown samples to this standard curve allows the quantification of initial copy numbers (Figure 5).

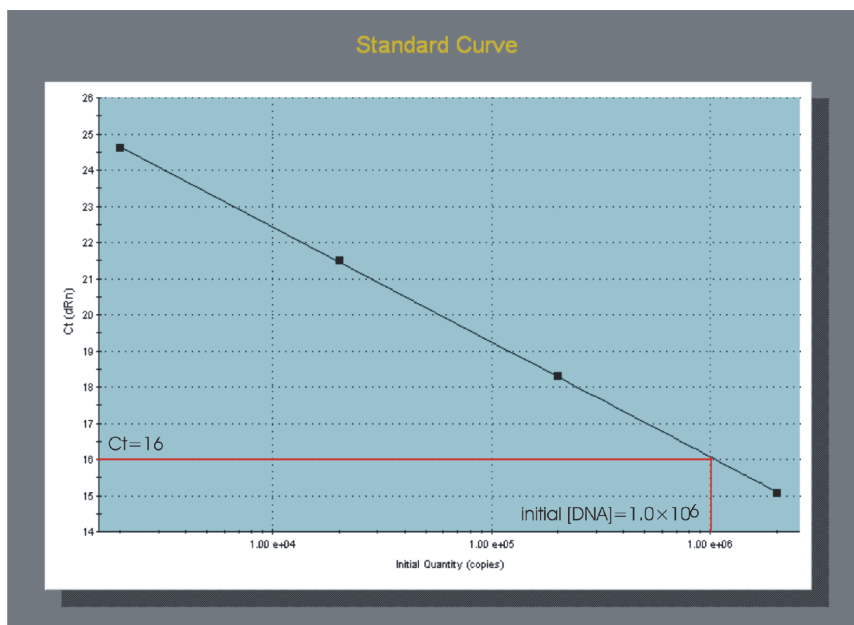


Figure 5
Illustration of the theory behind standard curve quantitation. The log of the initial template quantity is plotted against the Ct values for the standards. By comparing the Ct values of the unknowns to this Standard Curve plot, the initial template quantities for the unknown samples can be determined.

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.

In addition, the curve must be linear over the whole concentration range. The linearity is denoted by the R squared (Rsquared) value (R^2 or Pearson Correlation Coefficient) and should be very close to 1 (≥ 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 experimental 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 template 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[®] dyes). Include at least 2–3 no template control (NTC) wells and 2–3 no reverse transcriptase control wells (for QRT-PCR runs) with each run. Standard curve analysis is explained further in later sections.

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 reference gene or 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 from sample to sample and experiment to experiment. Normalizers are explained in more detail in later sections.

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).

QPCR Chemistry Options

The fluorescent reporter molecule used in real-time PCR reactions can be (1) a sequence-specific probe comprised 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:

- The level of sensitivity and accuracy required for the data analysis
- The budget available to support the project
- The skill and experience of the researcher in designing and optimizing QPCR assays
- The number of targets to be analyzed and whether a multiplex assay is appropriate

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: SYBR Green I DNA binding dye, TaqMan probes, Molecular Beacons, and Scorpions probes. Each QPCR chemistry option has advantages and drawbacks. Chemistries such as Amplifluor[®] primers, LUX primers, FRET probe pairs (also known as hybridization probes), or Invader[®] probes will work in the Mx system, however they are beyond the scope of this application guide.

DNA Binding Dyes

DNA binding dyes, such as SYBR Green I dye and EvaGreen[®] dye, are cost effective and easy to use, especially for researchers who

are new to QPCR techniques. These factors make DNA binding dyes a common choice for optimizing QPCR reactions.

When free in solution, these dyes display relatively low fluorescence, but when bound to double-stranded DNA, their 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 6). Compared to probe-based methods, SYBR Green I and EvaGreen assays are relatively easy to design and run. 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. These dyes will increase in fluorescence when bound to any double-stranded DNA, making the reaction specificity 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.

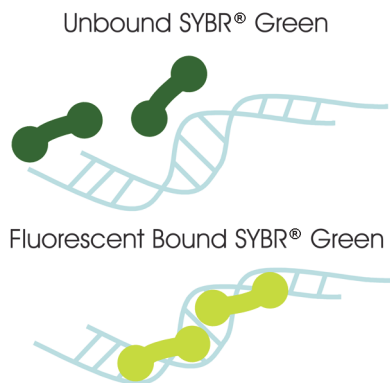


Figure 6
SYBR® Green I detection mechanism; double-stranded DNA in the reaction is bound by the dye. In the bound state, SYBR® Green I is 1000-fold more fluorescent than in the unbound state. As PCR amplification increases the amount of dsDNA present, the fluorescence signal increases proportionately.

A non-specific signal cannot always be prevented, but its presence can be easily and reliably detected by performing melt curve analysis on the PCR products from every run. Following the amplification reaction, the PCR products can be slowly melted while fluorescence from the dye is monitored. As the temperature increases, the DNA melts and the fluorescence intensity decreases. The temperature at which a DNA molecule melts depends on its

length and sequence; therefore, if the PCR products consist of molecules of homogeneous length and sequence, 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 T_m (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 specificity. Optimization of primers to use with SYBR Green I or EvaGreen chemistry is straightforward and provides a high level of QPCR experimental design success.

Probe-Based Chemistries

Compared to non-specific chemistries such as SYBR Green I dye, a higher level of detection specificity is provided by using an internal probe 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-based 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, a technique referred to as multiplex QPCR.

Linear Probes

Linear probes (i.e., 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 reporter dye, typically FAM, is attached to the 5' end of the probe and a quencher, such as TAMRA or a dark quencher (e.g. a Black Hole Quencher[®]), is attached at the 3' end. 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 7).

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, which is 7-10°C below the T_m 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.

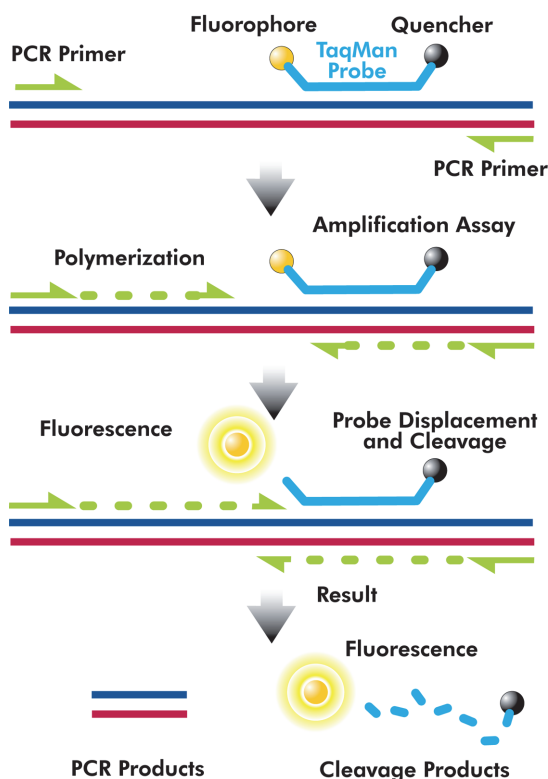


Figure 7
TaqMan® probe chemistry mechanism. These probes rely on the 5'-3' nuclease activity of *Taq* DNA polymerase to cleave a dual-labeled probe during hybridization to the complementary target sequence.

TaqMan chemistry can be used for 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 a specialized TaqMan probe (either MGB probes or LNA probes) or one of the structured probe chemistries described in the “Structured Probes” section (below).

Minor Groove Binder (MGB) TaqMan probes are similar to 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 T_m of the probe allows for the use of a much shorter probe (down to approximately 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.

Probes composed of locked nucleic acids (LNA's) are an alternative to TaqMan MGB probes that increase annealing stability without the use of a minor groove binder. An LNA is a modified RNA nucleotide with an extra bridge connecting the 2' and 4' carbons. This structure enhances base stacking and backbone pre-organization which significantly increases the annealing stability, thus allowing shorter oligo sequences to be used for the probe. Additionally, mismatched bases have been shown to have a larger effect on the T_m of an LNA probe compared to standard nucleic acid probes. When used for SNP detection, LNA probes normally contain a combination of LNA nucleotides and standard DNA nucleotides.

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. 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 was used as the quencher in this application, but BHQ's (Black Hole Quenchers) have become more common.

In the absence of target sequence, the stem-loop structure of the Molecular Beacon 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, which results in separation of the fluorophore and quencher, thus allowing the reporter fluorescence to be detected (Figure 8).

When the target consists of a nucleotide sequence that is not perfectly matched to the Molecular Beacon, the 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.

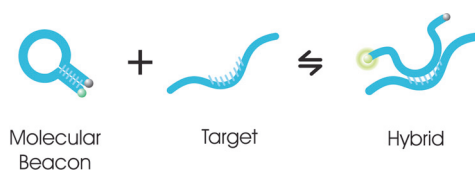


Figure 8
Molecular Beacon chemistry mechanism. The Molecular Beacon includes a hairpin-loop structure, with the loop complementary to a target sequence and the stem formed by the addition of internal complementary sequences. When hybridized to the target, the fluorophore and quencher are far enough apart to allow fluorescence to be detected.

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 9). 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.

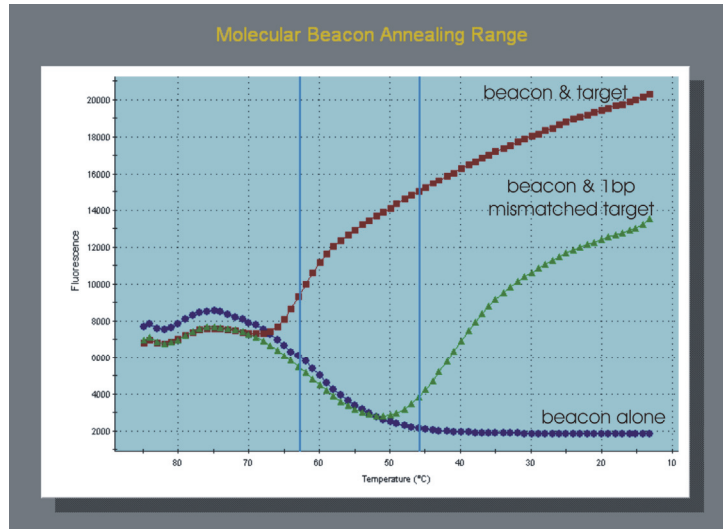


Figure 9
 Example of a Molecular Beacon melting curve. Temperature is decreasing left to right on the X axis, and fluorescence is plotted on the Y axis. The window between the two vertical blue lines represents a suitable annealing temperature range to discriminate two alleles with a 1-bp difference.

Scorpions, another type of structured probe chemistry, function 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 probes 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 typical 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.

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 and 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). The Ensembl genome database (www.ensembl.org) provides transcript structures with exon-intron borders, enabling the design of exon border spanning primers or probes when working with cDNA. For the design of primers and/or probes for Affymetrix microarray validation, the NetAffx analysis Center (www.affymetrix.com/analysis) is a valuable tool to identify GeneChip® array target regions on a given array.

After the sequence is obtained, a primer and probe design software program should be used in order to simplify and maximize success for the design process. Designer software packages are available both as freeware on the internet and through many oligonucleotide vendors. A representative list of primer design resources can be found in the *Useful Websites* section of this guide, or accessed from the MxPro software by selecting *QPCR Internet Links* from the Tools menu.

When using a software program to design primers and probes, it is important to set the concentration of monovalent ions (Na^+/K^+) and divalent ions (Mg^{2+}) to those that are used in your reaction for accurate melting temperature prediction. (The buffer conditions will generally be in the range of 50–100 mM monovalent cation and 1.5–5.5 mM Mg^{2+} .)

The region of the template sequence to be used for detection must be considered carefully. Compare the region of interest to the entire genome to ensure that the target sequence is unique (e.g., by performing an NCBI BLAST database search at www.ncbi.nlm.nih.gov/BLAST), and identify and avoid any potential secondary structures in the sequence (e.g., by using the mfold program at <http://mfold.bioinfo.rpi.edu>).

For detection of a coding sequence specific to RNA targets, it is advisable to design the probe to span exon-exon boundaries, thus preventing the detection of sequences from residual genomic DNA in the RNA sample. In circumstances where this is not an option, the RNA sample should be treated with DNase prior to the reverse

transcription (RT) reaction. This is an efficient approach and results in minimal loss of sample when carried out on a column-based purification system.

In QRT-PCR, consider the method of cDNA synthesis when designing primers if oligo dT priming is used. It is generally safe to assume that the RT reaction has transcribed between 500–1000 bases from the polyA site with quantitative linearity, so it is best to design the assay to target a sequence for amplification towards the 3' end of the gene. The presence of SNPs and splice variants within a sequence should also be considered, as these must either be avoided or targeted as required according to 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 70–150 bp in length for probe-based chemistries, and between 100–300 bp in length if SYBR Green I will be used. In order to maximize the efficiency of the PCR amplification, it is generally best to keep the target length relatively short. However, 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 fluorescence signal. When designing for SYBR Green I with the intention of moving to a probe-based chemistry later, keep in mind to use the lower range (i.e., 100–200 bp) for primer design.

General rules for primers used in all chemistries are that each primer should be between 15–30 bp in length and the theoretical T_m 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 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 should not contain an inverted repeat sequence that would allow it to fold on itself.

In general, the Gibbs free enthalpy (ΔG) of primer dimer and cross-primer dimer formation should be greater than –4 kcal/mol to ensure that primers do not form stable dimers. For multiplex reactions, it may be necessary to loosen the free enthalpy specification in order to allow for the design of the oligos required to work together in the same reaction. It is best to restrict the ΔG between each oligo pair to greater than –6 kcal/mol in a triplex reaction, and greater than –8 kcal/mol in a quadriplex reaction.

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 fluorescence quencher and should not be adjacent to the reporter dye.

Historically, TaqMan probes were situated 3–12 bp downstream of the primer on the same strand, but 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 T_m 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 extension at 60°C (Figure 10). For these assays, the probe is designed with a T_m 8–10°C higher than the T_m of the primers. Using the higher T_m for the probe ensures hybridization to the target before extension can occur from the primer, so there will always be a corresponding increase in fluorescence signal for every amplified copy that is produced.

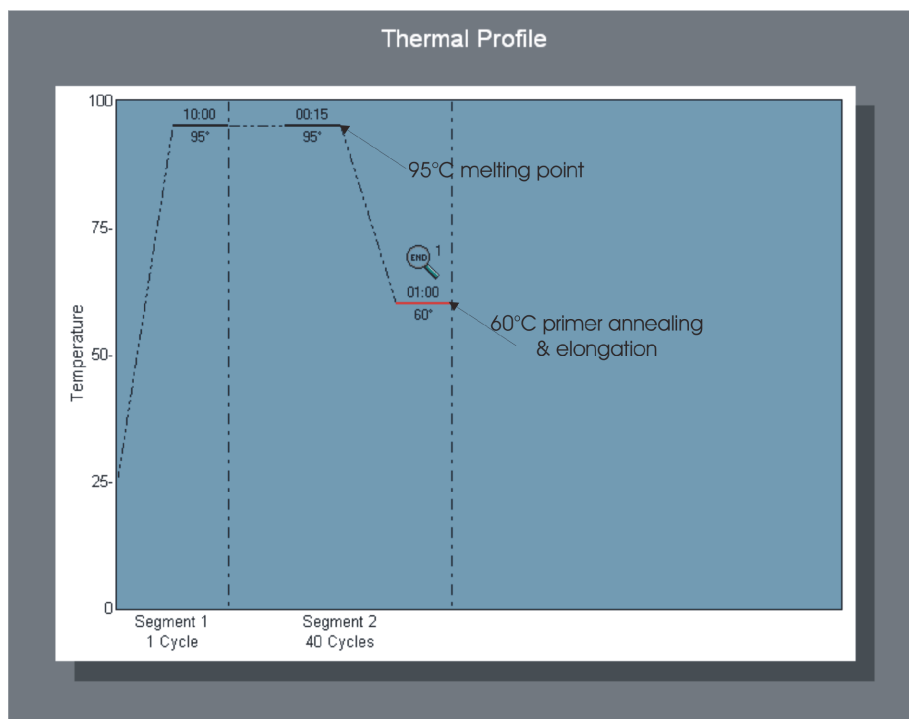


Figure 10
Typical two-step PCR thermal profile used for TaqMan® QPCR reactions.

Since TaqMan chemistry requires using the same thermal profile for each reaction, primers should always be designed with a T_m of approximately 60°C, and the hydrolysis probe with a T_m 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*).

For Molecular Beacons, the stem sequence should be designed to be 5–7 bp in length and have a similar T_m to that of the probe region in the loop. As a general rule, stem sequences that are 5 bp

long will have a T_m of 55–60°C, stems that are 6 bp long will have a T_m of 60–65°C, and stems that are 7 bp long will have a T_m of 65–70°C. Before having the Molecular Beacon probe synthesized, it is useful to use an oligo folding program [e.g., the mfold program (www.bioinfo.rpi.edu/~zukerm/rna/)] to verify that the sequence will form the desired stem-loop structure in your specific salt conditions and annealing temperature. 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. Additionally, Molecular Beacon probes should be designed to anneal at 7–10°C higher than the primers to allow hybridization before primer extension.

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 T_m of the stem-loop structure is 5–10°C higher than the T_m of the primer sequence to the target, and the ΔG value for the stem-loop confirmation is negative. The more negative the ΔG value, the more likely it is that folding will occur. Similar to Molecular Beacon probes, proper folding of the Scorpions probe should be verified using the mfold program listed above.

When designing probes, the combination and positioning of reporter dyes and quenchers is important. Make sure that the chosen quencher will efficiently suppress the fluorescence of your chosen reporter dye to ensure low background. Information on the recommended quenchers for each fluorophore is generally available from the companies that synthesize these probes, and general guidelines on the choice of dark quenchers are given in the *Dye and Quencher Choice* section of this guide.

When designing primers and probes for multiplex reactions, adhere to the following additional rules: (1) all amplicons should be of similar length (± 5 bp) as well as similar GC content ($\pm 3\%$) and (2) the primer set T_m 's, as well as the probe T_m 's, used in a multiplex assay should be within 1°C of each other.

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 recommends 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 are compatible, given the type of detection chemistry. In addition, when designing multiplexed reactions the spectral overlap between the fluorophores and quenchers for the different targets should be minimized to avoid possible crosstalk issues (Table 1).

Previously, the most common dye/quencher combination for a TaqMan probe was a FAM fluorophore with a TAMRA quencher. Today, dark quenchers have largely replaced fluorescent quenchers like TAMRA. 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 it is important to avoid emitted light from the quencher creating cross-talk signal with one of the reporter dyes.

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[®] Quenchers (Integrated DNA Technologies):

Iowa Black-FQ 420–620 nm
Iowa Black-RQ 500–700 nm

Table 1: Parameters of the Mx3000P and Mx3005P system filter sets

| Filter Set | Excitation Wavelength | Emission Wavelength |
|-------------------|------------------------------|----------------------------|
| Alexa Fluor 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 |
| Atto 425 | 440 | 492 |
| ANS | 330 | 492 |
| bis-ANS | 440 | 550 |
| SYPRO Tangerine | 517 | 568 |
| Nile Red | 585 | 665 |
| FR RAM | 492 | 580 |

*FR Cy5 is only available as a custom set.

A listing of TaqMan fluorophore and quencher combinations can be found at most oligo manufacturers' websites (see *QPCR References and Useful Websites* at the end of this guide for more information). Molecular Beacons have historically used DABCYL quenchers, which work with a wide range of fluorophores. However, Molecular Beacons can also be used with Black Hole Quenchers (BHQs).

In an Mx system, the choice of dyes will be limited by the 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 an optional reference dye. For triplex, FAM, HEX (JOE/VIC), and Cy5 with ROX as an optional reference dye are suggested. For quadriplex, we suggest FAM, HEX (JOE/VIC), Texas Red[®], and Cy5 dyes. Any fluorophores that have little to no spectral overlap are best suited for this type of application.

The Mx3005P system contains five filters to accommodate multiplexing four targets with the reference dye, performing a five-target qualitative assay, or simply providing greater dye flexibility between reactions. The most common combination of four filters is FAM, HEX, ROX, and Cy5 (and all equivalent dyes in these filter sets). The fifth filter set will most often be Cy3, as oligo manufacturers offer several dye choices which work well with this filter.

However, due the small spectral separation between HEX and Cy3 there can be some signal overlap with this pair. If running all five dyes simultaneously, the Atto 425 filter set is commonly used instead of Cy3. The Atto 425 filter set is in the far blue range of the spectrum and thus spectrally distant from all other filter sets. As mentioned above, dark quenchers can be especially useful when multiplexing. TAMRA can be an effective quencher, but the emission spectrum for TAMRA does have some overlap with other dyes such as ROX, HEX, and Cy3. With Dark Quenchers, background from the quenchers will not be an issue.

QPCR Reagent Choice

The Mx 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.

Other than the requirement imposed by the reference dye concentration, the Mx 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 Mx 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 Stratagene QPCR and QRT-PCR reagent products is located at the end of this guide. Table 2 and table 3 specifically highlight the Brilliant II and Brilliant III lines of reagent kits for QPCR.

For standard QPCR reactions using probe chemistries, the Brilliant II QPCR master mix provides all the buffer components in a pre-optimized solution. For SYBR Green I detection, the Brilliant II SYBR QPCR master mix comes with the SYBR Green I dye included. All Brilliant II kits are also available for use in both one-step and two-step QRT-PCR reactions.

Table 2: Reagents for Probe-Based Detection

| Format | DNA (cDNA) Quantification | RNA Quantification | 2-Step |
|---|--|--|---|
| Master Mix | Brilliant III Ultra-Fast QPCR Master Mix (Cat. No. 600880) | Brilliant III Ultra-Fast QRT-PCR Master Mix (Cat. No. 600884) | AffinityScript QPCR cDNA Synthesis Kit (Cat. No. 600559) plus Brilliant III QPCR Master Mix (Cat. No. 600880) |
| | Brilliant II QPCR Master Mix (Cat. No. 600804) | Brilliant II QRT-PCR Master Mix, 1-Step (Cat. No. 600809) | Brilliant II QRT-PCR, AffinityScript Master Mix, 2-Step (Cat. No. 600827) |
| | Brilliant II FAST QPCR Master Mix (Cat. No. 600845) | — | AffinityScript QPCR cDNA Synthesis Kit (Cat. No. 600559) plus Brilliant II FAST QPCR Master Mix (Cat. No. 600845) |
| Master Mix with ROX Dye Premixed (Low and High ROX for different instruments) | Brilliant II QPCR Low ROX Master Mix (Cat. No. 600806) | Brilliant II QRT-PCR Low ROX Master Mix, 1-Step (Cat. No. 600837) | AffinityScript QPCR cDNA Synthesis Kit (Cat. No. 600559) plus Brilliant II QPCR Low ROX Master Mix (Cat. No. 600806) |
| | Brilliant II QPCR High ROX Master Mix (Cat. No. 600805) | Brilliant II QRT-PCR High ROX Master Mix, 1-Step (Cat. No. 600838) | AffinityScript QPCR cDNA Synthesis Kit (Cat. No. 600559) plus Brilliant II QPCR High ROX Master Mix (Cat. No. 600805) |
| RNA Preparation and Amplification | — | SideStep II QRT-PCR Master Mix Kit, 1-Step (Cat. No. 400917) | SideStep II QRT-PCR Master Mix Kit, 2-Step (Cat. No. 400918) |

Table 3: Reagents for SYBR Green-Based Detection

| Format | DNA (cDNA) Quantification | RNA Quantification | 2-Step |
|---|--|--|--|
| Master Mix | Brilliant III Ultra-Fast SYBR [®] Green QPCR Master Mix (Cat. No. 600882) | Brilliant III Ultra-Fast SYBR [®] Green QRT-PCR Master Mix (Cat. No. 600886) | AffinityScript QPCR cDNA Synthesis Kit (Cat. No. 600559) plus Brilliant III Ultra-Fast SYBR [®] Green QPCR Master Mix (Cat. No. 600882) |
| | Brilliant II SYBR [®] Green QPCR Master Mix (Cat. No. 600828) | Brilliant II SYBR [®] Green QRT-PCR Master Mix, 1-Step (Cat. No. 600825) | Brilliant II SYBR [®] Green QRT-PCR, AffinityScript Master Mix, 2-Step (Cat. No. 600834) |
| | Brilliant II FAST SYBR [®] Green QPCR Master Mix (Cat. No. 600845) | — | AffinityScript QPCR cDNA Synthesis Kit (Cat. No. 600559) plus Brilliant II FAST QPCR Master Mix (Cat. No. 600845) |
| Master Mix with ROX Dye Premixed (Low and High ROX for different instruments) | Brilliant II SYBR [®] Green QPCR Low ROX Master Mix (Cat. No. 600830) | Brilliant II SYBR [®] Green QRT-PCR Low ROX Master Mix, 1-Step (Cat. No. 600835) | AffinityScript QPCR cDNA Synthesis Kit (Cat. No. 600559) plus Brilliant II SYBR [®] Green QPCR Low ROX Master Mix (Cat. No. 600830) |
| | Brilliant II SYBR [®] Green QPCR High ROX Master Mix (Cat. No. 600829) | Brilliant II SYBR [®] Green QRT-PCR High ROX Master Mix, 1-Step (Cat. No. 600836) | AffinityScript QPCR cDNA Synthesis Kit (Cat. No. 600559) plus Brilliant II SYBR [®] Green QPCR High ROX Master Mix (Cat. No. 600829) |
| RNA/DNA Preparation and Amplification | Brilliant SYBR [®] Green SideStep QPCR Master Mix (Cat. No. 400904) | SideStep II QRT-PCR Master Mix Kit, 1-Step (Cat. No. 400917) | SideStep II SYBR [®] Green QRT-PCR Master Mix Kit, 2-Step (Cat. No. 400909) |

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 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 and probe is how they are purified. It is best to consult the primer/probe manufacturer for guidance on the type and level of purification required for the oligo and application.

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. Aliquot the primers into volumes that will not require that they be thawed more than 20 times, and store probes 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 >5000 counts in the raw fluorescence signal. This digestion can be performed using 100 nM of probe in 25 μl 1 \times buffer with 10 U DNase or S1 Nuclease, incubated at room temperature for 30 minutes.

Reference Dye Considerations

A passive reference dye is a fixed concentration of inert fluorescent dye (usually ROX) that is added at the same concentration in all the samples, usually by including it in the master mix or by adding it to the buffer stock solution before it is aliquotted into the individual reaction tubes. Reference dye normalization is performed by dividing the raw fluorescence signal for each reporter dye at any given cycle by the raw fluorescence signal of the reference dye at the same cycle and then drawing the amplification plot based on these ratio values.

Historically, reference dye has been used to correct for sample to sample signal variation that is not due to the chemistry itself (e.g., aliquotting errors or deficiencies in the signal uniformity due to the instrument optical system). In the Mx system, the scanning read-head ensures that all wells receive the same level of excitation light and have the exact same light path to the detector so there is no need to correct for positional differences. Also, baseline correction algorithms generally correct for most variation due to aliquotting errors without the need for reference dye

normalization, which makes the use of a reference dye optional in the Mx system.

Running a reference dye can still have some value, because in some cases it can result in somewhat cleaner looking data. Also, even if normalization is not performed, it can be very useful in the troubleshooting process if you see any unexpected results in the amplification plots of your reporter dyes. If signal is particularly low or high, or if there is an odd shift in the fluorescence level, you can check to see if similar effects are seen in the reference dye profile, which should normally run flat. Being able to determine whether effects are seen only with your reporter dye or with both the reporter and reference dyes can often allow you to discriminate probe problems from potential instrument problems.

If ROX is used as a reference dye, from an instrument standpoint there is a difference in the concentration of reference dye that should be used in the Mx instrument vs. some other systems. The white light excitation in the Mx system and the system's dye-specific filters will excite and measure the fluorescence for ROX very efficiently. We designed the ROX filter set to be very sensitive so this dye channel can be used for actually detecting fluorophores labeled with ROX or Texas Red. 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 Mx system, the high concentration of ROX will create oversaturated signal on the ROX channel and result in the normalized data containing more noise than the non-normalized data. In the Mx system, ROX should be used at a final concentration of approximately 30 nM of free dye. If a master mix containing a final ROX concentration closer to 300 nM is used, it is recommended that the non-normalized baseline-corrected amplification plots (dR) be used for analysis rather than the normalized baseline-corrected plots (dRn).

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 Mx system and should not cause any difficulty in normalization.

Nucleic Acid Sample Preparation

DNA

There are many methods used to purify genomic and plasmid DNA, depending on sample type, sample amount, and budget. Pure, intact DNA is highly recommended for QPCR analysis. DNA purity can be measured by spectrophotometry; pure DNA has an A_{260}/A_{280} ratio of 1.8–2.0 in 10 mM Tris, pH 8.5. Intact DNA can be verified using gel electrophoresis and visual analysis of the gel image for lack of degradation.

RNA

Purification of undegraded RNA from biological sources is central to any investigation of gene expression and regulation studies, including those based on QRT-PCR. Traces of extraction components, DNA and protein contamination, and RNA degradation can dramatically affect the quality of QRT-PCR data. Therefore, we highly recommend starting with high quality, intact, DNA-free RNA.

Because ribonucleases are found in virtually all tissues and are often the leading cause of RNA degradation during RNA isolation, they represent a major challenge in isolating high-quality RNA. To avoid degradation caused by ribonucleases, RNA isolation protocols should involve cellular lysis tissue disruption with guanidine isothiocyanate, a strong protein denaturant. Complete lysis and homogenization is important in order to ensure that ribonuclease activity is eliminated. Additionally, proper handling of tissue prior to homogenization and RNA isolation, as well as using sterile RNase-free reagents and plasticware, will ensure recovery of full-length, intact RNA.

Total RNA vs. mRNA

Total RNA includes the full complement of RNA: mRNA, miRNA, tRNA, and rRNA. Messenger RNA (mRNA) constitutes 1–5% of the total RNA depending on a variety of factors (e.g., tissue type, disease state, etc.). Whether to use total RNA or mRNA is typically dependent on the preferences of the researcher. Several publications and a majority of microarray researchers support the use of total RNA to achieve quality gene expression data.^{1–5}

mRNA can be difficult to isolate, depending on tissue type, and mRNA isolation techniques produce very low yields. However, using mRNA can increase detection sensitivity. (Note: Histone mRNAs do not contain polyA tails, therefore when using a poly-T isolation method, these RNAs will be lost and the expression profiles of genes associated with histones will be compromised.)

Measuring RNA Quality

Absorbance

RNA purity can be measured using a spectrophotometer by determining the ratio of absorbance at 260 nm to the absorbance at 280 nm (A_{260}/A_{280}). Using a low salt buffer (e.g., water or 10 mM Tris) at neutral pH ensures the most accurate results. High salt concentrations and low pH conditions lower the A_{260}/A_{280} ratio and reduce the sensitivity to protein contamination.⁶ Pure DNA has an A_{260}/A_{280} ratio of 1.8–2.0 in 10 mM Tris at pH 8.5. Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1 in 10 mM Tris at pH 7.5. Contamination by phenol or urea will show absorbance at 230 nm or 270 nm, respectively. Protein contaminants have a high absorbance at 280 nm and therefore produce a low A_{260}/A_{280} ratio. Absorbance at 325 nm indicates contamination by particulates (e.g., from dirty cuvettes).

Gel Electrophoresis

The most common technique used to assess RNA quality is gel electrophoresis, which allows you to visualize discrete intact ribosomal bands and determine the degree of RNA degradation. Ribosomal RNA bands should be sharp with no smearing, and the ratio of 28S to 18S RNA should be 2:1. Figure 11 illustrates a 2:1 ratio of 28S:18S rRNA bands with virtually no degradation.

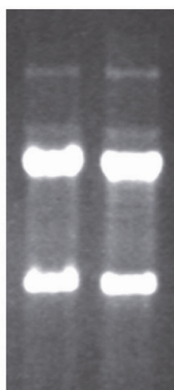


Figure 11

Gel electrophoresis analysis of a high-quality preparation of purified total RNA.

High-quality total RNA is indicated by sharp bands corresponding to 28S and 18S rRNA, at a ratio of approximately 2:1.

RNA Quality Analysis using the Agilent 2100 Bioanalyzer

According to MIQE recommendations (minimum information for publication of quantitative real-time PCR experiments), the assessment of RNA integrity is critical to ensure the relevance, accuracy, correct interpretation and repeatability of a QPCR experiment. The Agilent 2100 Bioanalyzer, with its RNA kit portfolio, has become the industry standard in RNA quality control tools for ensuring a RNA sample is of the highest integrity. The instrument uses electrophoretic separation on microfabricated chips to determine the quality of total RNA in a quantitative manner.

The output analysis provides the user with an image of the RNA fragments including the 28S and 18S bands, similar to gel electrophoresis. Additionally, it calculates the RNA integrity number (RIN) using a specific algorithm embedded in the Bioanalyzer software. The instrument software uses the entire electrophoretic trace (all regions and peaks) to calculate the integrity of samples (RIN) using a scale of 1 to 10, with 1 being the most degraded and 10 being the most intact.⁷ Figure 12 shows an electrophoretic trace of total RNA isolated using the Stratagene Absolutely RNA Kit, generated from the Agilent 2100 Bioanalyzer.

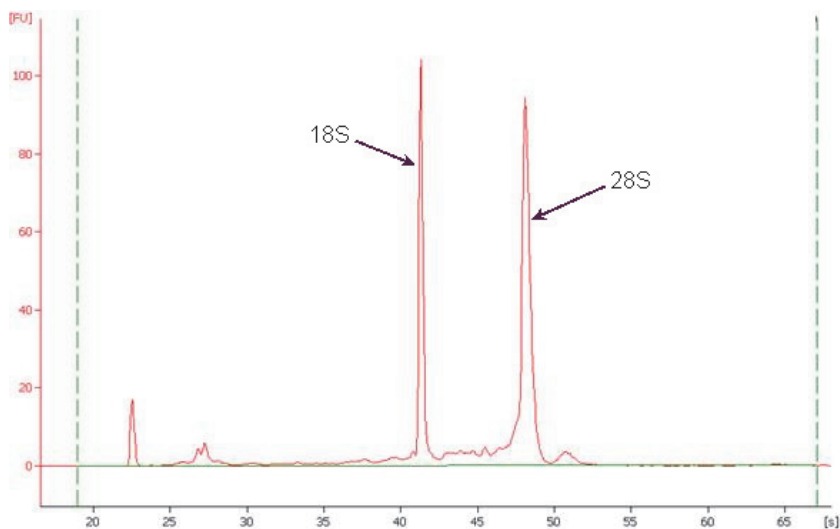


Figure 12
Agilent Bioanalyzer analysis of purified total RNA. The RNA is intact and undegraded, indicated by sharp peaks for the 28S and 18S ribosomal bands and a RNA Integrity Number of 9.6.

In addition to total RNA or mRNA analysis, the Agilent 2100 Bioanalyzer provides analysis to resolve small nucleic acids in the range of 6 to 150 nucleotides (see Figure 13). By consuming only 1 μ l of sample, even picogram amounts of purified small RNAs can be measured reproducibly and comparably within 30 minutes. After measuring the quality of total RNA extract by the RNA Nano or Pico kits, the high resolution of the Small RNA assay offers a detailed view on the 6–150 nucleotide range including miRNA content.

Measuring mRNA Integrity using QPCR

The best method to determine if messenger RNA is intact is to determine the ratio between 5'- and 3'-ends of a candidate gene using QRT-PCR analysis. Using this approach, first strand cDNA is synthesized using oligo dT primers, and the cDNA is used as template in two separate QPCR reactions with amplicons detecting either 5'- or 3'-proximal sequences.⁸

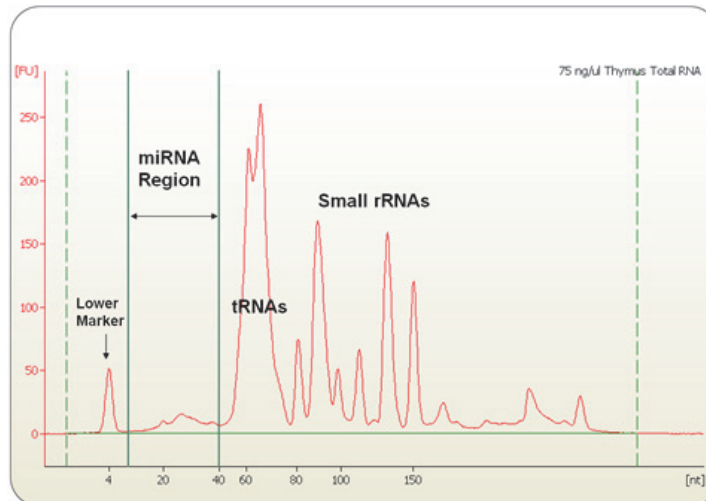


Figure 13

Electropherogram of total RNA sample showing the small RNA content of 75ng/μl Thymus total RNA (miRNA, tRNA, etc.). The miRNA area between 6 and 40 nucleotides is delineated by two vertical lines and the small RNA area by two dashed lines.

Storage of RNA

Total RNA may be stored at either -20°C or -70°C in double-distilled water. Poly(A)⁺ RNA (i.e., mRNA) should be stored at -70°C . Both total RNA and mRNA can be stored precipitated in either ethanol or isopropanol, depending on the extraction protocol used. It is best to store RNA in multiple small aliquots to prevent repeated freeze-thaw cycles which will degrade the RNA.

RNA from Paraffin-Embedded Tissues

The histopathology archive represents a vast, well-characterized source of specimens covering virtually every disease and is available for molecular biological investigation. This archive of formalin-fixed, paraffin-embedded (FFPE) tissue is becoming widely used for molecular genetic analysis. Archival specimens have become a source of material for extensive analysis of mRNA expression utilizing DNA microarrays, real-time QRT-PCR, and *in situ* hybridization and amplification techniques.¹⁰⁻¹³ However, the chemical nature of fixatives and fixation time can have significant effect on the ability to extract RNA from FFPE tissues. The process of formalin fixation also affects the structure of RNA molecules, resulting in the addition of monomethylol (CH_2OH) groups onto each RNA base at various rates, with an additional dimerization of adenine groups by methylene bridging.^{14, 15} These chemical modifications prevent RNA and DNA molecules from being amplified in subsequent analysis and therefore compromise accuracy. In addition, RNA extracted from FFPE tissues can be significantly degraded, which presents another challenge.

Important Considerations for using FFPE RNA

When isolating RNA from FFPE tissues, it is important to select appropriate reagents that work reliably, remove any chemical modifications imparted during the fixation process, and deliver high yields of pure, intact, DNA-free RNA. The Stratagene Absolutely RNA FFPE Kit offers reliable RNA preparation that removes the monomethylol groups from the RNA molecules, thereby ensuring high yields of useable RNA for QRT-PCR.

RNA isolated from FFPE tissue samples is often severely degraded, with most RNA fragments ranging from 100 to 400 nucleotides in length. It is important to design appropriate gene specific primers to amplify targets ≤ 100 bp to ensure accurate amplification and detection in QRT-PCR.

There can be a high degree of variability between blocks of tissue, even within 10-micron sections of paraffin tissue, due to differences in tissue harvest protocols, the nature of the tissue, fixation procedures, or the age or storage conditions of the archived material. This can result in a high degree of variability in QRT-PCR. To control for this variability, we suggest using a reference RNA, which can be used in two ways. First, the reference RNA can be used as a calibrator for relative quantification. The reference allows you to determine the relative quantity of gene expression levels of a normalizer gene and the gene of interest relative to the reference RNA. Second, because FFPE tissue RNA is precious, the reference RNA is an excellent source for assay optimization so that precious FFPE RNA is not wasted.

Using Cell Lysates in Real-Time QPCR

Nucleic acid purification can be tedious and time consuming, and can lead to the loss of precious RNA molecules, particularly of low-abundance messages, resulting in inaccurate gene expression measurements. Using cell lysates directly in QRT-PCR amplification circumvents RNA loss and degradation, and saves time. Additionally, using cell lysates such as those prepared using the Stratagene SideStep II Lysis and Stabilization Buffer offers the flexibility to lyse as many as 1×10^6 cells, or as few as 100 cells, and proceed directly to QRT-PCR.

Important Considerations for Using Cell Lysates

Primer Design: For QRT-PCR experiments, primers should be designed to prevent amplification of potentially contaminating genomic DNA. One approach is to include a primer that spans an exon-exon boundary in the target mRNA. This primer will not bind to genomic DNA sequences, where an intron interrupts the primer binding site. A second approach is to use primers that flank a large intron. Using this approach, a small amplicon (~ 150 bp) is amplified from the intron-less cDNA, but amplification of the large intron-containing genomic DNA amplicon does not occur under the

cycling conditions used. In all cases, whenever possible, design primers that avoid regions of secondary structure in the mRNA.

Cell Density: We recommend not exceeding 100 cells or 1 μl of undiluted lysate in a one-step QRT-PCR amplification. Typically, lysates prepared at 10^4 cells/ μl are serially diluted prior to their addition to one-step QRT-PCR reactions. For a cDNA synthesis reaction, up to 1 μl of lysate can be added, provided that less than 200 cell equivalents are added. For QPCR, add 2 μl (≤ 20 cell equivalents) of the cDNA synthesis reaction. Determining the lower limit for SideStep lysate addition to the cDNA synthesis reaction will be influenced by the abundance of the target and the sensitivity of the assay system used. Typically, a standard curve may be generated using at least four 2-fold serial dilutions of cell lysate in the range from 200 to 3.125 cell equivalents per μl (for example, for many cell lines/targets, a suitable standard curve would include 100, 50, 25, and 12.5 cell equivalents).

Reverse Transcription Considerations

The importance of the reverse transcription (RT) step in QRT-PCR is often overlooked, but it is worth bearing in mind that this step is critical for accurate and sensitive quantitation of RNA. While the QPCR itself is highly reproducible, the RT step is less so and appropriate optimization of the RT reaction is therefore advisable.

Converting RNA to cDNA with the AffinityScript QPCR cDNA Synthesis Kit

Stratagene AffinityScript Multiple Temperature Reverse Transcriptase is engineered to be highly thermostable, allowing reverse transcription across a range of temperatures. AffinityScript Multiple Temperature RT is ideal for challenging applications such as low RNA input amounts and low copy genes which demand the highest sensitivity. It has also been shown to effectively synthesize cDNA in the presence of strong inhibitors⁹. AffinityScript QPCR cDNA Synthesis Kit can be used for 1st strand cDNA synthesis in a 2-step protocol providing flexibility across a wide range of temperatures. AffinityScript RT minimizes the potential for primer-dimer formation or other non-specific PCR products and delivers the most reproducible results.

Reverse Transcription Priming

The choice of RT primer can have a marked effect on calculated levels of RNA expression. Priming strategies for the RT reaction fall into three categories: oligo(dT) priming, random priming or gene-specific priming.

Oligo(dT) Priming

As the name suggests, oligo(dT) primers are oligonucleotides comprised of thymine bases and are approximately 15 to 20 bases in length. Oligo(dT) primers are often supplied in RT kits, including the Stratagene AffinityScript QPCR cDNA Synthesis Kit. Oligo(dT) primers anneal to polyA sequences at the 3' end of mRNA transcripts and initiate synthesis of the cDNA strand from this position. Compared with random primers, oligo(dT) priming reduces complexity since cDNA synthesis will only occur on RNA transcripts having a polyA sequence. For example, the mammalian cellular RNA profile is approximately 80–85% ribosomal RNA (rRNA), 10–15% transfer RNA (tRNA) and 1–5% messenger RNA (mRNA). Although there is evidence that some organisms have polyadenylated rRNA, polyadenylation occurs primarily on mRNA. Consequently oligo(dT) strategies will tend to target the mRNA fraction. The downside of this specificity is, of course, that any transcripts lacking a polyA tail – for example, viral RNAs – will not be reverse-transcribed.

Oligo(dT) priming is considered by some to be the best method for achieving an accurate cDNA representation of the mRNA fraction. It also provides the opportunity to generate full length cDNA since

extension always proceeds from the 3' end of the RNA. However, the 3' dependence of priming can also be a limitation. As an RT enzyme extends away from the priming site, it may at any point detach from the RNA template strand resulting in a truncated cDNA. This tendency will be exacerbated by regions of secondary structure and long 3' untranslated regions. This results in a 3' bias in the resulting cDNA synthesis, with 5' regions under-represented. This can be an issue if the subsequent QPCR assay has been designed to detect the extreme 5'-end of a long RNA. The effect of long 3' polyA regions can be reduced by the use of anchored dT oligos which feature a G, C, or A as the final 3' base. This forces the oligo to anneal at the extreme 5' end of the polyA region and cDNA extension progresses from there. It should be noted that the fact that the RT priming site is fixed at the 3' end of the transcript also makes oligo(dT) priming a poor choice for fragmented RNA samples, such as those derived from archival sources.

Random Priming

Random primers are usually hexamers or nonamers which are supplied as a component of RT kits such as the Stratagene AffinityScript QPCR cDNA Synthesis Kit. The supplied random primers are a mixture of random sequences of a specified length consisting of the four DNA bases. Thus, for random hexamers, 4,096 combinations of A, T, G, and C are possible. For random nonamers, 262,144 different sequences are possible. During RT priming, random primers will anneal on any complementary site on an RNA molecule and cDNA strand synthesis will commence from this point.

An RNA transcript may have multiple priming sites along its length which correspond to various random primer sequences present. Consequently, random priming is not specific to one end of a transcript, unlike oligo(dT) priming described above. This removes the 3' bias problem, but random priming can result in truncated cDNA strands synthesized from primers annealing nearer to the 5' end of a transcript. Since random priming does not require the presence of a polyA sequence, it allows synthesis of cDNA for all RNA species, not just mRNA. This is an advantage when studying transcripts lacking a polyA tail, but does mean that the majority of cDNA yield is from rRNA. Thus, the RT reaction is not focused on the mRNA fraction and complexity is not reduced, unlike oligo(dT) priming. This could be problematic if the target transcript is of low abundance as this can result in disproportionate cDNA yield. In addition, this is likely to reduce the RNA concentration range over which cDNA yield is linear.

To its advantage, random priming may be less susceptible to secondary structure and fragmented RNA molecules. Multiple priming sites means it is more likely that a random RT primer will anneal in close proximity to a QPCR assay region wherever that region may be.

Combined Oligo(dT)/Random Priming

In some cases, using a combination of random primers and oligo(dT) primers in the RT reaction may result in higher RT efficiency and improved yield of cDNA. The precise stoichiometric ratio of random-to-dT oligonucleotides depends on the specific target transcripts and must be determined empirically.

Gene-Specific Priming

Gene-specific primers are designed by the researcher to specifically target the transcript of interest. The major advantage is a considerable reduction in complexity since the entire resources of the RT reaction are then focused on cDNA synthesis of one transcript. This has been shown to result in improved cDNA yields from low abundance transcripts and so may be particularly useful to those studying low-expressing genes. Conversely, the resultant cDNA prep can then only be used to study that one transcript and cannot be revisited to determine expression levels from other genes. This will introduce variability across studies examining multiple target genes since each gene-specific RT reaction is independent.

Given that gene-specific priming relies on a single priming site, this method is also susceptible to secondary structure. RT reactions are run at relatively low temperatures and significant secondary structure within the RT primer binding site can adversely affect the RT efficiency by inhibiting access of the RT primer to the template RNA molecule. Tools are available which aim to predict RNA folding at specified temperatures (e.g., the mfold program at www.bioinfo.rpi.edu/~zukerm/rna/). Such tools can only attempt to model a very complex process but may provide some indication of less problematic regions to which primers can be designed.

The gene-specific primer used may be one of the QPCR primers. If this is the approach taken, the amount of primer included in the reaction must be optimized. This will facilitate a robust RT reaction and ensure that sufficient primer is available for the downstream QPCR performance, particularly in one-step RT-PCR protocols.

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

Quantitative reverse transcription polymerase chain reaction (QRT-PCR) can be performed as either a one-step or a two-step procedure. In general, a one-step QRT-PCR is well-suited for high-throughput screening, especially when the sample material is not limited. A two-step format allows for amplification of multiple genes when the quantity of RNA template available is limited, the capability for more extensive optimization, and the ability to archive the 1st strand cDNA for future analysis. However, the selection of a one- or two-step procedure will ultimately be decided by the experimental objective.

One-step

One-step QRT-PCR is a quick and easy single tube reaction that converts RNA into cDNA, followed by QPCR amplification using gene-specific primers. It is defined by the fact that this reverse transcription and amplification are performed in a single reaction tube during a continuous thermal profile. See Figure 14 for a sample thermal profile for a one-step QRT-PCR.

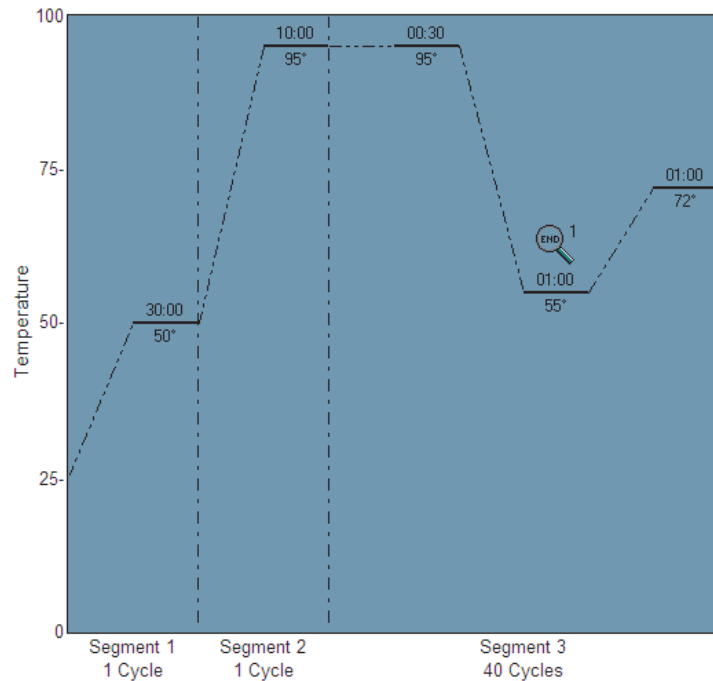


Figure 14
Typical thermal profile for one-step QRT-PCR. Segment 1 directs cDNA synthesis by reverse transcriptase using a 30-minute, 50°C synthesis protocol; Segment 2 directs a 95°C incubation, used for QPCR enzyme activation; Segment 3 directs 40 cycles of QPCR amplification.

This single-tube protocol has the advantage that additional manipulation of the generated cDNA is not required, thus the potential for contamination is greatly reduced. Additionally, since fewer pipetting steps are required, the one-step QRT-PCR is faster and reduces the potential for human error.

The disadvantage of using the one-step protocol is the inability to troubleshoot and optimize the reverse transcription reaction independent of the polymerase chain reaction, since the RT step and the QPCR step need to be optimized as a whole. The one-step reaction requires the same conditions for both enzymes, which may not be optimal for your particular assay. As an example, the gene-specific primers you have chosen may work well for QPCR amplification, but are not very effective at RT priming, resulting in a poorly performing assay.

As mentioned, one-step QRT-PCR is well suited for high-throughput screening especially when the sample material is not limited. However, these provisions are somewhat subjective. To illustrate why your experimental objective will ultimately decide your selection, consider the following example: Assume you need to screen 20 potentially transfected clones for the presence, absence, or relative expression of a transfected gene. The cell colonies are small (<200 cells) but will require a significant amount of time and resources to expand and characterize. This application would not be considered high-throughput and the sample material is quite limited. However, the experimental objective is simply to identify good candidate clones for future investigation. The time and expense of generating 20 separate 1st stand cDNA samples for archival purposes using a two-step QRT-PCR protocol is simply unnecessary, and hence one-step QRT-PCR is the fastest and simplest way to fulfill your experimental goal.

Two-step

As the name implies, two-step QRT-PCR is comprised of two reactions: one reaction for the conversion of RNA to cDNA and a separate second reaction for QPCR amplification of your gene of interest. See Figure 15 for sample thermal profiles for reverse transcription (RT) and QPCR.

The two-step approach allows for the use of optimal buffer conditions for the reverse transcriptase and for the DNA polymerase individually. cDNA can be generated using random primers, oligo dT primers, or a combination of both. Using this method, cDNA from a single RT reaction can be aliquotted into several different QPCR reactions to analyze several genes. A portion of the cDNA can also be archived for subsequent experiments. The most prominent advantages of the two-step QRT-PCR is that the maximum amount of information can be obtained from a single RT reaction because most RT reactions will yield enough cDNA to perform at least 20 QPCR reactions.

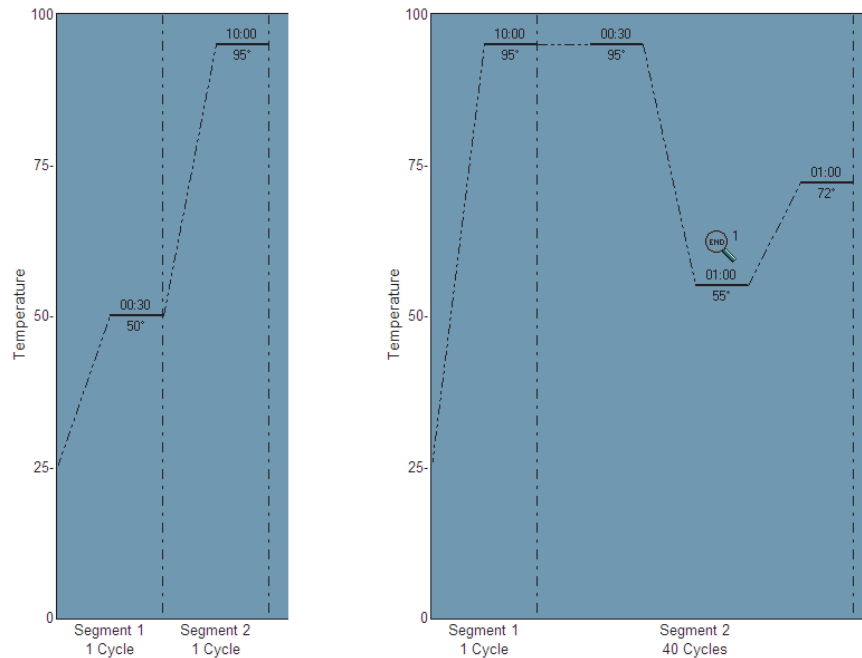


Figure 15

Typical set of thermal profiles for two-step QRT-PCR. Segment 1, in the first panel, directs cDNA synthesis by reverse transcriptase using a 30-minute, 42°C synthesis protocol. The right panel represents a separate thermal profile, in which Segment 1 directs a 95°C incubation, used for QPCR enzyme activation, and Segment 2 directs 40 cycles of QPCR amplification.

In the previous example of clone screening, we assumed that the objective was simply to screen for transfectants. If, however, you are checking for receptor status and there may or may not be a need to check for additional genes in the future, then the two-step procedure will provide archival cDNA, making this the preferred method.

Two-step QRT-PCR is much more flexible than the one-step protocol, but requires additional sample handling which takes time and increases the possibility of contamination and sample loss. It is also important to note that if you wish to employ a dUTP/UNG decontamination strategy to prevent carry-over contamination between experiments, then a two-step QRT-PCR is required so that the Uracil N-Glycosylase treatment can be performed prior to PCR amplification. The dUTP/UNG decontamination strategy could not be used in a one-step protocol because the UNG enzyme must be added to the reaction following the reverse transcription reaction in order to prevent it from digesting the template RNA.

Controls for Quantitative PCR Experiments

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 gene expression 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.

The specific controls that are needed will vary according to the experiment type, but there are certain controls that should be included in every run, such as No Template Controls (NTC). The two primary types of controls are positive controls and negative controls. Positive control samples should always show amplification, and if they fail to show expected amplification, it indicates some sort of problem with the assay or reagents. Negative controls should not show any amplification, and if an increase in fluorescence is seen, it indicates contamination, non-specific PCR product formation, or non-specific probe degradation. For qualitative experiments in particular, if your experimental parameters require that you avoid false positives you should always run negative controls, and if you need to ensure that you avoid false negatives you should always run positive controls.

Positive Controls

Positive controls will assist in identifying false negatives that might occur due to sample template 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 positive controls in all the amplification reactions validates the absence of detection of the target of interest as a true negative sample. In reactions where no positive control is run, if all your unknown samples come up negative, it is impossible to tell if these are true negatives or if some problem in your reagents caused amplification to fail.

Positive controls can be either completely different samples that are not related to your experimental samples (referred to here as exogenous positive controls), or they can be separate targets amplified from the same sample of nucleic acid (referred to here as endogenous positive controls). Exactly what type of positive control is used will control for different types or reaction failure.

An exogenous positive control is a source of the template that is known to contain the target of interest but which is independent of your experimental samples. Examples would be plasmid containing the gene of interest, in vitro RNA transcripts, previous PCR products, Human or Mouse QPCR Reference Total RNA, or DNA or RNA isolated from organisms known to contain or express the target of interest. Controls of this sort are very useful for just verifying that the amplification reaction is working and the

fluorescence signal is being generated and detected normally. They will not reveal if there are PCR inhibitors that are carried along with the nucleic acid in any of your unknown samples or if there is degradation of the nucleic acid in some samples.

The use of an endogenous positive control involves amplifying a second target from the same sample as the gene of interest. This can either be run in multiplex or in a separate tube containing an aliquot of the same template sample. If a normalizer gene is being used in the experiment (detailed below in the section *Sample Normalization using Reference Genes*) this will serve as a positive control of this sort. Any gene that is known to be present in the experimental sample (or any RNA known to be expressed, in the case of QRT-PCR) will work as a normalizer-type of positive control. Endogenous positive controls will alert you to general problems with the buffer or polymerase just as a positive control sample will, but since they must use a different primer/probe set than the gene of interest, they cannot be used to detect problems resulting from those reaction components.

Controls of the sort described so far will only provide you with a yes/no answer on whether amplification is taking place, and will not detect an RT or PCR inhibitor present in the sample preparation that is causing a low level of inhibition, and delaying Ct values without preventing amplification. Another type of positive control is an exogenous target that is spiked into the template sample, such as the Stratagene Alien QRT-PCR Inhibitor Alert target RNA. The Alien RNA Transcript is spiked into the purified RNA sample prior to reverse transcription and is amplified using Alien transcript-specific primers. QRT-PCR of the Alien control target is also run alone, in a separate tube, alongside the samples of interest. Measuring amplification of the control target side by side in both samples can be used to detect PCR inhibitors that influence the reaction Cts without preventing amplification outright. Figure 16 shows detection of QRT-PCR inhibition by guanidine using the Alien QRT-PCR inhibitor alert kit. This sort of positive control does not control for RNA isolation variances, which is controlled for using a normalizer gene.

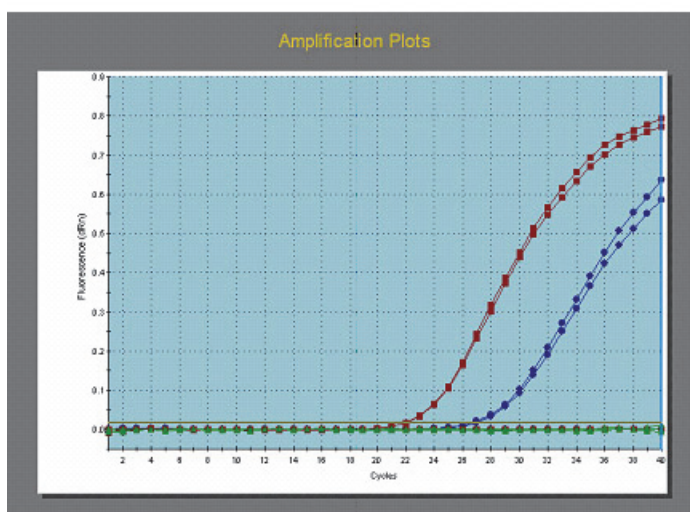
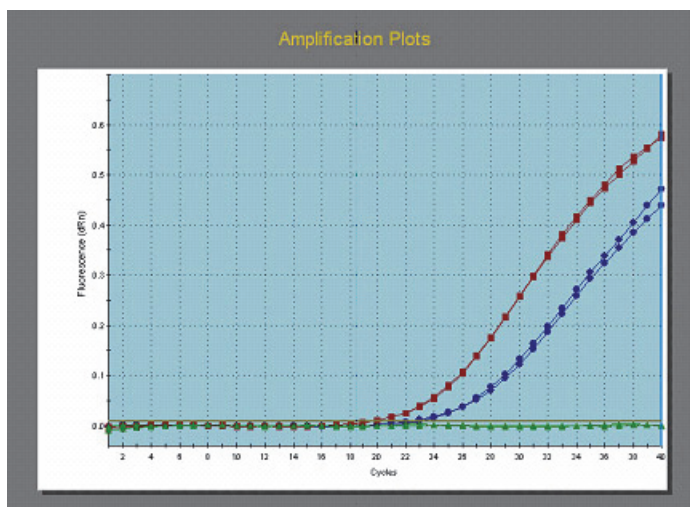


Figure 16
 Detection of inhibition by 10 μM guanidine using the Stratagene Alien QRT-PCR Inhibitor Alert. The upper panel shows amplification of the GAPDH target from human total RNA (50 ng Stratagene QPCR Reference Total RNA, Human) in the absence and presence of 10 μM guanidine (final concentration in the QRT-PCR reaction). The lower panel shows amplification of the Alien RNA target from samples containing a mixture of 10^5 copies of Alien RNA transcript and 50 ng human total RNA, in the absence and presence of 10 μM guanidine (final concentration in the QRT-PCR reaction). In the presence of 10 μM guanidine, a delay of 3 Ct values was observed for the GAPDH target, and a delay of 5 Ct values was observed for the Alien RNA target. Experiments were performed using the Stratagene Brilliant SYBR[®] Green 1-Step QRT-PCR Master Mix.

Positive controls can be used to provide consistent positive reference data points in a given experiment. A standard curve can be generated by simply performing a dilution series on the positive control material. If a standard curve of the gene of interest is performed in a given run and the standard template concentrations span the range of expected unknown sample concentrations, the MxPro 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 the Stratagene QPCR Human or Mouse Reference Total RNA. As described later,

standard curves can also be used to calculate the exact efficiency of amplification in your QPCR reaction. A decreased amplification efficiency or loss of linearity in the standard curve may indicate that a problem has developed with your reagents or that there is a contaminating inhibitor. In this case the standard curve will provide a useful indicator of whether or not the experimental data is valid, and to what degree it can be compared to samples performed in separate runs.

Negative Controls

Negative controls are often overlooked in experimental design, but they are one of the most important components of a QPCR assay. A negative control will typically be missing one of the components essential for the reaction to proceed, and thus it is expected to yield no shift in fluorescence. Depending on the type of negative control that is run, you can test for problems that might occur in the reaction at multiple steps. In addition to assay-specific negative controls, which use template samples from sources that are expected not to contain the sequence of interest, other common types of negative controls are no template controls (NTC), no reverse transcriptase controls (No RT), no amplification controls (NAC), and no probe controls (NPC).

NTCs 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 cycles, and preferably more than ten cycles, from the Cts of your least concentrated samples. If the Cts of the NTCs are less than five cycles delayed compared to samples containing template, the Cts of those samples should not be considered accurate since whatever is causing the fluorescence shift in the NTC wells could also be affecting the fluorescence in the unknown wells.

No RT controls are samples that are run exactly as the other QRT-PCR reactions, except that the reverse transcriptase enzyme is omitted. No RT controls should show no amplification in the subsequent PCR step since DNA polymerase cannot amplify an RNA template. Amplification occurring in the No RT control wells indicates that there is contaminating DNA template in the reaction. If amplification is observed in the No RT controls and not in the NTC reactions, contaminating genomic DNA is most likely present in the RNA sample.

Another common negative control is no amplification controls (NAC) which includes all the reaction components except for the DNA polymerase. This is useful if you suspect that an increase in fluorescence in your reaction is due to something other than actual amplification (e.g., degradation of your probe).

No probe controls (NPCs) are useful to test for background fluorescence signal, possibly due to contamination, but are rarely

used. When using a probe-based chemistry like TaqMan or Molecular Beacons, the fluorescence signal is generated by the fluorescent dye molecule on the probe. In reactions lacking probe, you should see the true background fluorescence level.

When performing qualitative PCR (i.e., generating a positive or negative determination of 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. The MxPro software bases a positive or negative call result on a p-value test, and a minimum of three negative data points are required to produce the population that the unknown wells will be compared to in order to determine if they are significantly amplified. 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. The first set of sample data is always the negative 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 user-specified confidence level, the well/dye is called as positive and signified with a plus sign (+). Otherwise, the well is called as negative (i.e., no difference detected) and signified with a minus sign (-).

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 and, therefore, the fluorescence from this dye should be constant throughout the amplification reaction. Because the concentration of the reference dye is identical in every well of the reaction, the fluorescence intensity for the reference dye should theoretically 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 aliquotting errors). This correction is not required, but if a reference dye is designated, it is performed automatically by the MxPro 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 Mx system.

Approaches to Normalizing Gene Expression

When performing gene expression experiments, there are two categories of events that influence the results: (1) actual biological differences between samples and (2) variability introduced by the techniques used. The goal of expression studies is to quantify the level of biological change and either eliminate or compensate for any effects due to technique. There are several ways to discriminate the biological signal from the discrepancies resulting from the techniques applied, and each method has advantages and disadvantages. It is important to choose the most appropriate method for your experiment, and when possible to use multiple levels of control in order to assure accurate results.

Variability in Starting Cell Number

In order to control for variability in cell number used in the preparation of each individual sample, the most direct method is to measure the quantity of cells used for the control and for the treated sample so the results can be expressed as quantity per cell. If you are working with cultured cells, cell number can be quantified with a hemocytometer. Cell number can also generally be determined when working with tissue isolated via laser capture microdissection (LCM), and can often be estimated based on the mass of the tissue sample. In some tissues, especially abnormal tissues such as from cancerous cells, exact cell number can be difficult to quantify.

Another method to control for variability in cell number is to quantify the starting template and express the results as fold variation per microgram of starting material. This method assumes equal efficiency of the reverse transcription for all samples and is often used in development studies. Starting concentrations of RNA can be quantified using RiboGreen or, if the concentration is sufficiently high, via absorbance spectrophotometry at OD₂₆₀. A technical note describing how to quantify RNA using RiboGreen in the Mx instrument can be found at www.genomics.agilent.com.

The DNA content of the cells can also be used to express the mRNA quantity results in terms of quantity of DNA. With this method you can either compare the mRNA quantity relative to the total DNA concentration or amplify one or more genomic targets alongside your RT-PCR reaction and compare the copy number of the mRNA target to the copy numbers of specific genomic DNA targets. Knowing the gene copy number per cell will allow you to express your mRNA level in terms of the cell number. This approach has gained more interest with the development of new products that allow for QPCR and QRT-PCR reactions to be done using cell lysate directly, without the purification step, thus removing the variation in the purification step from one sample to another (e.g., Stratagene SideStep Lysis Buffer). Kits of this sort avoid problems of correlation between DNA and RNA purification since the DNA and RNA are present in the same lysate without

any bias. If such a method is used, however, it is very important to design your RT-PCR primer sets to span exon-exon junctions to avoid amplification of genomic DNA. Like the previous methods mentioned, DNA quantity normalization does assume equal efficiency for the reverse transcription step.

Variability in the Reverse Transcription Reaction

One simple way to minimize the potential variation in the reverse transcription reaction is to perform three separate reverse transcriptions for each sample and pool the resulting RNA together prior to performing QPCR. If one of the three reactions is not as efficient as the others, the final results will be only slightly affected. If only one RT reaction is performed, the results are more sensitive to one inefficient reaction.

The reverse transcription efficiency can also be monitored using exogenous RNA spiked into the samples prior to the RT reaction (synthetic or *in vitro* transcribed RNA). The QPCR reaction is then designed to detect the exogenous sequence alongside the gene of interest, either in a separate or multiplex reaction. Since the same quantity of exogenous RNA is input into all samples, the same quantification results for each reaction are expected. Any difference in the results of this control from sample to sample would indicate a difference in the efficiency of the reaction between samples. We have developed “alien” RNA molecules (Alien QRT-PCR Inhibitor Alert) with a sequence that is not present in any available databases, which can be used to monitor the RT reaction efficiency without risking that the sequence will be homologous to any gene of interest.

Sample Normalization using Reference Genes

The most commonly used method to control for sample-to-sample variation that is not due to biological effects is based on the use of a reference gene. The reference gene is assumed to have equal levels of expression in each experimental sample.

In QRT-PCR experiments, especially those based on comparative quantification, it is important to include a normalizer gene (also referred to as 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 normalizer 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 commonly used housekeeping genes are GAPDH and β -actin, which are ubiquitously expressed, but there is evidence that their level of expression can vary considerably.^{16, 17} Alternative references like 18S or 28S rRNA have also been shown to be up- or down-regulated under different conditions¹⁷ 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 multiple references that have been empirically tested to be consistent across all experimental conditions in your assay. Initial data can be found in the literature or from microarray data. Software for normalizer validation can be found at www.gene-quantification.de/bestkeeper.html.

Because the expression level of the normalizer gene 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, RNA purification yield differences, 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. In order to minimize the error caused by minor fluctuations in expression of the normalizer gene, multiple normalizers may be used in a single experiment. This capability was added to the MxPro software with the release of version 4.1. When multiple normalizers are used in an experiment, the software normalizes the GOI expression levels by first normalizing the GOI quantity to each normalizer assay separately, and then calculating the geometric mean of all these normalized values. This mean value is then used for the Relative Quantity calculations. Figure 17 shows how to indicate on the Plate Setup screen of the MxPro software which assays to use as normalizers.

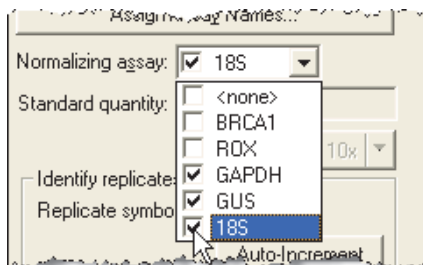


Figure 17
Selection of multiple normalizer assays in Plate Setup.

In addition to having unchanging expression levels, a normalizer also needs to have an expression level and an amplification efficiency that is similar to that of the GOI. During assay design, confirm that the amplification efficiency values are reproducible. If they are not, the normalization results cannot be considered reliable.

Any of the reference genes mentioned above (e.g., housekeeping genes, rRNA, etc.) 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 (e.g., an *in vitro* transcript) added in a defined amount to the extracted RNA. In this case, reference gene expression levels are no longer a concern, however RNA isolation variances must still be controlled for. For greatest control, endogenous and exogenous references can be combined in a single assay. Figure 18 describes the advantages of each type of normalizer for comparative quantification.

In order to validate the reference gene manually, extremely precise RNA quantification prior to the reverse transcription is necessary. Using the same starting quantity of total RNA (usually from 10–1000 ng), three distinct reverse transcription reactions are run and the products are pooled together. The QPCR reaction should then be performed in triplicate. The ideal reference gene should give the same Ct for every sample. A standard error of ± 0.5 Ct indicates that the level of resolution obtainable with this reference gene is approximately a 2-fold variation. A variation of this method has been proposed¹⁶ which reduces the effect of choosing a poor reference gene by using at least three different reference genes to generate a more stable normalization factor resulting from the geometric averaging of each individual reference gene. This method represents a solid approach for normalizing the results, however the main constraint of this method is the need to analyze at least three reference genes per sample, thus significantly increasing cost.

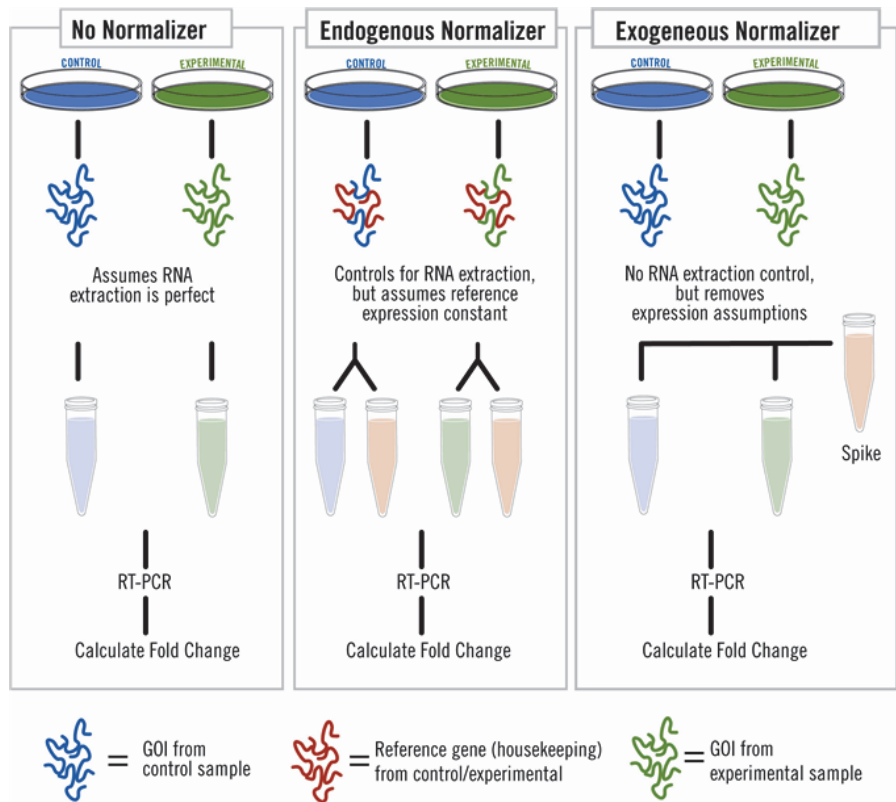


Figure 18
The use of exogenous and endogenous normalizers in QRT-PCR.

Assay Optimization

To ensure efficient and accurate quantification of the target template, QPCR assays should be optimized and validated for quantitative range and specificity. 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 (the recombinant plasmid must be linearized and purified), a purified PCR fragment, genomic DNA, a synthetic oligonucleotide, or a stock of cDNA from cell culture. When selecting a control template for assay optimization, the goal is to select a material that may be obtained in high abundance, that is most like the unknown samples you intend to analyze, and that contains the sequence of interest. If a linearized plasmid or oligonucleotide control template is used as the template, frequently this will require spiking in carrier material such as genomic DNA, yeast tRNA, or glycogen, to replicate sample complexity and avoid sample loss.

As an alternative, Stratagene offers a QPCR-specific universal reference RNA made from multiple cell lines that provides coverage of up to 85% of all genes, which can also be used as a template for optimization reactions. This is available for human and mouse systems.

Assays are most easily optimized by first evaluating the primer concentrations (forward and reverse) for a given template, across a range of concentrations. This requires the use of a standard curve, or a linear dilution series of starting material, to determine assay quality metrics. These metrics include QPCR efficiency, precision, sensitivity, and specificity, and can be assessed using a standard curve and SYBR Green I detection chemistry.

Following primer optimization, it may be necessary to further optimize the probe concentration (if the assay uses a probe-based detection chemistry) and the Mg^{2+} concentration. Completing the initial primer optimization step using SYBR Green I detection chemistry is generally sufficient to ensure an efficient reaction for other chemistries while conserving the probe to reduce cost.

Primer Optimization Guidelines

Depending on the QPCR chemistry being utilized for the assay, different ranges of primer concentrations can 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 are appropriate.

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 this entire range of primer concentrations. Starting out with a new primer set for a gene expression experiment, for example, one might try 300 nM of each forward and reverse primer, with a typical serial dilution. This would consist of five points of a five-fold serial dilution, starting with 100 ng of total RNA per reaction (or the equivalent cDNA amount). Inclusion of a negative control of just the primer (at each concentration) in the absence of template (NTC) will yield specificity information. A successful assay will have good linear Cts versus input amount of template, as indicated by the metrics described below, as well as a melt curve that predicts a single product in template positive samples, and a negative result for the NTC.

| | | Forward Primer [nM] | | | |
|---------------------|-----|---------------------|-----|-----|-----|
| | | 50 | 150 | 300 | 600 |
| Reverse Primer [nM] | 50 | | | | |
| | 150 | | | | |
| | 300 | | | | |
| | 600 | | | | |
| | | | | | |

Figure 19
Primer optimization matrix. For each primer pair, the Ct should be determined, and the dissociation curve should be analyzed to verify a single product for the template-containing samples and no product for the no-template controls (NTCs).

To demonstrate more complex primer optimization strategies, this guide will illustrate the optimization matrix for primer concentrations from 50–600 nM. These reactions should be run in duplicate with the appropriate negative controls for each concentration. Ideally, a middle concentration of template (5–10 ng RNA) should be used to assess each of these primer concentration pairs with SYBR Green I (Figure 19).

The ideal primer pair will yield the lowest average Ct, as well as a melt curve that shows a single product for the positive template sample and a negative result for the NTC.

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 independently 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.

Primer Optimization Data Analysis

Using the MxPro 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. It is recommended that a passive reference dye be used, as it tends to improve data quality. 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

During a 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 increases, fluorescence data are collected until the reaction reaches 95°C. The result is a plot of raw fluorescence data units, R, versus temperature (Figure 20). This view of the data may appear difficult to interpret at first, but the rapid linear decrease in fluorescence to background is where the major PCR product melts to its single stranded form.

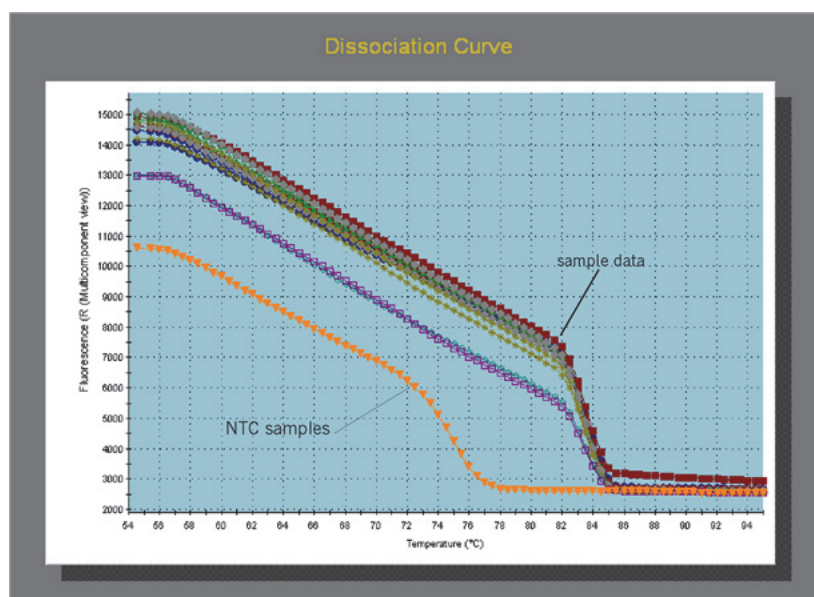


Figure 20
Raw fluorescence signal change plotted as a function of increasing temperature. The higher traces show a rapid melt between 82°C and 84°C. The NTC samples show a change in plot shape around 72°C.

Figure 21 shows the melt data as the negative first derivative of raw fluorescence, $R'(T)$, vs. temperature. This is the easier view to identify the T_m of each product, indicated by the peak in $R'(T)$. In analyzing the various combinations of primer in Figure 21, you can see one major peak at 78°C for the majority of the samples, but a few samples have peaks shifted to 79°C. The distinct melting peaks may indicate multiple PCR products in this assay. A fully optimized assay should contain only a single melt product. Banding by agarose gel analysis of the PCR product will determine if the appearance of more than one T_m indicates more than one product, or rather an artifact of the SYBR dye binding. Alternative splice forms, insertions, or deletions could create alternatively melting PCR products, from specific priming sites. The C_t s from such assays should be scrutinized, and no meaningful quantification should be based on these data.

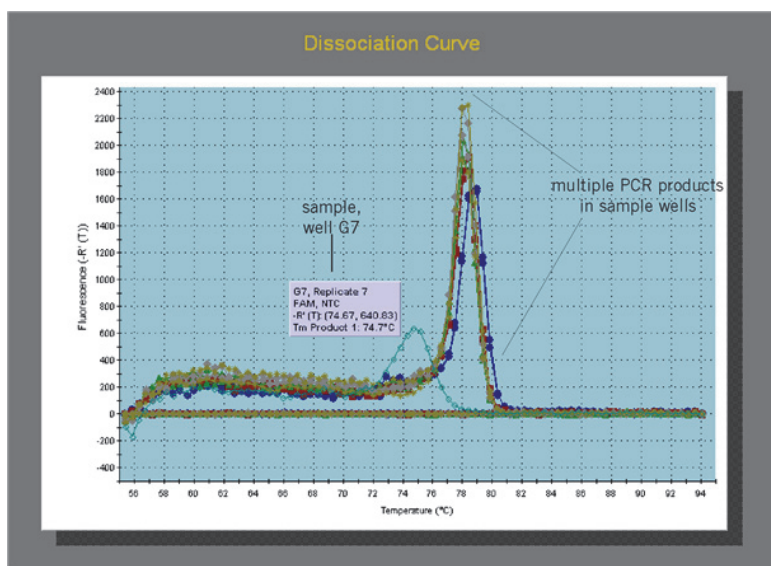


Figure 21

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.

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 22, 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. Slight, high cycle amplification and a small wide peak at a lower temperature by melt is an indication of primer-only amplification. 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 C_t s from these wells cannot be trusted as accurate.

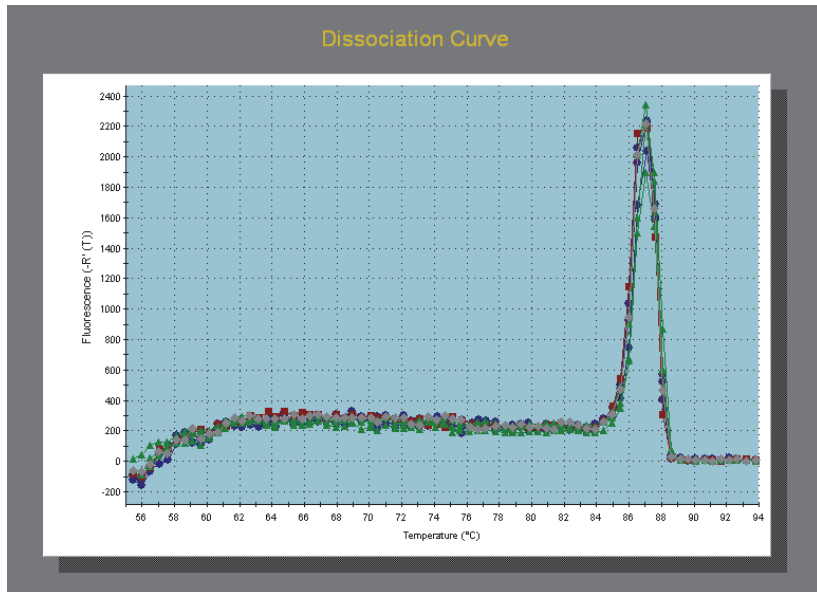


Figure 22
The first derivative of raw fluorescence plotted against an increase in temperature. The single melt peak at 86.5°C indicates a single PCR product is being amplified in these samples.

Choosing the Correct Primer Concentration

Figure 23 shows an example of a 50 nM–600 nM primer matrix in the presence of a linear hydrolysis probe. Based on Ct only, the primer concentration combination of 150:300 nM (forward:reverse) gives optimal but comparable results to other concentration pairs. It is best to get the lowest Ct values possible, but it is often as 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.

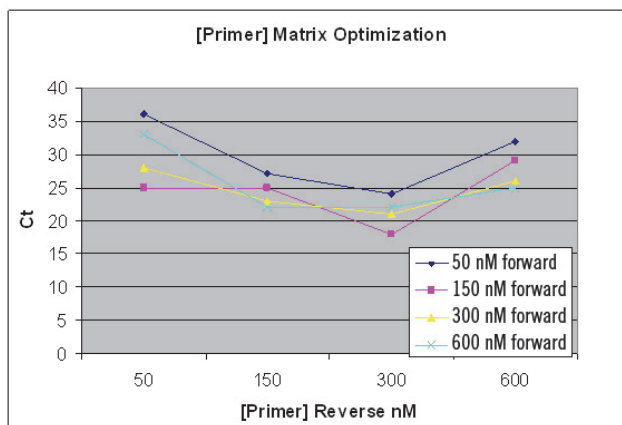


Figure 23
Analysis of primer optimization matrix data. Cts for each of the primer concentration pairs given in Figure 18 are plotted.

After analyzing the amplification plots, 150 nM forward primer concentration and 300 nM reverse primer concentration was chosen because this combination produced the lowest Ct and highest dRn. Additionally, when primers are individually optimized on the basis of concentration, there is a greater chance that they will function optimally in a multiplex format with other primers similarly optimized. Traditionally, primers could be optimized by changing annealing temperature in the assay thermal profile, however, this strategy is not appropriate for multiplex assays. Since a multiplex assay is run at one consistent thermal profile, it is unlikely that one optimal annealing temperature will be found where all primer concentrations function optimally. Thus, individual primer pair optimization on the basis of concentration using a constant thermal profile is favored.

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 using probe-based detection is nearly identical to that listed for the SYBR Green I procedure (see Figure 19). 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 like that of a SYBR Green I assay.

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 50–300 nM for linear hydrolysis probes and Molecular Beacons, while other QPCR chemistries, like Scorpions, might require concentrations as high as 500 nM.

Probe Optimization Data Analysis

Analysis of fluorescent probe optimization is similar to primer optimization analysis. Using the optimal primer concentrations, 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 24).

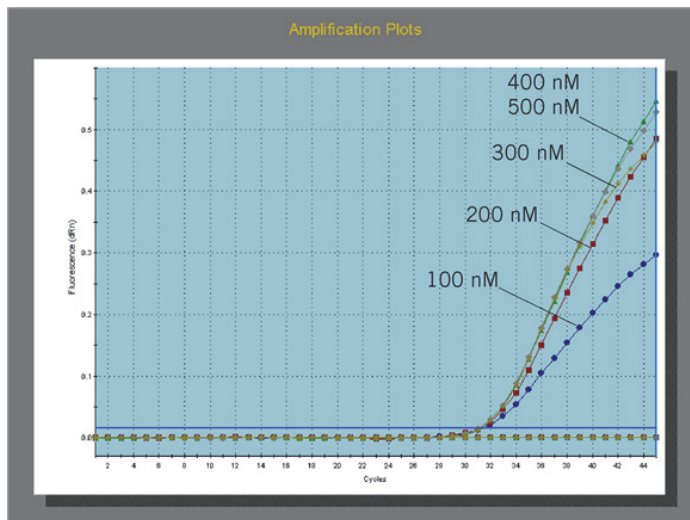


Figure 24

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 significantly with 100 nM probe, and decreased only slightly with 200 nM and 300 nM of probe. Based on these data, 200 nM of probe would be optimal.

Standard Curves for Analysis of QPCR Assay Performance

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, linear range of quantitation, 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). If the assay is intended to quantitate genomic DNA or copy number, such as with viral quantitation assays, a starting concentration of purified plasmid or PCR product in the 10–25 ng range is adequate. Be aware that not all points of a standard curve will conform to high data quality metrics as described below. Often, the high and low concentration points may not be in range, and elimination of these aberrant concentrations from analysis may result in a high quality assay, across a slightly lower linear quantitative range.

PCR Reaction Efficiency

The slope of the line of best fit drawn to the standard curve is used to determine reaction efficiency. The standard curve plots the log of starting template vs. PCR cycle number, and is generated by the MxPro software. A linear fit with a slope between approximately -3.1 and -3.6 , equivalent to a calculated 90–110% reaction efficiency, is typically acceptable for most applications requiring accurate quantification. If the amplification reaction is not efficient at the point being used to extrapolate back to the amount of starting material (usually the C_t is used for this purpose), then the calculated quantities may not be accurate. Since the PCR reaction is based on exponential amplification, if the efficiency of PCR amplification is 100%, the amount of total template is expected to double with each cycle. This assumption allows the reliable calculation of quantity from C_t , and thus $\sim 100\%$ QPCR efficiency needs to be assessed and verified prior to running valuable samples.

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. R_{sq} 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 R_{sq} will be 1.00. As the data fall further from the line, the R_{sq} decreases. As the R_{sq} decreases it is more difficult to determine the exact location of the standard curve plot thus decreasing the accuracy of quantification. An R_{sq} value >0.985 is acceptable for most assays.

Sensitivity

The slope and R_{sq} 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 (100% Efficiency), the R_{sq} is below 0.985, and the data points indicate an upward trend in the standard curve plot at the lower template concentrations, this may indicate the reaction is reaching the threshold of sensitivity, i.e., more cycles are required to amplify ever decreasing amounts of template. 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 interpolation from that part of the standard curve, or C_t s from that range should not be used in further analysis.

Standard Curve Examples

Figures 25 and 26 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 (96.8% Efficiency) which is well within the acceptable range, and an Rsq value of 1.0.

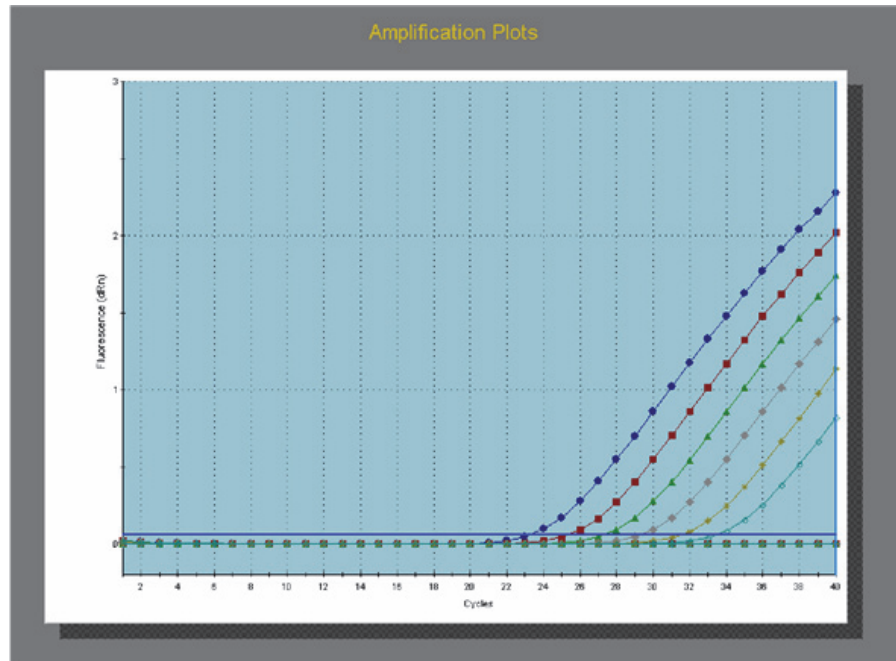


Figure 25
Amplification plots of standards in a four-fold dilution series over three orders of magnitude.

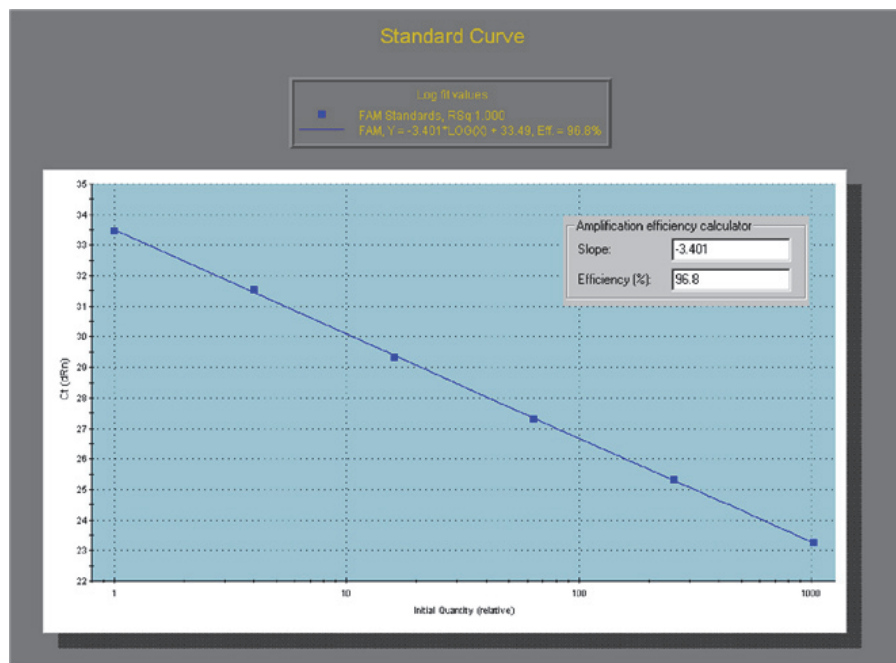


Figure 26
Standard curve generated with data from Figure 24, with slope and Rsq indicated.

Further Optimization

If the assay is still not performing well after the probe and primer concentrations are optimized, you can try altering the Mg^{2+} concentration in the reagents by adding extra $MgCl_2$ from the Stratagene core reagents kits. Increasing the Mg^{2+} concentration tends to favor hybridization, and therefore excessive Mg^{2+} can promote the formation of primer-dimers as well as template specific priming. While primer-dimers are not detected by sequence specific probe chemistries, they can cause the reactions to be inefficient and therefore less sensitive and detect fewer positive samples.

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 up to 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 $MgCl_2$. In SYBR Green I assays, primer-dimer formation and detection can contribute to the overall signal. Therefore, for SYBR Green I assays it is best to use 1.5 mM to 2.5 mM $MgCl_2$ to avoid excessive dimer formation.

When multiplexing, a low standard Mg^{2+} concentration can also be used to avoid cross reactivity of primers and probes.

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 allows the maximum amount of data to be generated from each assay. It does require more up front expenditure in the form of probes, and more time to design and optimize reactions that will all work together in the same tube, but the long term savings in reagent costs, plasticware, and 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, T_m , 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 suggests a lower probability that the two primer oligos will energetically favor hybridization, over

hybridization to the specific template. 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, in both singleplex and multiplex, to ensure that the reactions do work together. 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.

If the multiplex assays do not appear to be working well together, it 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 \times solution to a 1.5 \times solution. For this purpose, Stratagene offers a Brilliant Multiplex QPCR Master Mix that is optimized for the simultaneous amplification of multiple targets.

In assay situations such as a duplex for a gene of interest with a normalizing housekeeping transcript, where it is expected there may be very different template concentrations, a primer-limiting optimization can be performed. This is the case where one reaction in particular has a very early Ct or a very large dR compared to the other reactions, and has a tendency to dominate use of the reagents during the assay. One can decrease the primer concentration in that dominant reaction to prevent this from affecting the other reactions, or primer-limit the rate of product formation and reagent use. 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 produces 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, MgCl₂ 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 optimization steps outlined above 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. Immediately after the run completes, it is best to remove the sample tubes from the instrument and examine them. 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.

When first analyzing your amplification plots, you should follow these steps:

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

Raw Fluorescence Values

To begin, select the Analysis tab in the upper right corner, and the Analysis Selection/Setup button in the upper left corner. When first viewing the file, on the right side of the screen make sure The Amplification Based Threshold and Adaptive Baseline are turned on and the Moving Average is turned off. Also make sure that Replicates are being treated individually. Eventually you can turn the moving average back on and treat the replicates collectively if you wish, but these options can mask anomalies in the data or poor replicate uniformity, so on a first pass of the data it is best to turn them off. All wells that you want to be included in the analysis should also be highlighted on this page (usually the whole plate). On this same page in the upper right corner, you can select which data collection point will be used for the results if more than one collection point was set on the thermal profile.

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. For each reporter dye, verify that the baseline signal level for all the amplification plots is above 3000 RFU. If the baseline is below 3000, it will be running very close to background, which can cause excessive signal noise that will make accurate baseline correction difficult. Also, ensure the exponential phase of amplification is below 35,000 RFU. In a real-time QPCR reaction, it will generally not matter if the endpoint fluorescence is outside the linear range of detection, but the region of the curves that will determine the

Ct should definitely fall in the range of 3000–35,000 RFU in order to get accurate results. The signal is completely saturated with a fluorescence value at 65,536 fluorescence units and a signal level above 35,000 is approaching saturation and can cause problems (such as spiking, or poor signal uniformity). At signal levels below 3000 RFU the plots will be running too close to the background noise to give good results.

If a reference dye was run, make sure the reference dye profiles are all flat, do not contain spiking, and make sure the fluorescence values are not too high or too low. The signal level should be between 3000 RFU and 45,000 RFU. If the reference dye profiles contain noise or if the signal is saturated, you would be best off analyzing the data in the other dye channels in the non-normalized (dR) view as opposed to dRn.

Setting the Baseline

Fluorescence intensity data (Amplification plots) can be described as a two-component function: a linear component (background) and an exponential component that is actually due to PCR amplification. To isolate the exponential component, the linear contributions to fluorescence can be estimated and subtracted. This is what is referred to as “baseline correction.” It is a three-step process that must be carried out for each amplification plot before a common threshold can be established and the plots can be compared to one another.

1. Identify the range of cycles during which all contributions to fluorescence are strictly linear (no exponential increase in fluorescence).
2. Within this range of cycles, draw a best-fit straight line to the data (a function predicting the contribution of the linear components throughout the reaction).
3. Subtract the predicted background fluorescence indicated by this best-fit line from the measured fluorescence intensity at each cycle.

The resulting curve sets all the amplification plots to a common baseline with any shift in fluorescence above this corresponding to the change in fluorescence due to DNA amplification. The MxPro software performs steps 2 and 3 automatically when the amplification plots are viewed in the baseline corrected (dR) or normalized and baseline corrected (dRn) fluorescence views. However, there are a few options for determining which cycles to use to estimate the contribution from the background fluorescence in step 1.

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 fluorescence increase is found, the cycle prior to the increase is defined as the Baseline End Cycle.

As can be seen in Figure 27, 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.

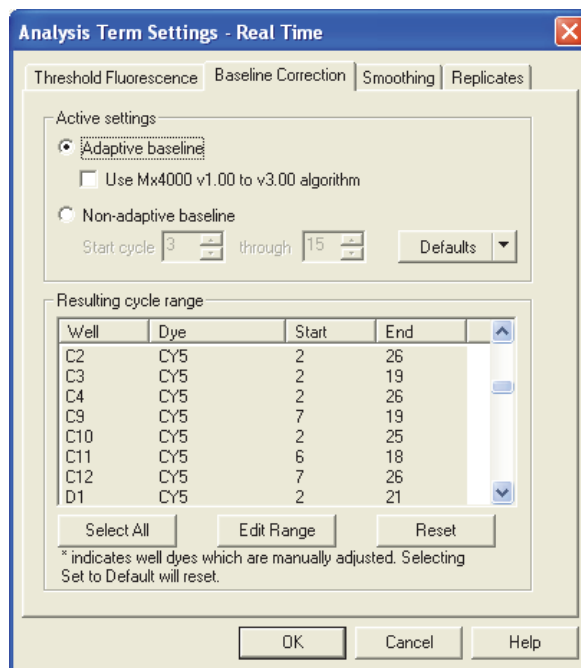


Figure 27
A view of the baseline correction screen in the Analysis Term Settings window. When the Adaptive Baseline is turned on, the software will set the baseline cycle range independently for each amplification plot.

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 cross the threshold at a different cycle than they would have if the baseline had been set more accurately. 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.

When first viewing the amplification plots, it is recommended to use the Adaptive Baseline. Change the fluorescence to dR (baseline corrected fluorescence) and verify that the baselines for the reporter dyes remain flat and the curves are not tilted over. If the curves look abnormal in the dR view, it may be necessary to manually adjust the baseline range for those profiles. To do this, select Options on the Menu bar and choose Analysis Term Settings. On the Analysis Term Settings Window, select the Baseline Correction tab, highlight the well and dye you want to adjust, and push the Edit Range Button. You will then need to enter the cycle range in which this profile is flat, above the tailing and below where it starts to ramp up. After adjusting these wells, push the OK button to close this window.

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 are 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- and background-based thresholds) and a third which is 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.

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. Alternatively, 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.

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 fluorescence 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.

Dissociation Curves (Only for SYBR[®] Green I)

As mentioned previously, when the detection chemistry is based on double-stranded DNA detection, such as SYBR Green I, you should run a melting 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 $-R_n'(T)$, 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 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 is 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. Since primer dimers will typically have a lower melting temperature, the temperature at which the peak occurs can generally be used to discriminate peaks caused by primer dimers from peaks due to amplicon contamination. 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 can help decrease the incidence of this type of problem.

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.

Replicate Agreement

If replicate samples were run, verify that the replicate wells are tightly grouped. If any well in a replicate set is an obviously anomalous point (e.g., the Ct is coming up very late/not at all, or there is excessive spiking) you should go back under Analysis Selection/Setup and assign this well its own replicate symbol or deselect it. This will prevent it from interfering with the calculations for the other replicate wells. Consistently poor replicate uniformity could indicate possible problems in the experimental setup and will definitely affect the accuracy of your results.

Standard Curve Quantification

After amplification, given that both the standards and experimental samples are amplifying efficiently, the Ct's 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%.

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

Figure 28
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.

A typical plate setup for a standard curve can be seen in Figure 28. The Ct values from each standard well will be used to create a standard curve. Figure 29 represents a typical standard curve constructed over three orders of magnitude (40 copies to 20,000 copies) on an Mx instrument.

Data from a standard curve run can be viewed in multiple formats including: Standard Curve, Initial Template Quantity, and Plate Sample Values.

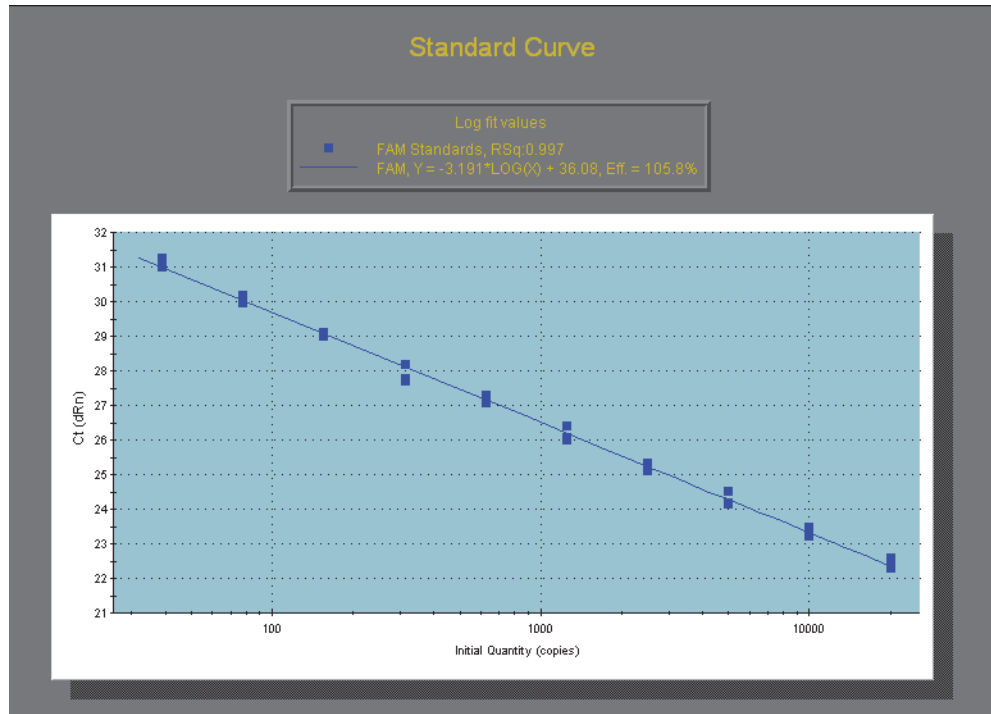


Figure 29 Standard curve demonstrating a two-fold dilution series, from 20,000 to 40 copies. At each standard dilution a one-cycle change in Ct value is observed. This direct correlation between fold-decrease in standard concentration and increased Ct value demonstrates that the doubling efficiency of this assay is approximately 100%.

In the standard curve view, as seen in Figure 29, 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:

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

In experiments where a standard curve is run, the slope should be in the range of -3.10 to -3.59, which would correlate to a 90–110% efficiency range. The RSq value for the standard curve should be 0.985 or higher. R squared indicates how well the data points fit to a straight line, indicating both the agreement between your replicates and the linear range of the assay. If points are dropping off the linear at one end of the standard curve, this would indicate those concentrations are outside the linear range of detection for that assay, and further assay optimization may be necessary to accurately quantify sample concentrations in that range. Adjusting the threshold may help improve the slope and R squared to a certain extent as well.

Relative or Comparative Quantification

In studies where the experimental objectives only require the determination of relative copy number of a target, especially when dealing with large number of targets such as in high-throughput gene expression studies, comparative quantitation is commonly used instead of the standard curve method of analysis. This method allows you to calculate relative quantities of targets without the necessity of setting up a standard curve for each assay. Results are calculated as “relative quantity to the calibrator”, where the calibrator sample is assigned an arbitrary quantity of “1” and all the other samples are expressed in terms of their fold difference to this sample. Normally the calibrator will be something such as an uninduced sample, a zero-timepoint, or wild-type sample which you use as a point of reference to judge the relative copy number for all the other samples. This method has evolved since it was first introduced to account for differences in the efficiency of amplification between the gene of interest and normalizer, thus significantly increasing the accuracy of the calculations.

$\Delta\Delta\text{Ct}$ Method

The earliest method of comparative quantitation is commonly referred to as the $\Delta\Delta\text{Ct}$ method.¹⁷ This form of analysis utilizes the equation:

$$\text{Relative quantity to the calibrator} = 2^{-(\Delta\Delta\text{Ct})}$$

where $\Delta\Delta\text{Ct} = (\text{Ct}_{\text{GOI}} - \text{Ct}_{\text{norm}})_{\text{unknown}} - (\text{Ct}_{\text{GOI}} - \text{Ct}_{\text{norm}})_{\text{calibrator}}$

The $\Delta\Delta\text{Ct}$ method relies on two assumptions. The first is that any change in Ct value for the normalizer has an equivalent effect on relative quantity as that corresponding change in Ct for the GOI. In other words, this equation assumes an efficiency of 100% (E=1) for both assays and ignores any previously determined efficiency calculations. The second assumption is that efficiency of an assay is consistent from one run to the next. Any run-to-run variance is not included in calculations.

The $\Delta\Delta Ct$ method is often referred to as an approximation method and requires a validation step to confirm that efficiencies of your normalizer and GOI are similar.

Figure 30 shows how the ΔCt values are calculated for this method.

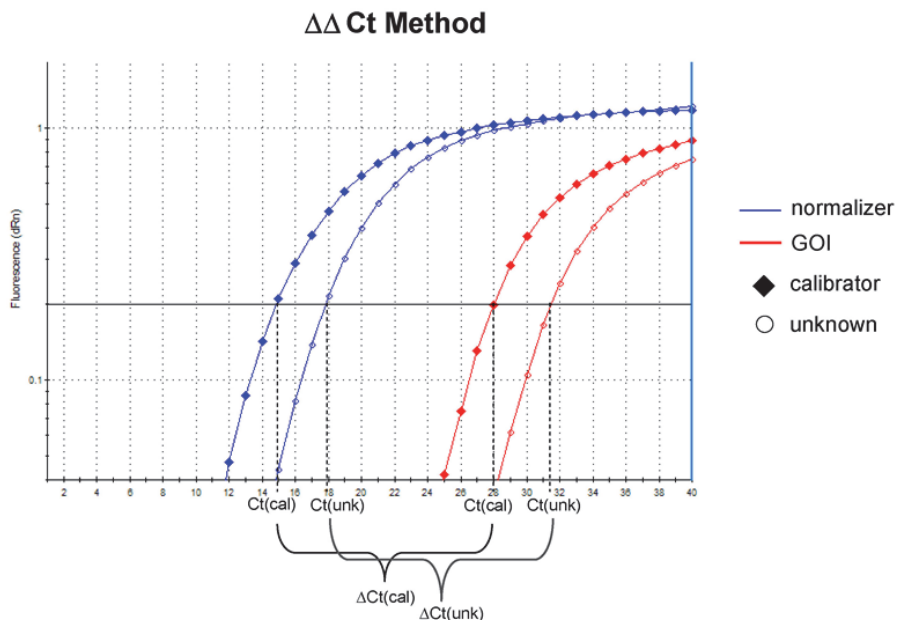


Figure 30
Calculation of ΔCt using $\Delta\Delta Ct$ method. ΔCt is determined by calculating the difference between the Ct of the normalizer assay and Ct of the GOI for each sample.

Efficiency-corrected Comparative Quantitation

Efficiency-corrected comparative quantitation is an enhancement of the previously existing comparative analysis method. Introduced in 2001,¹⁸ this system allows the incorporation of different efficiencies for each assay into the mathematical model. The relative quantity is first calculated separately for each assay, following normalization of GOI assays to assigned normalizers. To do this, the following equation is used:

$$\frac{(1 + E_{GOI})^{\Delta Ct_{GOI}}}{(1 + E_{norm})^{\Delta Ct_{norm}}}$$

where:

E_{GOI} = efficiency of the target assay

E_{norm} = efficiency of the normalizer assay

$\Delta Ct = (Ct_{calibrator} - Ct_{unknown})$

The use of this method eliminates the assumption of equal efficiencies for your GOI and normalizer assays, however, run-to-run variance, as with the $\Delta\Delta C_t$ method, will not be incorporated into the calculation. If precise results are required for an experiment, averages of efficiencies for a given assay determined from standard curves run at separate times can be used to control variance.

Comparative Quantitation Module in the MxPro Software

With the MxPro software (the operational and analysis software for the Mx QPCR instruments), relative quantity calculation can be automated using the Comparative Quantitation module. By selecting “Comparative Quantitation” under the experiment type dialog box, the application will allow the assignment of the following options that are unique to this experiment type:

Calibrator Well Type – At least one sample well must first be selected as a calibrator. All of the unknown wells will be compared to the calibrator sample. If more than one calibrator well is run on the same plate, the results of all the calibrator wells will be averaged before the comparison is performed, unless you assign a separate dye name or assay name to each.

Normalizer – Although not necessary, one assay can be assigned as a normalizer to normalize all other assays. If no normalizer is assigned, relative quantities will be calculated for each assay without any normalization. If a normalizer gene is used, there will generally be one normalizer corresponding to every sample, including the calibrator(s).

Association symbols – If GOI assays and a normalizer assay are run in separate tubes, wells containing GOI assays for each biological sample must be associated to the corresponding wells containing the normalizer assay for that sample. This can be completed by the assignment of an association symbol, an arbitrary letter common to all wells of that sample. If the GOI assay and normalizer assay are multiplexed in the same well, an association symbol is not necessary. As of version 4.1, the MxPro software allows multiple normalizers to be associated with a single GOI. For more information, refer to the section "Sample Normalization using Reference Genes."

Assay efficiencies – Individual assay efficiencies can be entered under the “Analysis Selection/Setup” tab on the “Analysis” page by selecting the Analysis Term Settings button. If no efficiencies are entered, the software will use the default of $E=1$ for all assays to calculate relative quantities.

Figure 31 illustrates the use of these Plate Setup functions in an MxPro Comparative Quantitation experiment.

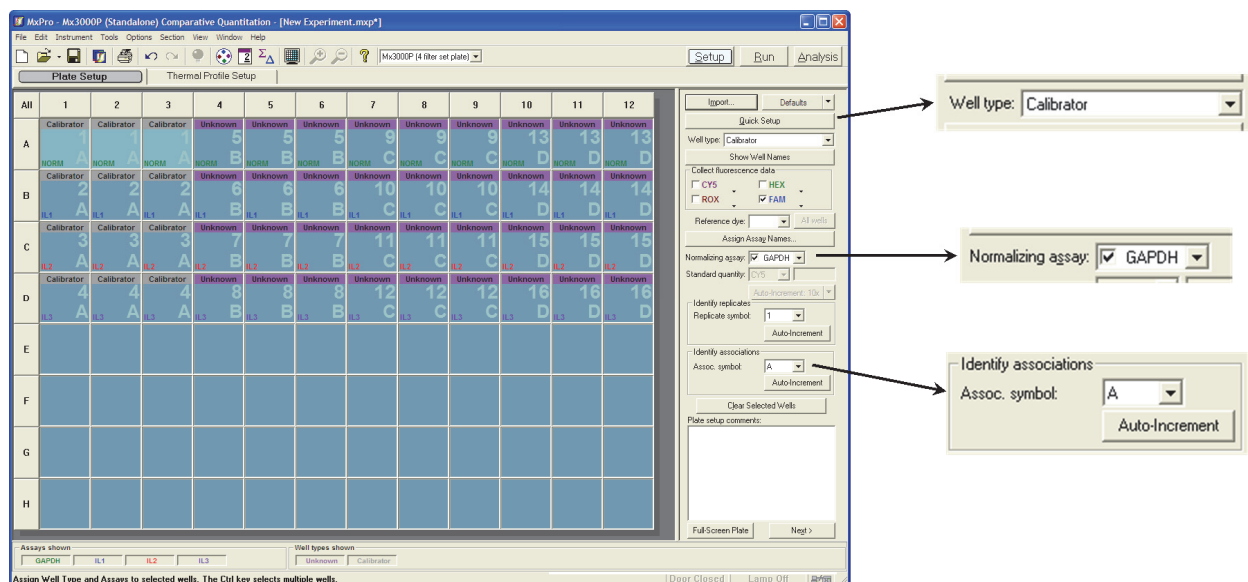


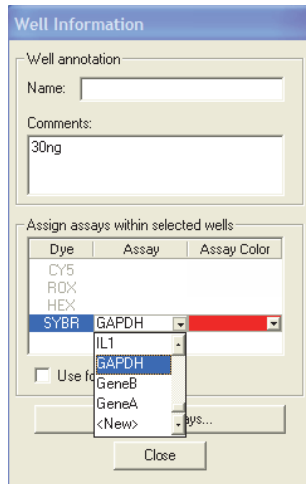
Figure 31
 Plate setup for a Comparative Quantitation experiment in the MxPro software. Plate Setup screen features that are important for setting up this experiment type are expanded at right.

Examples of Plate Setup for Comparative Quantitation Experiments

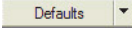
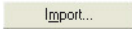
Example 1: Using the Comparative Quantitation module with a SYBR® Green assay:

1. Assign all sample wells with a well type of “Unknown.” (The calibrator well will be reassigned at the completion of plate setup.)
2. With all wells selected, check the box next to the SYBR filter set. If SYBR is not shown, use the pull-down arrow next to one of the currently selected filter sets and switch to SYBR.
3. If a reference dye was used in your master mix, check the box next to the filter set corresponding to that dye, and then select the dye in the Reference dye pull-down menu.

- Select the “Assign Assay Names” button. This feature will allow different assays to be assigned across a plate, regardless of the number of dyes used. (This is an important function in all QPCR experiments as different assays will be assigned separate thresholds for each assay amplification profile, each assay standard curve will be plotted separately, and unknowns will only be compared to calibrators with the same assay name for determining relative quantities.) Click the “SYBR” selection under the “Dye” column of Well Information dialog box. One assay at a time, select wells for your GOI and normalizer assays. A name and a defined color can be assigned to each assay with this tool. A pull-down menu in the Assay column can be used to select any previously-created assays. (It is beneficial to assign different colors to each assay as these are conserved throughout the results section and can help with visualization of data.)



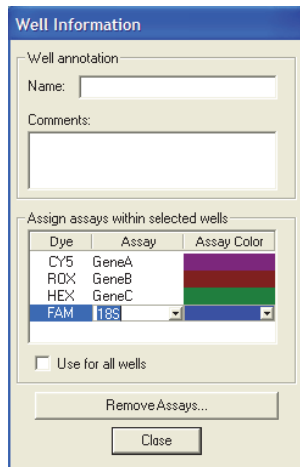
- Select the wells containing the normalizer assay and, under the Normalizing assay: GAPDH pull-down menu, select the assigned assay name.
- Assign replicate symbols to all technical replicates. The application cannot calculate and display technical error without this step.
- Assign association symbols using the Assoc. symbol: A tool to link GOI wells to the corresponding Normalizer well containing the same sample. Multiple GOI assays can be linked to a single Normalizer.
- Redefine the well type of the appropriate samples as “Calibrator”. Well type: Calibrator

- The default thermal profile for a comparative quantitation experiment does not contain a dissociation segment. You may add a dissociation segment to the end of the default thermal profile, using the dissociation segment of a SYBR Green sample experiment files as a model. Alternatively, the comparative quantitation experiment default profile can be changed in the  drop-down menu to a thermal profile containing a dissociation segment, or a profile containing a melt curve can be imported for use in the current experiment using the  button on the top right section of the thermal profile tab.
- After data is collected, the melt curve data must be linked to the dissociation curve plot. This is done in Analysis Selection/Setup tab on the Analysis page. In the upper right corner, under the “Select Data Collection Ramp/Plateau” box, select the Dissociation button. Under this dialog box, the dataset containing the dissociation data can be selected.

Example 2: Using the Comparative Quantitation module with a multiplex assay:

In this example, three GOI assays are multiplexed with one normalizer assay labeled with the FAM fluorophore.

- Assign sample wells with the appropriate well type of “Unknown” or “Calibrator.”
- Assign each assay in the experiment using the Well Information dialog box.



In this experiment, no association symbols are necessary because the normalizer assay and the GOI assays are multiplexed in the same well. After the well information has been added, the Plate Setup screen will appear as shown below.

| | | | | | | | | |
|------------|------------|------------|---------|---------|---------|---------|---------|---------|
| Calibrator | Calibrator | Calibrator | Unknown | Unknown | Unknown | Unknown | Unknown | Unknown |
| GeneA | GeneA | GeneA | GeneA | GeneA | GeneA | GeneA | GeneA | GeneA |
| GeneB | GeneB | GeneB | GeneB | GeneB | GeneB | GeneB | GeneB | GeneB |
| GeneC | GeneC | GeneC | GeneC | GeneC | GeneC | GeneC | GeneC | GeneC |
| NORM | NORM | NORM | NORM | NORM | NORM | NORM | NORM | NORM |

Setting Efficiencies

Efficiencies are determined by running a standard curve for each assay prior to processing experimental samples. In essence, the software will be applying all future Ct values to this previously run standard curve, emphasizing the importance of accurate efficiency estimation. It is suggested to run at least 6 dilutions in triplicate to obtain this level of accuracy. The efficiency values determined for each assay may be entered using the Analysis Term Settings dialog box, as shown in Figure 32.

Error Calculation of Relative Quantity

Technical error is unavoidable, but can be controlled through assay optimization and improvement in technique. Ultimately, the goal of any experiment is to obtain the most precise value that the technology allows, however, calculating precision and accuracy can be complicated in comparative quantitation experiments.

To correctly propagate error of relative quantity to the calibrator, the MxPro software uses a novel algorithm to best visualize the accuracy of the results. Based on the standard error on the mean of fluorescence rather than basic standard deviation of replicate Ct values, error bars in the relative quantity section of the software show statistical accuracy of a single “treated collectively” value, rather than the mean and variance of a group of values. If error is acceptable, this single value can be used for more advanced statistical analysis of biological variance in the system.

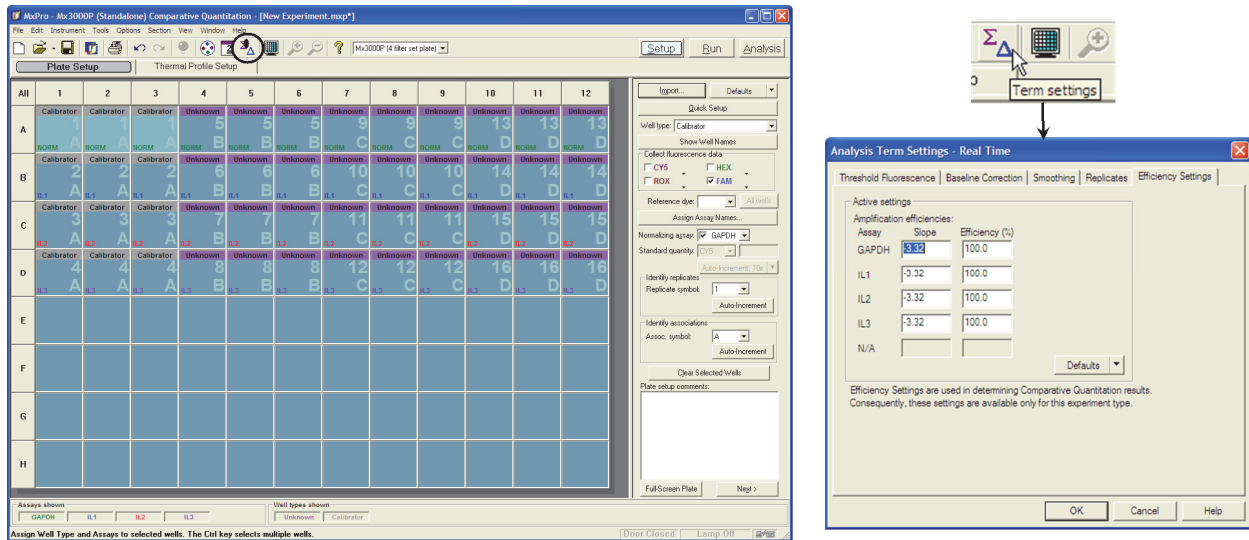


Figure 32

Entering amplification efficiencies for a Comparative Quantitation experiment in the MxPro software. The Analysis Term Settings dialog box may be accessed from the Analysis Selection/Setup screen. This dialog box includes an Efficiency Settings page, where the amplification efficiency for each assay used in the experiment may be entered.

Because each assay is given a different efficiency in the relative quantity calculation, variation in Cts may affect relative quantities differently. It is because of this that each contributor must first be calculated separately before pooling technical variance. When calculating normalized relative quantity, inaccuracies in the determination of both the normalizer and the GOI Ct value must be incorporated into the error calculation individually for each sample.

Because results obtained by comparative quantitation are “relative quantity to the calibrator” both the inaccuracies in the unknown relative quantity as well as those of the calibrator calculation must also be pooled. If the calibrator is an exogenous control used to normalize across plates, this error will represent variability in that normalization factor where the use of an endogenous calibrator will show inter-experimental variability.

Calculating Relative Quantity to a Calibrator Using Collectively Treated Amplification Plots

To really understand the meaning of the relative quantity charts and the error bars, it is important to look at how the instrument actually generates the relative quantity values and the error based on the amplification data that is collected. Error bars can only be generated for the relative quantity chart when replicate samples are run and when the replicates are treated collectively.

Ct Determination of Amplification Plots when Treated Collectively

When replicate samples are run and the software is set up to treat replicates collectively, the amplification plots are collapsed by taking the average of all wells in the replicate group at each cycle and then graphing a single plot for the entire replicate group. For each replicate amplification plot a Ct is calculated based on threshold crossing point.

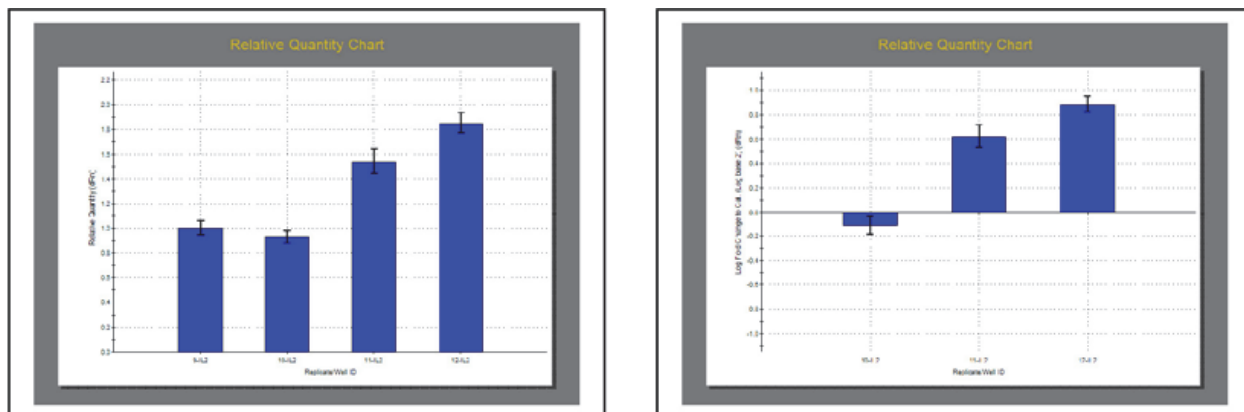


Figure 33
Relative Quantity Charts. Relative quantity to the calibrator results can be displayed as linear relative quantity, assigning a value of “1” to the calibrator (left) or displayed as log(base2) relative quantity to the calibrator where the calibrator is assigned a value of “0” (right).

In most cases, the point at which the plot crosses the threshold will not be exactly at one of the collection points, but rather will fall between two collection points. In determining the precise Ct value based on the amplification plot, the software identifies the data point at the cycle immediately before the amplification plot crosses the threshold and the data point at the cycle immediately after the amplification plot crosses the threshold. A log-based graph is then interpolated between the two points and cycle at threshold fluorescence is determined using the following equation:

$$Ct = C_0 + \left(\frac{\log\left(\frac{dR(C_0)}{\text{threshold}}\right)}{\log\left(\frac{dR(C_0)}{dR(C_1)}\right)} \right)$$

where C_0 is the cycle immediately before the amplification plot crosses the threshold, C_1 is the cycle immediately after the amplification plot crosses the threshold, $dR(C_0)$ and $dR(C_1)$ are the background subtracted fluorescence values (or the background subtracted, reference dye normalized reference values) for C_0 and C_1 respectively, and “threshold” is the defined fluorescence threshold value.

If a normalizer assay is assigned during plate setup, relative quantity of an unknown sample to a calibrator (RQ) is calculated as follows:

$$RQ = \frac{(1+E_{GOI})(Ct_{GOI}(\text{calibrator}) - Ct_{GOI}(\text{unknown}))}{(1+E_{norm})(Ct_{norm}(\text{calibrator}) - Ct_{norm}(\text{unknown}))}$$

where E_{GOI} is the efficiency of the gene of interest assay and E_{norm} is the efficiency of the normalizer assay.

Calculating Relative Quantity to the Normalizer

To accurately calculate error associated with relative quantities to the calibrator, error calculation of the quantity of the unknown sample as well as the calibrator sample must first be calculated separately. To execute this, we can introduce the concept of relative quantity to the normalizer (Q). This value provides an intermediary for relative quantitation prior to assigning a calibrator and scaling all unknown samples to that value. To calculate Q, the following equation is used:

$$Q = \frac{(1+E_{norm})Ct_{norm}}{(1+E_{GOI})Ct_{GOI}}$$

Because error will be based on varying threshold cycles, by transforming this equation into log space, Ct is removed from the exponent. The following equation shows the calculation of the log of the relative quantity to the normalizer (Q).

$$\text{Log}(Q) = \text{Ct}_{\text{norm}} * \log(1 + E_{\text{norm}}) - \text{Ct}_{\text{GOI}} * \log(1 + E_{\text{GOI}})$$

Error of Q is calculated by plotting + and - error amplification plots around each Ct of plots treating collectively. SEM (standard error to the mean) is calculated for the cycle immediately before the amplification plot crosses the threshold as well as the cycle immediately after the amplification plot crosses the threshold. Fluorescence data of all individual plots contributing to the collective plot is used to define this error.

SEM is calculated by determining the standard deviation (σ) of all fluorescence data (dR or dRn) contributing to the amplification plot treated collectively and dividing by the square root of the number of replicate data points (n).

$$\text{SEM} = \frac{\sigma}{\sqrt{n}}$$

SEM amplification plots are added using upper and lower range of error, and Ct values are determined for these plots using the log interpolation equation above.

The three Ct values extracted from each replicate set (when treated collectively) are the Ct, Ct+1sem, and the Ct-1sem. Notice that Ct+1sem results in a lower Ct value where Ct-1sem yields a higher Ct value. These Ct values can be used to determine upper and lower error for the relative quantities to the normalizer (Q).

Two values contribute to error associated with determining relative quantities to the normalizer:

- Error associated with calculating GOI quantity
- Error associated with calculating normalizer quantity

Because contribution of total error is dependant on assay efficiency, accurate error propagation requires separate calculation of error from each of these two components. Likewise, it is important to understand that these two components have opposite effects on calculation of Q. As Ct of the GOI increases, Q will decrease, however, an increase in the Ct of the normalizer will result in an increase in Q. Therefore, the upper error of Q can be calculated using the Ct_{GOI}+1sem and Ct_{norm}-1sem while the lower error can be calculated using the Ct_{GOI}-1sem and Ct_{norm}+1sem. To determine the upper and lower limits of Q, we can use the error of each of these values (-1sem, +1sem for both the GOI and the normalizer) and use the following equations to propagate the error for incorporating the efficiencies of each:

Log of upper limit (logQ) =

$$\text{Log}(Q) + \sqrt{(+1\text{sem}_{\text{GOI}} * \log(1+E_{\text{GOI}}))^2 + (-1\text{sem}_{\text{norm}} * \log(1+E_{\text{norm}}))^2}$$

Log of lower limit (logQ) =

$$\text{Log}(Q) - \sqrt{(-1\text{sem}_{\text{GOI}} * \log(1+E_{\text{GOI}}))^2 + (+1\text{sem}_{\text{norm}} * \log(1+E_{\text{norm}}))^2}$$

Calculating Error of Relative Quantity to a Calibrator

The relative quantity to the calibrator (RQ) can be calculated using the (Q) values. Each unknown sample value (Q_{unknown}) is divided by the relative value ($Q_{\text{calibrator}}$) of the calibrator sample.

$$\text{RQ}_{\text{unknown}} = \frac{Q_{\text{unknown}}}{Q_{\text{calibrator}}}$$

To accurately calculate error of RQ, error of Q for the unknown sample as well as error for the calibrator sample must be incorporated.

Upper limit (RQ) =

$$(\text{RQ}_{\text{unknown}}) * 10^{\sqrt{(\text{Log}(Q_{\uparrow})_{\text{unk}} - \text{Log}(Q)_{\text{unk}})^2 + (\text{Log}(Q)_{\text{cal}} - \text{Log}(Q_{\downarrow})_{\text{cal}})^2}}$$

Lower limit (RQ) =

$$\frac{(\text{RQ}_{\text{unknown}})}{10^{\sqrt{(\text{Log}(Q)_{\text{unk}} - \text{Log}(Q_{\downarrow})_{\text{unk}})^2 + (\text{Log}(Q_{\uparrow})_{\text{cal}} - \text{Log}(Q)_{\text{cal}})^2}}}$$

The RQ value is used by the MxPro software in generating the relative quantity charts, and the upper and lower RQ limits are used to generate the error bars on these bar graphs (Figure 34).

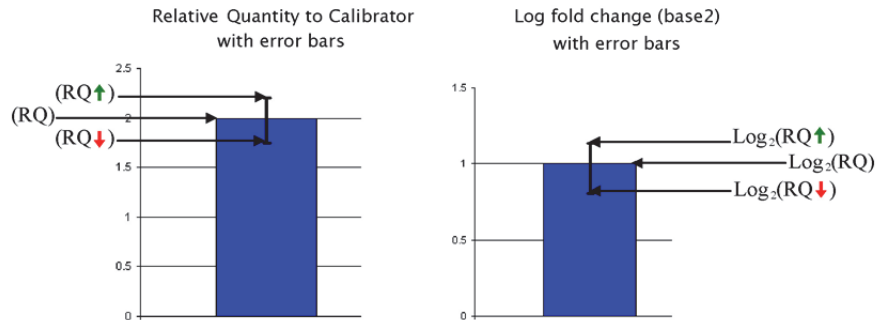


Figure 34
Derivation of error bars on Relative Quantity Charts.

Qualitative PCR

In addition to Quantitative PCR, the Mx 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 MxPro 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 (R_{post}), the normalized final fluorescence ($R_{\text{n, post}}$), the shift in raw fluorescence ($R_{\text{post}} - R_{\text{pre}}$), the shift in normalized fluorescence ($R_{\text{npost}} - R_{\text{npre}}$), or the ratio of the raw fluorescence shift ($R_{\text{post}}/R_{\text{pre}}$).

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. 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 Term 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 MxPro software, so the data would have to be exported and statistically evaluated elsewhere.

Using the Plate Read/Allele Discrimination Experiment type, it is also possible to run genotyping experiments. It is possible to do this with real-time QPCR, but end point analysis will generally be sufficient. Since only one or the other allele form will be present in homozygotes and both allele forms will be present in equal quantities in heterozygotes, this type of test really just involves running two Qualitative QPCR tests to determine if just one or the other or both allele targets are present in a sample. This test can be performed by using a primer/probe set specific to each allele run either side by side on the same sample or in multiplex. This can be performed just like running a standard Quantitative Plate Read experiment using either endpoint fluorescence alone or fluorescence readings from both pre- and post-PCR fluorescence readings. The MxPro software will make positive and negative calls for each allele target. The results can also be viewed on a dual color scatter plot, which makes it easier to see how the populations are grouped.

Other Applications of Plate Reads and Endpoint QPCR

In addition to qualitative PCR, it is possible to use the Plate Read functionality for other applications. Although it is not as accurate as real-time quantitation, it is possible to perform quantitative QPCR based on the endpoint fluorescence in plate read experiments. Using the Quantitative Plate Read experiment type, you can run standards that can be used to generate a standard curve based on the endpoint fluorescence, and the MxPro software will calculate concentrations for any unknown samples based on these standard curves.

Using the Plate Read experiment types, you can also essentially use the Mx instrument as a fluorescence plate reader, although it

can only be used to read fluorescence in standard PCR tubes or plates, so it would not be possible to read fluorescence in a 96 well microtiter plate. Using this sort of fluorescent plate scan, though, you can use the instrument for applications such as quantifying RNA concentration using RiboGreen dye. An application note for this particular procedure can be found online at www.genomics.agilent.com.

Multiple Experiment Analysis

Multiple experiment analysis is a feature that allows the results from two or more post-run experiments to be analyzed and displayed together in a multiple-experiment file known as a project. The data from the experiments in a project may be grouped together based on assay or maintained as separate data sets. The analysis and display of the data depend on how you choose to define the associations among the experiments of the project and how you choose to view the project results.

Applications

Multiple experiment analysis in MxPro was designed for the following applications:

- In Quantitative PCR, Comparative Quantitation, SYBR Green, EvaGreen and Quantitative Plate Read projects, multiple experiment analysis may be used to determine the quantity of a gene of interest (GOI) in an **Unknown** sample using a standard curve generated from **Standard** samples that were run on a separate plate of the same experiment type. This application allows you to compare Unknown sample data from multiple experiments to the same standard curve, thereby eliminating the need to run **Standards** with every experiment that includes amplification of that GOI. This application is particularly useful with Quantitative PCR experiments where a standard curve is ordinarily run with every plate. See detailed instructions on this application in *How-To Examples*.
- In a Comparative Quantitation project, the quantity of a gene of interest (GOI) may be normalized to a **Normalizer** target that was run on a separate plate. This capability is particularly useful when running many different GOI assays that all use the same **Normalizer**. See detailed instructions on this application in *How-To Examples*.
- Results from multiple experiments of the same type may be displayed side-by-side while still treating the experiments independently. This capability facilitates comparisons between experiments.

Considerations for Multiple Experiment Analysis

There are several things to be careful of when analyzing data from several different plates together as one project. When samples are set up together on the same plate using the same stock of master mix, you can be reasonably confident that the efficiency of amplification and the background will be the same for all the reactions on that plate with the same primers, and a common threshold for all those samples will yield Cts that can be directly compared to one another to give meaningful results. If samples are run on different plates, at different times, and possibly using different stocks of reagents, however, it raises the question of how

equivalent are the results from each run, and whether the Ct values can be directly compared.

From the standpoint of amplification efficiency, you have to make the assumption that your efficiency is not changing from reaction to reaction if you plan to directly compare Ct values between runs. The best way to monitor efficiency is by running a standard curve, but if you run a standard curve on every plate you really are not going to be comparing Ct values between runs, you will be comparing calculated quantities based on standard curve quantification. The best way to ensure consistent efficiency is to always use good lab techniques and high-quality reagents. It is also not advisable to change to different lots of reagents between experiments that are to be directly compared. During assay design and optimization, it is a good idea to run several standard curves on different days just to make sure that your efficiency does stay consistent before you start working with your samples of interest. For comparative quantitation, the MxPro software will only allow you to compare unknown samples to a calibrator that is run on the same plate, but it will allow you to normalize to a gene target that is run on a different plate. This avoids the potential problem of reaction efficiency changes between the same reaction performed on different plates, because you are only comparing the delta-Ct values between runs rather than directly comparing Ct values. If Standard Curve Quantification is used, the MxPro software will allow comparison of unknown wells on one plate to a standard curve run on a separate plate, which is a direct comparison of Ct values across plates and is therefore the accuracy of the quantification using that method will be very dependant on the amplification efficiency remaining the same between the runs.

Threshold Determination in Multiple Experiment Analysis

One of the major concerns when directly comparing the Ct values between experiments, is the manner in which the threshold is set. If you consider a single amplification plot, raising the threshold will give a later Ct, and lowering the threshold will give an earlier Ct. So, samples with the same starting concentration of template that have identical efficiency of amplification will have different Ct values if the threshold is set differently. Obviously, then, you don't want to vary the way the threshold is set between runs where you will be directly comparing the Ct values. The problem, however, is what does it mean to set the threshold the same?

To consider this question on thresholds, it helps to first examine the subject of baseline correction. In any single experiment, if you view the raw amplification plots in the raw fluorescence view (R view) you will normally see that all the plots are at slightly different places on the Y axis, which makes it impossible to compare all the curves to a common threshold unless you first correct them so that they all have a common starting point. The process of baseline correction involves defining a range of cycles for each amplification plot where the plot is flat prior to the shift in fluorescence due to amplification. A best-fit straight line is

drawn based on the data points in that cycle range, and then that line is subtracted from every point on the curve. This has the effect of dropping the flat portion at the beginning of the plot to zero. After this is done for all the plots, a common threshold can be drawn to produce Ct values for all the curves that are directly comparable. The point at which the threshold is set is defined in terms of its height above the baselines.

The problem with using this same method with data from different runs is that the signal level of a curve in the baseline range is not an absolute value. The baseline signal is a function of the total noise in the system. Background fluorescence in the samples that is not due to amplified signal and electronic noise from the instrument both contribute to produce this baseline signal level. If you assume this level of noise is always the same, then it is valid to compare Ct values from different experiments by simply setting the threshold at the same height above the baseline in both experiments. If the background noise varies between experiments, however, then just setting the threshold at the same height above the baseline in both experiments does not actually place it at the same point on the curves (see Figure 35 for an illustration).

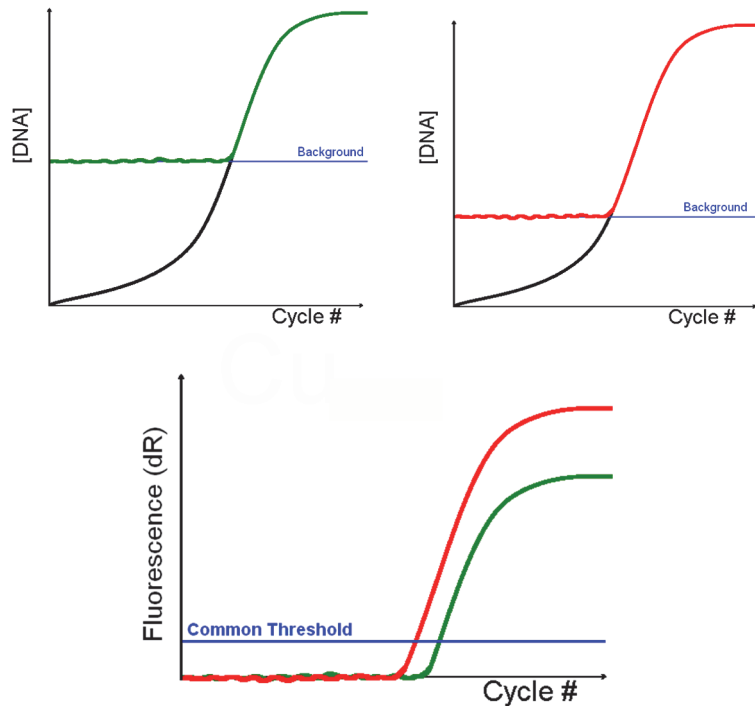


Figure35

Consider two identical amplification reactions, both starting with the same concentration of template. A plot of the actual DNA concentration in the reaction vs. the cycle number will appear as the black lines in the graphs above. If these runs are performed in separate amplification reactions where the background signal levels are different, as indicated by the blue lines, then the actual amplification plots that are seen when the fluorescence data is collected will appear as the red and green lines below. Baseline correcting these amplification plots will produce dR plots as shown in the bottom graph. In this example, you can see that these plots, which were originally identical, actually will give different Ct values when a threshold set at the same height above the baseline is used in both experiments.

If you do feel that you have similar background signal levels between runs, it can be acceptable to just set the threshold levels the same for each assay in all the experiments that are part of a project. Since this will not always be the case, however, the MxPro software will not force you to do this. In version 4.1 of the MxPro software, at the time a Multiple Experiment project is created you will be offered three different ways to set the threshold. The first option is to set an individual threshold for each experiment file using the background-based threshold algorithm, which sets the threshold for each experiment at 10x the standard deviation in the background. This was chosen as the default in preference over the Amplification-Based Threshold Algorithm, because while Amplification based threshold will ensure the threshold crosses all the plots in an experiment within the exponential phase of amplification, it will tend to set very different thresholds between experiments where different concentration ranges have been run. Since the background based threshold is based on noise levels in the baseline it will tend to set more equivalent thresholds from experiment to experiment, however it does not guarantee that you will get equivalent threshold settings between runs.

The second choice for setting the threshold in a multiple experiment project is to set a common threshold in all the experiment files. To accomplish this, the software rescales each experiment to make the threshold of each the same. When this method is selected, a common threshold value is calculated for all the assays using data from all wells selected for analysis in the project. The threshold-scaling factor used for each experiment in the project is then used to generate scaled fluorescence data for each experiment. Because threshold and fluorescence data are scaled in the same proportion, Ct values are not affected.

The third method available in the software for setting thresholds is to use an Inter-Run Calibrator sample to set different but equivalent thresholds in all the experiments. This is probably the most valid way to ensure equivalent thresholds between all the experiments in a project. When this method is used, a replicate sample is run in the same well position on all the plates that will be analyzed together. This could be a standard of known concentration, but the exact concentration does not necessarily have to be known. The important thing is that it has the same template concentration in every run. The thresholds in each experiment file are then adjusted by the software so that in each experiment the control well has the exact same Ct. This ensures equivalent threshold settings for that assay. If multiple assays are run (such as multiple genes of interest or normalizers), there will be a separate threshold for each, so you will need a separate Inter-Run Calibrator well for each assay.

Use of this sort of inter-experiment control is normally the most accurate way to set thresholds when performing multiple experiment analysis. If it is not possible to run this sort of control, using the common threshold for all the experiments will usually be sufficient unless there is some reason to suspect differences in

background noise between experiments, in which case the background based threshold algorithm run individually for each experiment may be better.

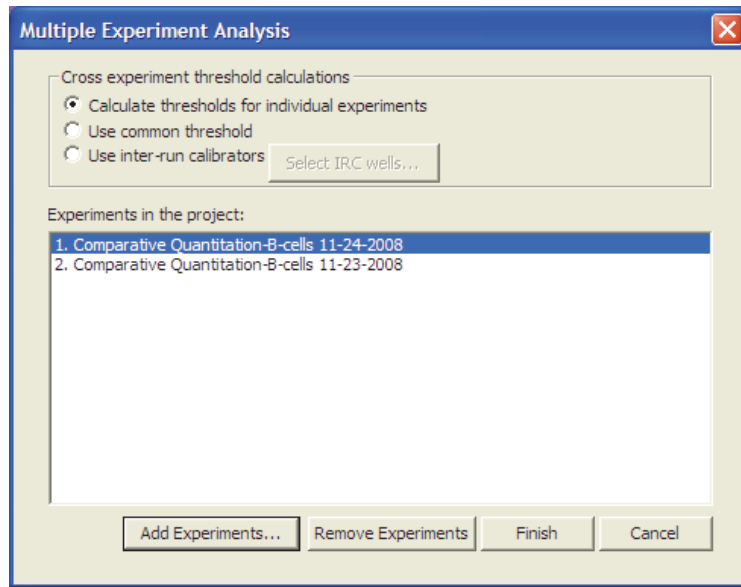


Figure 36

In the window that opens when a Multiple Experiment Project is created, up to 12 separate run files can be imported, and at this time the manner in which the thresholds will be set is selected. If you wish to change the way the Threshold is set after the project is created, you can select the Cross experiment threshold calculation button on the control panel under the Project Selection/Setup screen.

In most respects the analysis screens in a MEA project file will be very similar to the corresponding analysis screens in an individual experiment file, but there are a few differences that can be important.

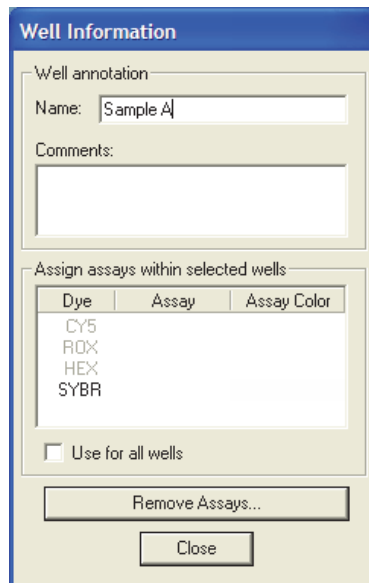


Figure 37

The well name assignments that serve to associate normalizer and GOI wells across plates in an MEA project can be changed in the Well Information Dialog Box.

If normalization is performed across plates, with the normalizer gene unknown and calibrator wells on one plate and the GOI unknown and calibrator wells corresponding to the same samples on a different plate, the normal Association Symbol assignments will not work across plates. In order to associate which of the normalizer wells come from the same template sample as each of the GOI wells, you should assign the same Well Name to both. In an MEA project, the well name will function just like the Association Symbol letter assignments normally do within a single plate experiment file.

In the analysis of the Project, in order to analyze the data from the different plates all together, you also have to make sure that the Data View Type is set to *Consolidated* or *By Assay* rather than *By Experiment*. If the *By Experiment* view is used, the analysis will treat each plate individually.

Multiple Experiment Analysis Applications: How-To Examples for MxPro

Application 1: How to Find the Initial Template Quantity in an Unknown Sample Using Standards from a Separate Experiment

This example will walk you through the process of using multiple experiment analysis to calculate the initial template quantity of a gene of interest (GOI) in an Unknown sample by comparing its Ct (or final fluorescence in a plate-read experiment) to a standard curve for that GOI target that was generated on a separate plate of the same experiment type. Any project type that uses a standard curve to calculate initial template quantity (i.e. Quantitative PCR, Comparative Quantitation, SYBR Green, EvaGreen and Quantitative Plate Read projects) may be used for this application.

Step 1: Creating the Project

Create a multiple experiment analysis project containing both experiments (the experiment with the Unknown samples and the experiment with the Standard samples). One way to quickly create a new project is to open either of the two experiment files, click the **New Project (Multiple Experiment Analysis)** icon on the toolbar to open the Multiple Experiment Analysis dialog box, then add the second experiment to the project using the **Add Experiments** button. After adding the second experiment, select the appropriate method for calculating threshold fluorescence under **Cross experiment threshold calculations**. For more information on threshold calculation options for Projects, see *Threshold Determination in Multiple Experiment Analysis*.

Step 2: Assigning Cross-Plate Relationships

Assign the identical assay name to the Unknown samples and the Standard samples. The software can only identify cross-plate relationships between Unknowns and Standards if both well types

have the same assay name assigned to the same dye position. The identical assay name indicates that these reactions amplified the same target.

To assign custom assay names, navigate to the **Plate Setup** tab in the Setup section of the software. Double-clicking on a well opens the **Well Information** dialog box where you may assign an assay name in the appropriate dye position (see Figure 37). For wells designated as Unknowns, the assay name will appear in that dye position in any plate view screens. For Standard wells, the standard quantity appears in the dye position instead of the assay name.

Step 3: Selecting Analysis Settings

Once assay names have been designated through Plate Setup, the next step is to go to **Analysis Selection/Setup** (in the Analysis section) and define the settings for analysis of the project. Because only one experiment may be displayed at a time, use the buttons just underneath the toolbar to toggle between the two experiments.

The analysis settings that need to be assigned are:

- The wells and assays to be included in analysis
- The data collection point to be used for analysis
- The treatment of replicate wells
- Any adjustments desired for the advanced settings (accessible through the **Analysis Term Settings** dialog box)

Step 4: Viewing Project Results

To view the project results screens, navigate to the **Project Results** tab in the Project section of the software. Several project results screens are available depending on the type of experiments that make up the project. Under the **Area to analyze** menu on the command panel, select the **Standard curve** option. The Quantitative PCR, Comparative Quantitation, SYBR Green, EvaGreen and Quantitative Plate Read project types all have the Standard curve option.

The Standard curve is a plot of the initial template quantity in the Standard wells (X-axis), versus the Ct (or final fluorescence in a plate-read project). Quantities in any Unknown wells/replicates included in the analysis may also be plotted on the Standard curve (depending on the Data view type) based on their measured Ct/fluorescence values.

To view the initial template quantities in the Unknown samples, select *By Assay* as the **Data view type** in the Standard curve screen. In this view, data from both experiments in the project are grouped together based on assay name and a separate graph is displayed for each assay included in the analysis. Thus, all the Ct values for one assay in all Standard and Unknown wells are plotted on the same graph.

Place the cursor over the data point for an Unknown well/replicate to display the calculated initial template quantity (Figure 38). This quantity is calculated from the Standard data for that same assay.

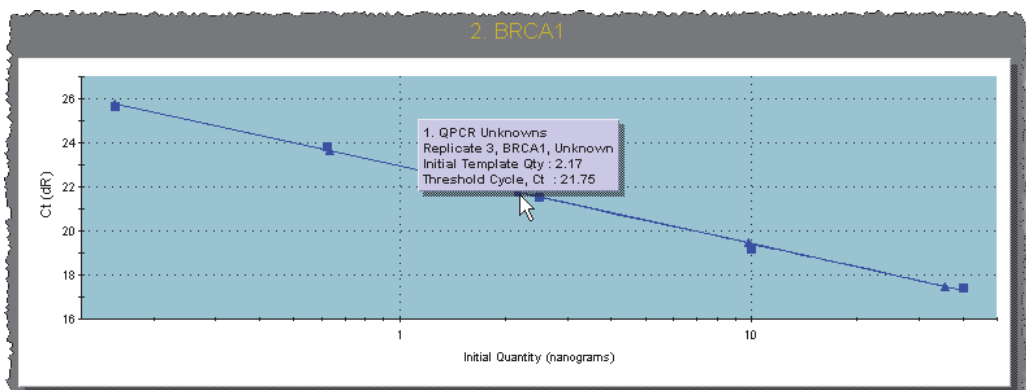


Figure 38
The initial template quantity in an Unknown well is displayed in the tooltip when the cursor is placed over a data point.

Application 2: How to Normalize GOI Data to an Assay from a Separate Experiment

The Comparative Quantitation experiment type is used to compare amounts of mRNA of a gene of interest (GOI) in *treated vs. untreated* or *normal vs. diseased* cells or tissues. The control sample is referred to as the Calibrator, and the test samples are referred to as the Unknowns. To obtain the most accurate results, one or more Normalizer target (e.g. a housekeeping gene) needs to be amplified from each Calibrator and Unknown template sample.

Using multiple experiment analysis, the data from GOI assays can be normalized to data from a Normalizer assay that was run in a separate Comparative Quantitation experiment. This example will walk you through the process for setting up a project in which the GOI assay and a single Normalizer assay are on different plates.

Step 1: Creating the Project

Create a multiple experiment analysis project containing both Comparative Quantitation experiments (the experiment with the GOI assays and the experiment with the Normalizer assay). One way to quickly create a new project is to open either of the two experiment files, click the **Multiple Experiment Analysis** icon on the toolbar to open the **Multiple Experiment Analysis** dialog box, then add the second experiment to the project using the **Add Experiments** button. After adding the second experiment, select the appropriate method for calculating threshold fluorescence under **Cross experiment threshold calculations**. For more information on threshold calculation options for Projects, see *Threshold Determination in Multiple Experiment Analysis*.

Step 2: Assigning Cross-Plate Relationships

Once the new project has been created, well names and assay names need to be assigned in order to establish template and

target identities across both plates. Assign identical well names to wells containing the same template sample on each plate, and assign a unique assay name to each target. This allows the software to normalize the GOI results to the Normalizer results for each template sample, and distinguish between different targets that were detected with the same dye.

To assign well names and assay names, navigate to the Plate Setup tab in the Setup section of the software. Double-click on the well to open the **Well Information** dialog box. Type an appropriate well name in the **Name** field. All wells from both experiments that contain the same template should be given the same well name. For the GOI assays, type in an assay name for each target in the appropriate dye position in the **Well Information** dialog box. All wells that are detecting the same target with the same dye should have the same assay name assigned to that dye position.

Step 3: Selecting Analysis Settings

Once the appropriate well and assay names have been designated, the next step is to go to Analysis Selection/Setup (in the Analysis section) and define the settings for analysis of the project. Because only one experiment may be displayed at a time, use the buttons just underneath the toolbar to toggle between the two experiments.

The analysis settings that need to be assigned are:

1. The wells and assays to be included in analysis
2. The data collection point to be used for analysis
3. The treatment of replicate wells
4. Any adjustments desired for the advanced settings, e.g. specifying the amplification efficiencies for the GOI and Normalizer targets (accessible through the **Analysis Term Settings** dialog box)

Step 4: Viewing Project Results

To view the project results screens, navigate to the **Project Results** tab in the Project section of the software. Under the **Area to analyze** menu, select the **Relative quantity chart** option. The **Relative quantity chart** screen is particularly useful for viewing the relative quantity of a GOI when the Normalizer target is on a different plate. Each bar on the chart represents the quantity of a GOI target from a single Unknown well/replicate relative to its quantity in the Calibrator sample.

From the **Data view type** menu, select either *By Assay* or *Consolidated*. In either of these views, the results for each assay are grouped across all experiments in the project based on assay name and the GOI data are normalized to the designated Normalizer based on matching well names. Place the cursor over any of the bars on the graph to display the exact value of the Y-axis coordinate (i.e. the relative quantity; see Figure 39). The relative quantity appears at the bottom of the tooltip box. In Figure 39, the relative quantity is 0.967.

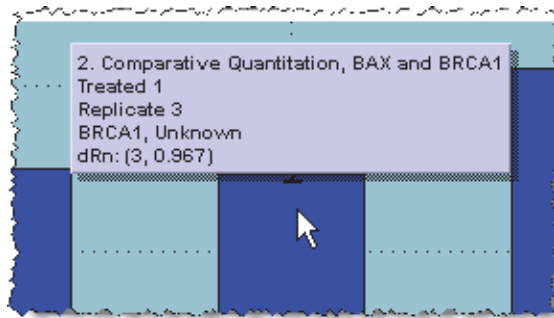


Figure 39

The relative quantity in an Unknown well is displayed in the tooltip when the cursor is placed over a bar on the Relative Quantity chart.

Appendix

Primer Optimization Reaction Example

1. Prepare master stock of primers at 100 μM , aliquot and store at -70°C .
2. Dilute primers to 10 μM working solutions and store at -20°C .
3. Dilute forward and reverse primers according to the directions below (both 1 μM and 5 μM primer stocks are required to set up this primer optimization matrix), for a total of 52 reactions (25 primer combinations 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 ddH₂O 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 ddH₂O 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 ddH₂O 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 ddH₂O to a final volume of 21.6 μl

Set 5: Reverse Primer 900 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 ddH₂O 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 ddH₂O

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 ddH₂O

4. Prepare the final reactions using Stratagene Brilliant SYBR[®] Green QPCR master mix (supplied at 2 \times concentration with a separate tube of undiluted Passive Reference Dye) as indicated below. Note: Do not add primers.

| Reagent Master Mix | 1 \times 60 μl reaction | 27 \times 60 μl |
|--|--------------------------------------|------------------------------|
| 2 \times SYBR Green I QPCR MM | 30 μl | 810 μl |
| Passive Reference Dye (1:200) | 0.9 μl | 24.3 μl |
| dH ₂ O | 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.

- Mix and pulse centrifuge to collect the master mix.
- 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.
- Completely cap the tubes or plate wells, label and spin if necessary, place the tubes/plate in the thermal block of the Mx3000P system.
- In the MxPro 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 $^{\circ}\text{C}$ plateau. If necessary, change the annealing temperature to match the calculated T_m of the primers, in most cases this should be 55 $^{\circ}$ –60 $^{\circ}\text{C}$.
- 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 1 μM probe stock to set up the probe matrix.
4. Set up the reaction master mix as indicated in step 3 of the Primer Optimization Example above.
5. Pipette out the probe matrix as indicated below:

| | 50 nM | 100 nM | 200 nM | 300 nM | NTC 1 | NTC 2 |
|------------------------|-------|--------|--------|--------|-------|-------|
| ddH ₂ O | 17 | 14 | 8 | 2 | 17 | 2 |
| Probe, 1 μM | 3 | 6 | 12 | 18 | 3 | 18 |

6. Prepare the reagent master mix as indicated below:

| Reagent Master mix | 1 \times 60 μl reaction (μl) | 12 \times 60 μl |
|---|--|------------------------------|
| 2x QPCR MM | 30 μl | 360 μl |
| Passive Reference dye (1/200) | 0.9 μl | 10.8 μl |
| dH ₂ O | 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.

7. Add 40 μl of the reaction master mix to each probe condition tube and mix gently.
8. Pipette 25 μl 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.

References

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Fast Track Education Program

The Fast Track QPCR Education Program provides up-to-date training tools targeted to a broad spectrum of QPCR users. Introductory education on the technology as well as advanced application-focused training can be found at the Stratagene Fast Track website. This site consists of a series of specific web-based seminars covering topics from “An Introduction to the Stratagene’s Mx QPCR Software” to “Critical Components of Assay Design.” Experimental setup and methods of data analysis are also discussed in a few of the more advanced web-seminars. This source of training is consistently updated by Stratagene and provides an excellent source of instruction to new QPCR users as well as ongoing support with evolving projects utilizing this technology.

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 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. This can be performed in separate tubes or in multiplex if the different probes are each labeled with spectrally distinct dyes.

Allele Discrimination (Plate Read) Plate read measurements of fluorescence 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 fluorescence 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 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 T_m of 80°C or higher correspond to the larger PCR products, and are usually assigned to a 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 range of fluorescence signal (from the lowest to the highest in the experiment) in which there is a direct linear relationship between actual fluorescence and reported signal. This range lies between the background noise on the lower end and the point where the detector starts to become saturated on the higher end. 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 (T_m), 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 fluorescence can be detected.

Plate-Read (Endpoint) Experiments A single measurement of the fluorescence taken at the completion of the amplification reaction. Results are generally recorded as either a positive or negative call on whether amplification occurred. Quantitation based on endpoint fluorescence is generally not as accurate as a real-time quantitative PCR assay.

Qualitative Detection Determination of the presence or absence of template of interest based on either C_t values or endpoint fluorescence.

Quantitative PCR Analysis Determination of either the starting concentration of a template of interest or the relative ratio of the quantity of a template in two different samples. This is based on either product measurement after the PCR reaction is complete or monitoring fluorescence intensity during the PCR reaction at each cycle in a closed-tube system. 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, Black Hole Quencher).

Real-Time Experiments QPCR 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,

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 from cycle to cycle during the reaction, and if the reaction was aliquotted properly it will be at the same concentration in every sample. To normalize, the fluorescence signal of the reporter dye at each cycle is divided by the fluorescence signal of the reference dye in that tube at the same cycle, and normalized results are displayed as a ratio of the signal from the two dyes. 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 of the product of interest. 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 any 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 a perfectly complementary target oligo in Molecular Beacon melting curve experiments.

NAC (No Amplification Control) In this sample type the polymerase 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 template(s).

Negative Dye Control Negative dye control wells contain all the PCR reagents primers and probes, and all the target templates except the target detected by the defined dye. These wells, in combination with NTC wells, are used to calculate final +/- calls in Quantitative Plate Read experiments.

Positive Dye Control A sample type that is known to contain the template that will be detected with one specific dye channel.

Standard A sample type containing reaction mixture with known concentration of the target nucleic acid. Wells of this type are used for generation of a Standard Curve, and 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 a particular 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 signal level. The region specified is typically in the cycle range before exponential amplification occurs. The Background Cycles are used for the background-based 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 a specified range of cycles (the baseline range) 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 In Comparative Quantitation, the sample quantities are defined in terms of fold-change compared to a reference sample (calibrator). 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 the fluorescence of all wells with the same replicate symbol at every cycle to generate a common amplification plot for the whole replicate group and 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 either the endpoint fluorescence values or the 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 in the Unknown wells prior to amplification. 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. The multicomponent view of the amplification plots (also known as the Raw fluorescence or R) refers to the non-normalized and non-baseline corrected view of the plots.

p-value The probability that the mean of one set of sample data is different from the mean of another set of sample data. In a qualitative PCR assay, if the user-defined confidence level for calls is set to 99%, a positive call (+) for an Unknown well means that at most 1% of the time a measurement of sample identical to the negative 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 line to the data points that were used to calculate it. 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 MxPro 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 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 background-based 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 reaches a user or instrument defined threshold value. The Threshold should be set at a point that is at a statistically-significant level above the background signal and it should cross all the amplification plots within the exponential phase of amplification. 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 fluorescence reading

Rn fluorescence reading normalized to the reference dye

dRn baseline subtracted fluorescence reading normalized to the reference dye

Rpre the initial fluorescence reading

Rpost the final fluorescence reading

R_{n, pre} the fluorescence before thermal cycling normalized to the reference dye in a plate-read experiment

R_{n, post} the fluorescence after thermal cycling normalized to the reference dye in a plate-read experiment

R_{post}-R_{pre} the total change in fluorescence in a plate-read experiment

R_{n, post}-R_{n, pre} the total change in normalized fluorescence in a plate-read experiment

R_{post}/R_{pre} 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

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

dR_n 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 and Useful Websites

QPCR References

The Ccr4–Not Deadenylase Subunits CNOT7 and CNOT8 Have Overlapping Roles and Modulate Cell Proliferation

Aslam, A. et al
Molecular Biology of the Cell
Vol. 20(17), 3840–3850, September 2009

Real-Time PCR-Based Ready-to-Use Multi-Target Analytical System for GMO Detection

Querci, M. et al
Food Analytical Methods
Vol. 2(4), 325–336, December 2009

Microarray-based global mapping of integration sites for the retrotransposon, intracisternal A-particle, in the mouse genome

Takabatake, T. et al
Nucleic Acids Research
Vol. 36(10), e59, June 2008

Real-time multiplex PCR assay for detection of *Yersinia pestis* and *Yersinia pseudotuberculosis*

Matero, P. et al
Acta Pathologica, Microbiologica et Immunologica Scandinavica
Vol. 117(1), 34–44, January 2009

Malate dehydrogenase is negatively regulated by miR-1 in *Trichomonas vaginalis*

Lin, W. et al
Parasitology Research
Vol. 105(6), 1683–1689, November 2009

For the most current list of QPCR references, please visit
www.genomics.agilent.com

Useful Websites

Oligonucleotide and Assay Design Resources

www.genomics.agilent.com
Search for info on QPCR and links to application notes

www.gene-quantification.info/
Gene Quantification Homepage

<http://frodo.wi.mit.edu/primer3/>
Primer 3 Homepage

www.idtdna.com/Scitools/Applications/Primerquest/
Primerquest Homepage

<http://www.idtdna.com/order/order.aspx>
IDT Ordering Page

www.premierbiosoft.com/stratagene/stratagene.html
Stratagene Beacon Designer software

Oligonucleotide Properties Calculators

<http://mfold.bioinfo.rpi.edu>

Zucker Mfold

www.idtdna.com/ANALYZER/Applications/OligoAnalyzer/

IDT Oligoanalyzer

www.rnature.com/oligonucleotide.html

RNATure

Miscellaneous

www.rdml.org/miqe.php

MIQE: Minimum Information for Publication of Quantitative Real-Time PCR Experiments

www.ncbi.nlm.nih.gov/RefSeq/

RefSeq (Reference sequence database)

www.ncbi.nlm.nih.gov/spidey/

Spidey (cDNA to genomic alignment tool)

www.molecular-beacons.org/

All about Molecular Beacons

<http://iubio.bio.indiana.edu/>

General Molecular Biology Links and Tools

www.tataa.com/

TATAA Biocenter

<http://medgen.ugent.be/rtprimerdb/index.php>

Real-time PCR Primer and Probe Database

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

Product Listings & Ordering Information

| | Reactions per Kit | Reaction Volume | Catalog No. |
|--|-------------------|-----------------|-------------|
| QPCR Systems | | | |
| Mx3000P QPCR System 4-color system (110v) with notebook computer | | | 401403 |
| 4-color system (110v) with desktop computer | | | 401405 |
| 4-color system (230v) with notebook computer | | | 401406 |
| 4-color system (230v) with desktop computer | | | 401407 |
| Mx3005P QPCR System 5-color system (110v) with notebook computer | | | 401449 |
| 5-color system (110v) with desktop computer | | | 401456 |
| 5-color system (230v) with notebook computer | | | 401457 |
| 5-color system (230v) with desktop computer | | | 401458 |
| MxPro ET (Electronic Tracking) QPCR Software | | | |
| New and existing Mx QPCR System users | | | 401467 |
| Multi-user license agreement for six users | | | 401468 |
| QPCR and QRT-PCR Reagents | | | |
| Brilliant III Ultra-Fast QPCR Master Mix ^a | 400 rxn | 20 µl | 600880 |
| Brilliant III Ultra-Fast QPCR Master Mix, 10-pack ^a | 4000 rxn | 20 µl | 600881 |
| Brilliant III Ultra-Fast SYBR Green QPCR Master Mix ^{b,c} | 400 rxn | 20 µl | 600882 |
| Brilliant III Ultra-Fast SYBR Green QPCR Master Mix, 10-pack ^{b,c} | 4000 rxn | 20 µl | 600883 |
| Brilliant III Ultra-Fast QRT-PCR Master Mix ^a | 400 rxn | 20 µl | 600884 |
| Brilliant III Ultra-Fast QRT-PCR Master Mix, 10-pack ^a | 4000 rxn | 20 µl | 600885 |
| Brilliant III Ultra-Fast SYBR Green QRT-PCR Master Mix ^{b,c} | 400 rxn | 20 µl | 600886 |
| Brilliant III Ultra-Fast SYBR Green QRT-PCR Master Mix, 10-pack ^{b,c} | 4000 rxn | 20 µl | 600887 |
| Brilliant II QPCR Master Mix ^a | 400 rxn | 25 µl | 600804 |
| Brilliant II QPCR Master Mix (10 pack) ^a | 4000 rxn | 25 µl | 600815 |
| Brilliant II QRT-PCR Master Mix, 1-Step ^a | 400 rxn | 25 µl | 600809 |
| Brilliant II QRT-PCR Master Mix, 1-Step (10 pack) ^a | 4000 rxn | 25 µl | 600818 |
| Brilliant II QRT-PCR Core Reagent Kit, 1-Step ^a | 400 rxn | 25 µl | 600810 |
| Brilliant II QRT-PCR Core Reagent Kit, 1-Step (10 pack) ^a | 4000 rxn | 25 µl | 600819 |
| Brilliant II QRT-PCR, AffinityScript Master Mix, 2-step ^a | 400 rxn | 25 µl | 600827 |
| Brilliant II QPCR Low ROX Master Mix ^a | 400 rxn | 25 µl | 600806 |
| Brilliant II QPCR Low ROX Master Mix (10 pack) ^a | 4000 rxn | 25 µl | 600817 |
| Brilliant II QPCR High ROX Master Mix ^a | 400 rxn | 25 µl | 600805 |
| Brilliant II QPCR High ROX Master Mix (10 pack) ^a | 4000 rxn | 25 µl | 600816 |
| Brilliant II QRT-PCR Low ROX Master Mix, 1-Step ^a | 400 rxn | 25 µl | 600837 |
| Brilliant II QRT-PCR Low ROX Master Mix, 1-Step (10 pack) ^a | 4000 rxn | 25 µl | 600841 |
| Brilliant II QRT-PCR High ROX Master Mix, 1-Step ^a | 400 rxn | 25 µl | 600838 |
| Brilliant II QRT-PCR High ROX Master Mix, 1-Step (10 pack) ^a | 4000 rxn | 25 µl | 600842 |
| Brilliant II SYBR Green QPCR Master Mix ^{b,c} | 400 rxn | 25 µl | 600828 |
| Brilliant II SYBR Green QPCR Master Mix (10 pack) ^{b,c} | 4000 rxn | 25 µl | 600831 |
| Brilliant II SYBR Green Low Rox Master Mix ^{b,c} | 400 rxn | 25 µl | 600830 |
| Brilliant II SYBR Green High Rox Master Mix ^{b,c} | 400 rxn | 25 µl | 600829 |
| Brilliant II SYBR Green High Rox Master Mix (10 pack) ^{b,c} | 4000 rxn | 25 µl | 600832 |
| Brilliant II SYBR Green Low Rox Master Mix (10 pack) ^{b,c} | 4000 rxn | 25 µl | 600833 |
| Brilliant II SYBR Green QRT-PCR Master Mix, 1-Step ^{b,c} | 400 rxn | 25 µl | 600825 |
| Brilliant II SYBR Green QRT-PCR Master Mix, 1-Step ^{b,c} | 4000 rxn | 25 µl | 600826 |
| Brilliant II SYBR Green QRT-PCR AffinityScript Master Mix, 2-step ^{b,c} | 400 rxn | 25 µl | 600834 |
| Brilliant II SYBR QRT-PCR Low ROX Master Mix, 1-Step ^{b,c} | 400 rxn | 25 µl | 600835 |
| Brilliant II SYBR QRT-PCR High ROX Master Mix, 1-Step ^{b,c} | 4000 rxn | 25 µl | 600836 |
| Brilliant II SYBR QRT-PCR Low ROX Master Mix, 1-Step (10 pack) ^{b,c} | 4000 rxn | 25 µl | 600839 |
| Brilliant II SYBR QRT-PCR High ROX Master Mix, 1-Step (10 pack) ^{b,c} | 4000 rxn | 25 µl | 600840 |
| Brilliant II FAST SYBR Green QPCR Master Mix ^{b,c} | 400 rxn | 25 µl | 600843 |
| Brilliant II FAST SYBR Green QPCR Master Mix (10 pack) ^{b,c} | 4000 rxn | 25 µl | 600844 |
| Brilliant II FAST QPCR Master Mix ^a | 400 rxn | 25 µl | 600845 |
| Brilliant II FAST QPCR Master Mix (10 pack) ^a | 4000 rxn | 25 µl | 600845 |

(Table continues on next page)

| Sample Preparation | | |
|---|-----------|--------|
| Absolutely RNA FFPE Kit | 50 preps | 400809 |
| Absolutely RNA FFPE Kit (w/o deparaffinization reagents) | 50 preps | 400811 |
| Absolutely RNA Microprep Kit | 50 preps | 400805 |
| Absolutely RNA 96 Microprep Kit | 2 plates | 400793 |
| Absolutely RNA 96 Microprep Kit | 10 plates | 400794 |
| Absolutely RNA Miniprep Kit | 50 preps | 400800 |
| Absolutely RNA Nanoprep Kit | 50 preps | 400753 |
| Messenger RNA Isolation | | |
| Absolutely mRNA Purification Kit | 10 preps | 400806 |
| cDNA Synthesis for Two-Step QRT-PCR | | |
| AffinityScript Multiple Temperature Reverse Transcriptase | 10 rxn | 600105 |
| AffinityScript Multiple Temperature Reverse Transcriptase | 50 rxn | 600107 |
| AffinityScript Multiple Temperature Reverse Transcriptase | 200 rxn | 600109 |
| Universal QPCR Control RNAs | | |
| AffinityScript QPCR cDNA Synthesis Kit | 50 rxn | 600559 |
| Stratagene QPCR Reference Total RNA, Human | 25 µg | 750500 |
| Stratagene QPCR Reference Total RNA, Mouse | 25 µg | 750600 |

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