qPCR Technical Guide

Detection Methods

Primer and Probe Design

Instrumentation

Applications Guide

SIGMA-ALDRICH

Table of Contents

Introduction ... 1

Quantitative PCR: How does it work? ... 2

qPCR Detection Methods ... 3
- Dye-Based Detection ... 3
  - DNA Binding Dyes – How They Work ... 3
  - Melt/Dissociation Curves ... 3
  - Advantages and Disadvantages of Dye-Based Detection ... 4
- Probe-Based Detection ... 5

Primer and Probe Design ... 6
- Linear Probes ... 6
  - Hydrolysis Probes (Dual Labeled Fluorescent Probes or TaqMan) ... 6
  - Hybridization Probes ... 6
- Structured Probes ... 7
  - Molecular Beacons Probes ... 7
  - Scorpions Probes ... 7
- Modifications ... 8
  - Locked Nucleic Acid ... 8
  - Minor Groove Binders ... 9
- Template, Primer Design, Probe Design, Dye Choice and Quenchers ... 9
  - Template Considerations ... 9
  - Primer Design Considerations ... 9
  - Probe Design Considerations ... 10
  - Dye Choice in Probe Design ... 10
  - Quenchers in Probe Design ... 10
  - Primer and Probe Design Software and Web Sites ... 11

Instrumentation ... 12
- Considerations ... 12
  - Components ... 12
  - Sensitivity ... 12
  - Specificity of Detection ... 12
  - Dynamic Range ... 12
  - Detection Linearity ... 12
  - Software ... 12
- High-Throughput qPCR Instruments ... 13
  - Applied Biosystems (ABI) ... 13
  - Roche Applied Science ... 13
  - Stratagene ... 14
  - Techne ... 14
  - Bio-Rad Laboratories ... 15
- Low-Throughput qPCR Systems ... 16
  - Cepheid ... 16
  - Corbett Research ... 16
  - Roche Applied Science ... 16

qPCR Applications Guide ... 17
- Quality of the Template ... 17
- Level of Quantitative PCR Controls ... 17
  - Absolute Quantitation ... 17
  - Relative Quantitation ... 17
  - Qualitative Analysis ... 17
  - Summary ... 17

Optimizing qPCR ... 18
- Guidelines for Optimizing Both qPCR and qRT-PCR ... 18
  - Check Primer Design for Primer-Dimer Potential ... 18
  - Optimize Primer Concentrations ... 18
  - Optimize Probe Concentration ... 19
  - Validate Performance with a Standard Curve ... 20
  - Prepare a Melt Curve ... 21
  - Set the Threshold Value ... 21
- Additional Guidelines for Quantitative Reverse Transcription PCR (qRT-PCR) ... 22
  - Verify RNA Quality ... 22
  - Confirm that Primers Span or Flank Long Introns ... 22
  - Conduct No-Reverse Transcriptase (no-RT) Controls ... 23
  - Optimize Reverse Transcription ... 24
- Additional Optimization for Multiplex Reactions ... 26
  - Check Primer Design ... 26
  - Optimize Primer Concentrations ... 26
  - Optimize Mg^{2+} Concentration ... 26

qPCR Reagent Selection Table ... 28

Troubleshooting ... 29
- Fluorescence Issues ... 29
- Dissociation/Melting Curves ... 30
- Standard Curve ... 31
- qRT-PCR Specific ... 33
- Multiplex ... 33
- Product Specific ... 33

Appendix 1: Traits of Commonly Used Fluorophores ... 34

Appendix 2: How to Optimize Your Quantitative Real-Time RT-PCR ... 35
- Introduction ... 35
- Tissue Sampling and RNA Extraction ... 35
- RNA Quantity and Integrity ... 35
- Reverse Transcription ... 36
- Elevated Fluorescence Acquisition ... 36
- Crossing Point Data Evaluation ... 37

References ... 38

Introduction

The routine study of DNA became practical with the invention of the polymerase chain reaction (PCR) by Kary Mullis in 1983. With the advent of PCR, it was possible to multiply a given DNA segment from complex genetic material millions of times in a few hours using simple equipment. PCR provided researchers with the ability to generate enough genetic material to study gene function and the effects of mutations, offering new possibilities in basic research and diagnostics.

Despite these advances, quantitation of DNA or RNA in cells remained a difficult task until 1993 when Russell Higuchi, *et al.*,[1] introduced real-time, or kinetic, monitoring of DNA amplification. Higuchi's experiments revealed that the relationship between the amount of target DNA and the amount of PCR product generated after a specific number of amplification cycles is linear. This observation formed the basis for real-time quantitative PCR (qPCR).

Numerous qPCR detection chemistries and instruments are now available to answer a wide range of questions. For instance, qPCR can be used to measure viral load or bacterial pathogens in a clinical sample, to verify microarray data, for allelic discrimination or to determine RNA (via cDNA) copy numbers to analyze the level of gene product in a tissue sample.

The Quantitative PCR Technical Guide from Sigma-Aldrich is intended to provide new users with an introduction to qPCR, an understanding of available chemistries, and the ability to apply qPCR to answer research questions. The guide also contains numerous tips and tools for the experienced qPCR user.

Quantitative PCR: How does it work?

Real Time quantitative PCR (qPCR) is very similar to traditional PCR. The major difference being that with qPCR the amount of PCR product is measured after each round of amplification while with traditional PCR, the amount of PCR product is measured only at the end point of amplification.

The concept of qPCR is simple: amplification products are measured as they are produced using a fluorescent label. During amplification, a fluorescent dye binds, either directly or indirectly via a labeled hybridizing probe, to the accumulating DNA molecules, and fluorescence values are recorded during each cycle of the amplification process. The fluorescence signal is directly proportional to DNA concentration over a broad range, and the linear correlation between PCR product and fluorescence intensity is used to calculate the amount of template present at the beginning of the reaction. The point at which fluorescence is first detected as statistically significant above the baseline or background, is called the threshold cycle or Ct Value.

The Ct Value is the most important parameter for quantitative PCR. This threshold must be established to quantify the amount of DNA in the samples. It is inversely correlated to the logarithm of the initial copy number. The threshold should be set above the amplification baseline and within the exponential increase phase (which looks linear in the log phase). Most instruments automatically calculate the threshold level of fluorescence signal by determining the baseline (background) average signal and setting a threshold 10-fold higher than this average.

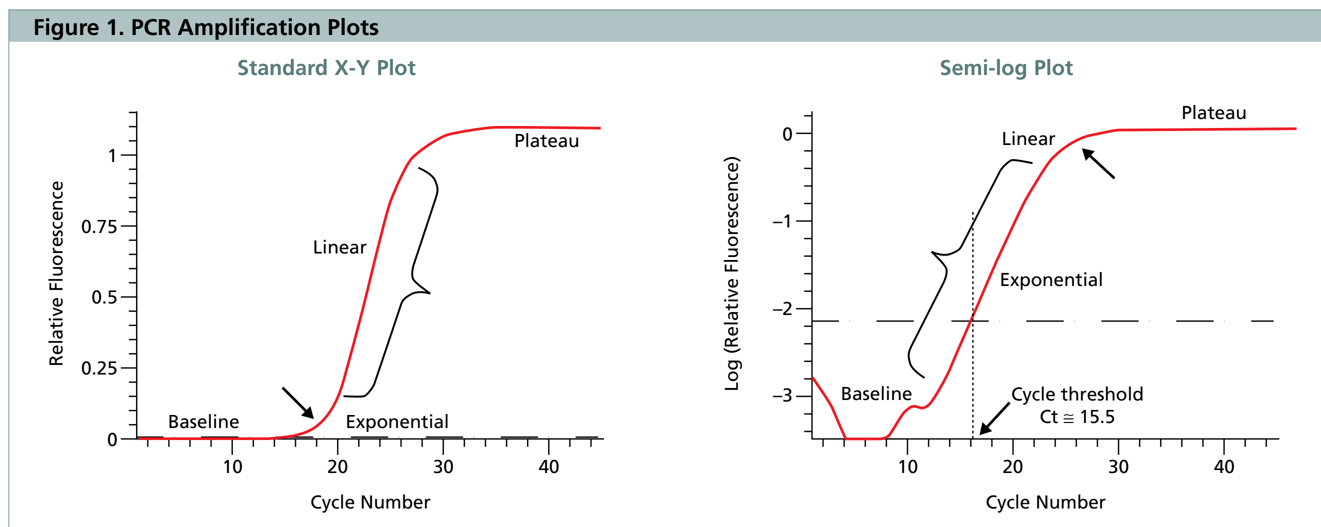
In theory, an equal number of molecules are present in all of the reactions at any given fluorescence level. Therefore, at the threshold level, it is assumed that all reactions contain an equal number of specific amplicons. The higher the initial amount of sample DNA, the sooner the accumulated product is detected in the fluorescence plot, and the lower the Ct value.

There are two ways to graph qPCR fluorescence data: a standard X-Y plot of fluorescence versus cycle number and a semi-log plot of log fluorescence versus cyle number **(Fig. 1)**. Since PCR is a geometric amplification, ideally doubling every cycle, a linear plot of the data should show a classic exponential amplification as it does in the standard X-Y plot. A logarithmic plot of a successful geometric reaction will result in a straight line in the exponential region of the graph. The slope of this portion of the semi-log plot can be used to calculate the efficiency of the PCR.

Both plots can be broken into different regions showing the phases of PCR amplification. The different graphing techniques emphasize different reaction phases. During a typical qPCR experiment, the initial concentration of template is extremely low; therefore the resulting product-related fluorescence is too low to be detected. The background signal is shown as baseline in Figure 1. After the yield has reached the detection threshold, shown as the dotted line, the reaction course can be followed reliably through the exponential phase, which is best tracked in the semi-log plot. Once the reaction reaches significant product inhibition, or limiting reagent, the reaction reaches a linear phase, which is best tracked in the linear plot. After this point, the reaction is at the maximum yield, or the plateau phase.

There are two main methods used to perform quantitative PCR: dye-based, or non-specific detection, and probe-based, or specific detection. Both methods rely on calculating the initial (zero cycle) DNA concentration by extrapolating back from a reliable fluorescent signal.

Figure 1. PCR Amplification Plots



qPCR Detection Methods

Both dye-based and probe-based qPCR detection methods utilize a fluorescent signal to measure the amount of DNA in a sample. Fluorescence results from the molecular absorption of light energy by fluorescent compounds, fluorophores, at one wavelength and the nearly instantaneous re-emission at another, longer wavelength of lower energy. The fluorescence signature of each individual fluorophore is unique in that it provides the wavelengths and amount of light absorbed and emitted. During fluorescence, the absorption of light excites electrons to a higher electronic state where they remain for about 1-10 × 10^{-8} seconds and then they return to the ground state by emitting a photon of energy. The intensity of the emitted light is a measure of the number of photons emitted per second.

Most fluorophores are either heterolytic or polyaromatic hydrocarbons. Fluorescence intensity depends on the efficiency with which fluorophores absorb and emit photons, and their ability to undergo repeated excitation/emission cycles. The maximal absorption and emission wavelengths, and the excitation coefficients, of the most common fluorophores are listed in Appendix 1: Traits of Common Fluorophores.

Figure 2 below shows the emission curves of a selection of common fluorophores.

Dye-Based Detection

Dye-based detection is performed via incorporation of a DNA binding dye in the PCR. The dyes are non-specific and bind to any double-stranded DNA (dsDNA) generated during amplification resulting in the emission of enhanced fluorescence. This allows the initial DNA concentration to be determined with reference to a standard sample.

DNA Binding Dyes – How They Work

DNA binding dyes bind reversibly, but tightly, to DNA by intercalation, minor groove binding, or a combination of both. Most real-time PCR assays that use DNA binding dyes detect the binding of the fluorescent binding dye SYBR® Green I, or the more stable binding dye SYBR® Gold, to DNA. A selection of commonly used DNA binding dyes is presented in Table 1.

Table 1. DNA Binding Dyes

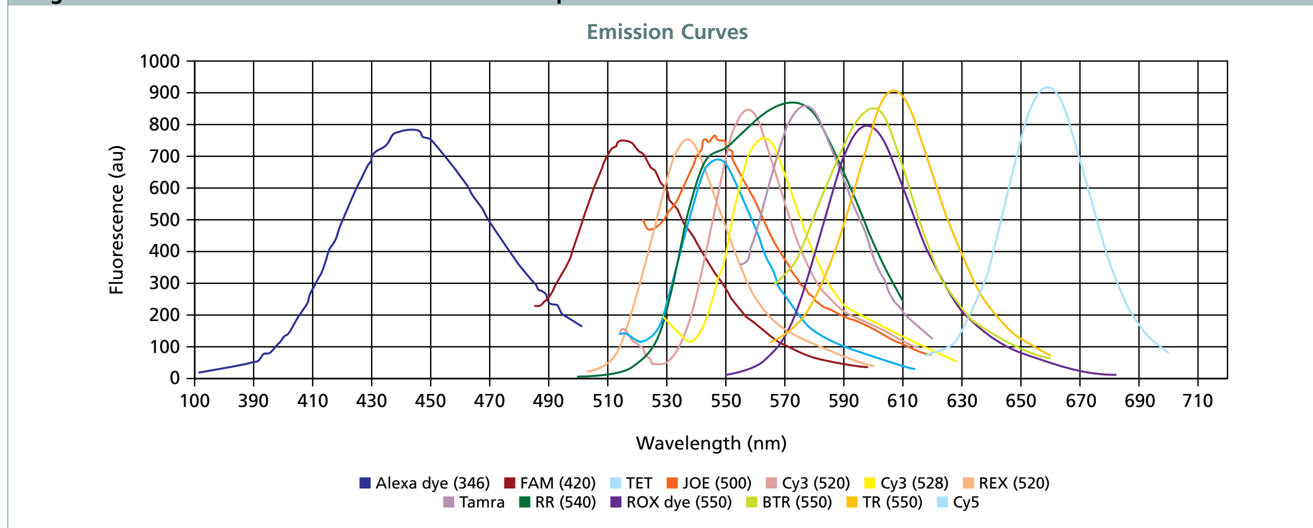
DNA Binding Dyes
SYBR® Green I
SYBR® Gold
YoYo™-1
Yo-Pro™-1
BOXTO
BEBO
Amplifluor™
Quencher-labeled primers I
Quencher-labeled primers II
LUX™ primers

Prior to binding DNA, these dyes exhibit low fluorescence. During amplification, increasing amounts of dye bind to the double-stranded DNA products as they are generated. For SYBR Green I, after excitation at 497 nm (SYBR Gold 495 nm), an increase in emission fluorescence at 520 nm (SYBR Gold 537 nm) results during the polymerization step followed by a decrease as DNA is denatured. Fluorescence measurements are taken at the end of the elongation step of each PCR cycle to allow measurement of DNA in each cycle. See Figure 3, for an illustration of how a dye based assay works. Assays using SYBR Green I binding dye are less specific than conventional PCR with gel detection because the specificity of the reaction is determined entirely by the primers. However, additional specificity can be achieved and the PCR can be verified by melt or dissociation curves.

Melt/Dissociation Curves

Melt curves allow a comparison of the melting temperatures of amplification products. Different dsDNA molecules melt at different temperatures, dependent upon a number of factors including GC content, amplicon length, secondary and tertiary

Figure 2. Fluorescent Emission of Common Fluorophores



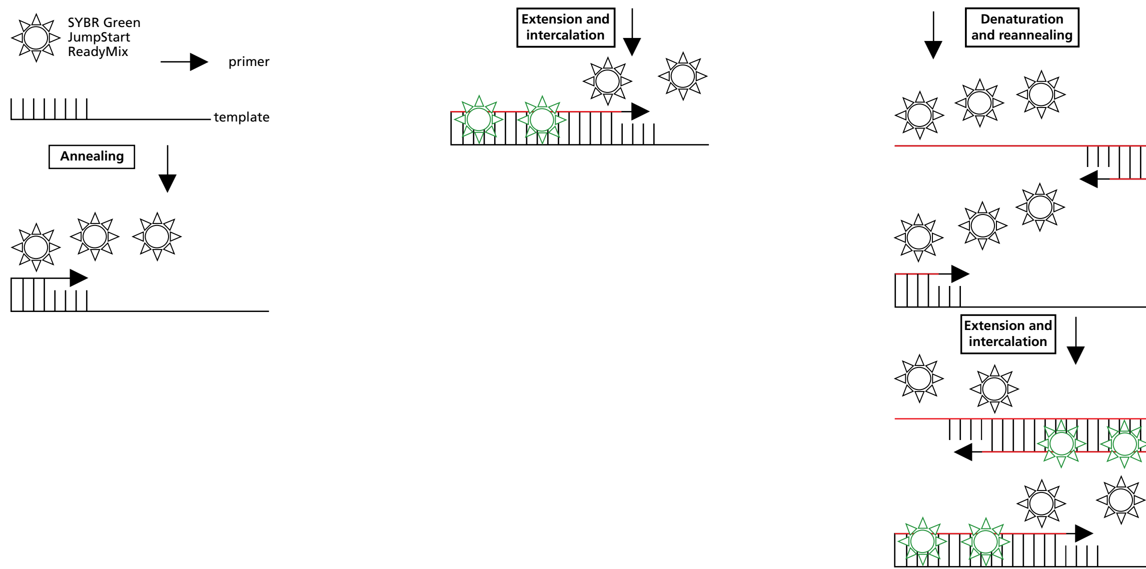
qPCR Detection Methods

Figure 3. Dye-Based qPCR using SYBR Green I Binding Dye

1) The PCR reaction contains enzyme, dNTPs, buffer and SYBR Green I, primers and template. SYBR Green I has no significant fluorescence in the presence of single-stranded DNA.

2) As the reaction progresses, double-stranded products are generated. The SYBR Green I intercalcates into these products and begins to fluoresce.

3) When enough products have accumulated the fluorescence rises above background. This is called the threshold cycle of Ct. The Ct value is used to quantitate the starting amount of template.



structure, and the chemical formulation of the reaction chemistry. To produce melt curves, the final PCR product is exposed to a temperature gradient from about 50 °C to 95 °C while fluorescence readouts are continually collected. This causes denaturation of all dsDNA. The point at which the dsDNA melts into ssDNA is observed as a drop in fluorescence as the dye dissociates. The melt curves are converted to distinct melting peaks by plotting the first negative derivative of the fluorescence as a function of temperature (-dF/dT). Products of different lengths and sequences will melt at different temperatures and are observed as distinct peaks. It is important to note that the populations are not necessarily homogeneous and may contain multiple PCR product species. However, if the PCR assay is fully optimized, it is possible to produce a melting profile that contains only a single peak representing the specific product expected from the primer pair. In this situation, SYBR Green I may be useful for mutation detection as amplicons that differ by a single nucleotide will melt at slightly different temperatures and can be distinguished by their melting peaks. This makes it possible to distinguish homozygotes, a single peak, from heterozygotes, two peaks.[2] Please see **www.corbettresearch.com** for further information about high resolution melt analysis for genotype analysis using dsDNA binding dyes.

Advantages and Disadvantages of Dye-Based Detection

Advantages

- Are the most economical format for detection and quantification of PCR products
- Are an affordable and ideal method for optimizing qPCR reactions
- Uses conventional PCR primers
- Does not require an expensive probe to identify a specific target
- Are useful for generating melt curves, providing an economical solution for genotype analysis

Disadvantages

- Bind non-specifically to any double-stranded DNA
- Cannot be used to compare levels of different targets

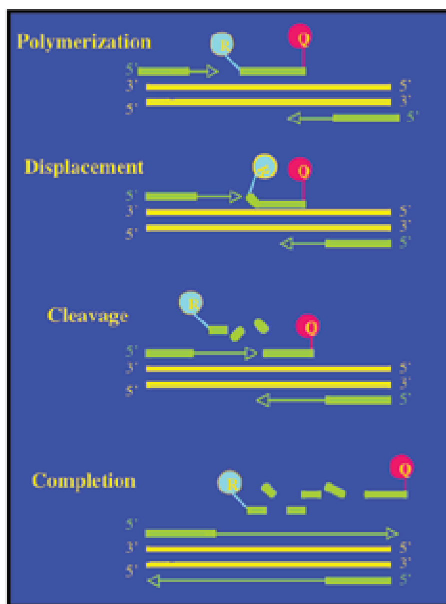
Some of the DNA binding dyes will bind to single-stranded DNA (ssDNA). They also bind indiscriminately to any dsDNA, resulting in non-specific fluorescence and overestimation of the actual product. Non-specific binding results in fluorescence readings in the “no template controls” (NTC) due to dye molecules binding to primer dimers and misprimed products. For RT-PCR assays, separate reverse-transcription, PCR, and DNase treatment can dramatically reduce non-specific priming and provide more accurate quantification when using SYBR Green I binding dye.

Another concern is that multiple dye molecules may bind to a single amplified molecule. Signal intensity is dependent on the mass of dsDNA in the reaction. Even in reactions with the same amplification efficiencies, amplification of a longer product will generate more signal than a shorter one, leading to the implication that there are more copies of the longer template than the shorter one. If amplification efficiencies are different, quantification will be even more inaccurate.

Probe-Based Detection

Probe-based quantitation uses sequence specific DNA-based fluorescent reporter probes. Sequence specific probes result in quantification of the sequence of interest only and not all dsDNA. The probes contain a fluorescent reporter and a quencher to prevent fluorescence. Common fluorescence reporters include derivatives of fluorescein, rhodamine and cyanine. Quenching is the process of reducing the quantum yield of a given fluorescence process. Quenching molecules accept energy from the fluorophore and dissipate it by either proximal quenching or by Fluorescence Resonance Energy Transfer (FRET, see Fig. 4).[3] Most reporter systems utilize FRET or similar interactions between the donor and quencher molecules in order to create differences in fluorescence levels when target sequences are detected. The fluorescent reporter and the quencher are located in close proximity to each other in order for the quencher to prevent fluorescence. Once the probe locates and hybridizes to the complementary target, the reporter and quencher are separated. The means by which they are separated varies depending on the type of probe used. Separation relieves quenching and a fluorescent signal is generated. The signal is then measured to quantitate the amount of DNA. The main advantage to using probes is the specificity and sensitivity they afford. Their major disadvantage is cost.

Figure 4. Fluorescence Resonance Energy Transfer



Denaturation: Double-stranded DNA is heated to 94 °C-98 °C. During this period, the double-stranded DNA helix melts open into two single-stranded templates.
Annealing: The reaction is cooled to 45 °C-65 °C. Probes labeled with both a Reporter molecule and a Quencher molecule and sequence-specific primers anneal to the single-stranded DNA template. During this cycle, DNA polymerase attaches to the primed template and begins to incorporate complementary nucleotides (dATP, dCTP, dGTP, TTP). This process is very slow because the polymerase is inefficient at these lower temperatures.
Extension: The temperature is raised slightly during the extension cycle to 65 °C-75 °C. The optimal temperature for Taq DNA polymerase is 72 °C. During this phase, DNA polymerase extends the sequence-specific primer with the incorporation of nucleotides that are complementary to the DNA template. The DNA polymerase then displaces the Reporter molecule from the probe resulting in fluorescence. The fluorescence accumulates as cycling of PCR continues and is measured at the end of each PCR cycle. The intensity of fluorescence generated by the Reporter molecule above background level (Ct value) is measured and used to quantitate the amount of newly generated double-stranded DNA strands.
Replicated DNA: After repeating the denaturation, annealing and extension cycles approximately 35-40 times, analysis can begin. The Ct values can be used to quantitate starting amounts of DNA or to establish a standard curve for gene expression studies or other comparative analysis.

Probe-based quantitation can also be used for multiplexing: detecting multiple targets in a single reaction. Different probes can be labeled with different reporter fluorophores allowing the detection of amplification products from several distinct sequences in a single qPCR reaction. See Appendix 2 for an Applications Note on Multiplexing.

Many probe-based detection systems are available and they are all variations on a single theme with significant differences in complexity, cost, and the results obtained. Therefore, it is important that the chemistry selected is appropriate for the intended application. For an overview of available specific chemistries, see Table 2.

Chapter 3 contains additional information on available probes and primers for specific quantitation to aid in the understanding and selection of the ideal chemistry.

Table 2. Probe-Based Chemistries and Modifications

Probe-Based Chemistries	
Linear Probes	**Structured Probes**
Hydrolysis probes	Molecular Beacons
Hybridization probes	Scorpions™
Probe Modifications	
Locked Nucleic Acids (LNA)	Minor Groove Binders (MGB)

Primer and Probe Design

Various primer and probe formats are available for performing qPCR assays. The vast majority of assays use the 5′ nuclease format. This format functions by release and generation of a fluorescent signal due to the inherent nuclease activity of the polymerase used. The assay requires a pair of PCR primers and a probe labeled with 5′ reporter and 3′ quencher molecules. While this method is the most widely used, there are several other formats available that may offer advantages in certain situations. Examples include the SYBR Green intercalating dye detection method, the Molecular Beacon technology and probes with a more complex structure, such as the Scorpions. Finally, increasing use is also being made of modifications in primer and probe design. Locked Nucleic Acids® (LNAs) and Minor Groove Binders (MGB™) provide significant advantages in certain assays that may not be as amenable to the more traditional approaches. The methods available fall into two different categories: linear probes and structured probes.

Linear Probes

The major advantages of linear probes are that the absence of secondary structure allows for optimum hybridization efficiency, and they are extremely simple to design and use. The most common linear probes are described below. All probes included in this section are FRET based.

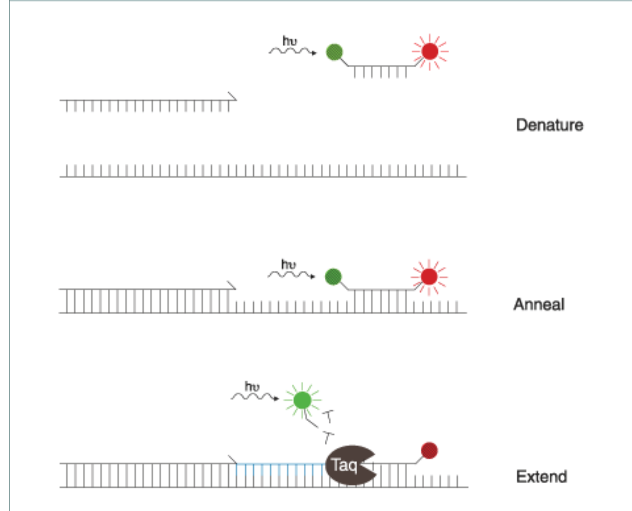
Hydrolysis Probes

Also known as Dual Labeled Fluorescent Probes (DLFP) or TaqMan®

Developed by: Roche Molecular Systems

How They Work: The TaqMan method relies on the 5′-3′ exonuclease activity of *Thermus aquaticus (Taq)* DNA polymerase to cleave a labeled probe when it is hybridized to a complementary target. A fluorophore is attached to the 5′ end of the probe and a quencher to the 3′ end. If no amplicon complementary to the probe is present, the probe remains intact and low fluorescence is detected. If the PCR results in a complementary target, the probe binds to it during each annealing step of the PCR. The double-strand-specific 5′-3′ nuclease activity of the *Taq* enzyme displaces the 5′ end of the probe and then degrades it. This process releases the fluorophore and quencher into solution, spatially separating them, and leads to an irreversible increase in fluorescence from the reporter. Reaction conditions must be controlled to ensure that the probe hybridizes to the template prior to elongation from primers. The probe is usually designed to hybridize at 8-10 °C above the Tm of the primers and to perform the elongation step at a lower temperature to ensure maximum 5′-3′ exonuclease activity of the polymerase. Since this also reduces enzyme processivity, short amplicons are designed. Figure 5 illustrates how these probes work.

Figure 5. Function of Hydrolysis Probes



Advantage: This is the most popular qPCR chemistry and relies on the activity of *Taq* DNA polymerase.

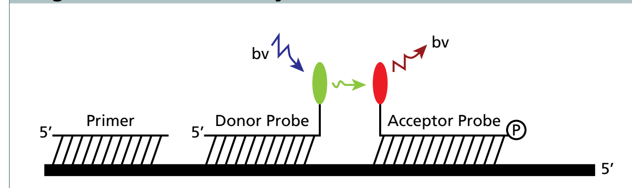
Uses: Undoubtedly the chemistry of choice for most quantification applications and for those requiring multiplexing.

Hybridization Probes

Developed by: Developed specifically for use with the Idaho Technology/Roche capillary-based instrument, but can be used with many real-time instruments.

How They Work: Two probes are designed to bind adjacent to one another on the amplicon. One has a donor dye at its 3′ end, FAM for example. The other has an acceptor dye on its 5′ end, such as LightCycler® Red 640 or 705, and is blocked at its 3′ end to prevent extension during the annealing step. Both probes hybridize to the target sequence in a head-to-tail arrangement during the annealing step. The reporter is excited and passes its energy to the acceptor dye through FRET and the intensity of the light emitted is measured by the second probe. Figure 6 illustrates how these probes work.

Figure 6. Function of Hybridization Probes



Hybridization probes produce fluorescence when both are annealed to a single strand of amplification product. The transfer of resonance energy from the donor fluorophore (3′-fluorescein) to the acceptor fluorophore (5′-LC Red 640) is a process known as fluorescence resonance energy transfer.

Advantages: Since the probes are not hydrolyzed, fluorescence is reversible and allows for the generation of melt curves.

Uses: Can be used for SNP/mutation detection, where one probe is positioned over the polymorphic site and the mismatch causes the probe to dissociate at a different temperature to the fully complementary amplicon.[4] As stated above, they can also be used to generate melt curves.

Structured Probes

Thermodynamic analysis reveals that structurally constrained probes have higher hybridization specificity than linear probes.[5,6] Structurally constrained probes also have a much greater specificity for mismatch discrimination due to the fact that in the absence of target they form fewer conformations than unstructured probes, resulting in an increase in entropy and the free energy of hybridization. The following outlines the traits of the two most common structured probes.

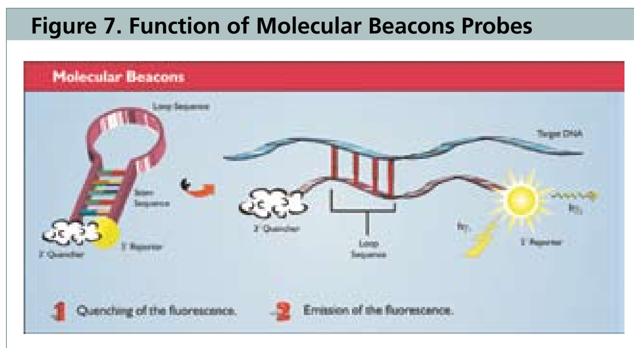
Molecular Beacons Probes

Developed by: Public Health Research Institute in New York

How They Work: They consist of a hairpin-loop structure that forms the probe region and a stem formed by annealing of complementary termini. One end of the stem has a reporter fluorophore attached and the other, a quencher. In solution, the probes adopt the hairpin structure and the stem keeps the arms in close proximity resulting in efficient quenching of the fluorophore.

During the annealing step, the probe is excited by light from the PCR instrument ($h\upsilon_1$). Molecular Beacons hybridize to their target sequence causing the hairpin-loop structure to open and separate the 5′-end reporter dye from the 3′-end quencher. The quencher is no longer close enough to absorb the emission from the reporter dye. This results in fluorescence of the dye and the PCR instrument detects the increase of emitted energy ($h\upsilon_2$). The resulting fluorescent signal is directly proportional to the amount of target DNA. If the target DNA sequence does not exactly match the Molecular Beacon probe sequence, hybridization and fluorescence does not occur. When the temperature is raised to allow primer extension, the Molecular Beacons probes dissociate from the targets and the process is repeated with subsequent PCR cycles. See Figure 7 for an illustration of how these probes work.

Figure 7. Function of Molecular Beacons Probes



Advantage: Greater specificity for mismatch discrimination due to structural constraints.

Disadvantage: The main disadvantage associated with Molecular Beacons is the accurate design of the hybridization probe. Optimal design of the Molecular Beacon stem annealing strength is crucial. This process is simplified with the use of specific software packages such as Beacon Designer from Premier Biosoft or by contacting a member of the design team at **www.designmyprobe.com** for assistance.

Uses: Molecular Beacons[7] probes have become popular for standard analyses such as quantification of DNA and RNA.[8] They have also been used for monitoring intracellular mRNA hybridization,[9] RNA processing,[10] and transcription[11] in living cells in real-time. They are also ideally suited to SNP/mutation analysis[12] as they can readily detect single nucleotide differences[13,14] and have been reported to be reliable for analysis of G/C-rich sequences.[15] The sensitivity of Molecular Beacons probes permits their use for the accurate detection of mRNA from single cells.[16] They have been used in fourplex assays to discriminate between as few as 10 copies of one retrovirus in the presence of 1 × 10^5 copies of another retrovirus.[17]

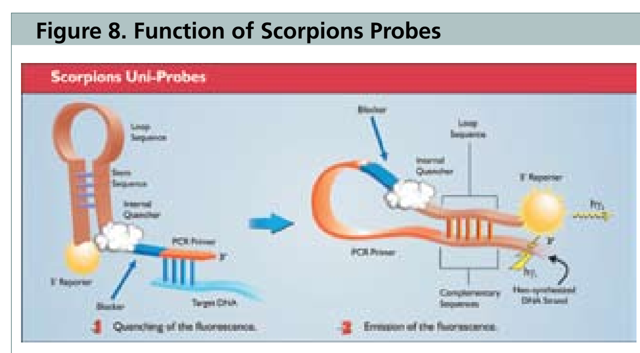
Scorpions™ Probes

Developed by: DsX, Ltd.

How They Work: The Scorpions uni-probe consists of a single-stranded bi-labeled fluorescent probe sequence held in a hairpin-loop conformation (approx. 20 to 25 nt) by complementary stem sequences (approx. 4 to 6 nt) on both ends of the probe. The probe contains a 5′-end reporter dye and an internal quencher dye directly linked to the 5′-end of a PCR primer via a blocker. The blocker prevents *Taq* DNA polymerase from extending the PCR primer. The close proximity of the reporter dye to the quencher dye causes the quenching of the reporter's natural fluorescence.

At the beginning of the real-time quantitative PCR reaction, *Taq* DNA polymerase extends the PCR primer and synthesizes the complementary strand of the specific target sequence. During the next cycle, the hairpin-loop unfolds and the loop region of the probe hybridizes intra-molecularly to the newly synthesized target sequence. The reporter is excited by light from the real-time quantitative PCR instrument ($h\upsilon_1$). Now that the reporter dye is no longer in close proximity to the quencher dye, fluorescence emission may take place ($h\upsilon_2$). The significant increase of the fluorescent signal is detected by the real-time PCR instrument and is directly proportional to the amount of target DNA. See Figure 8 for an illustration of how these probes work.

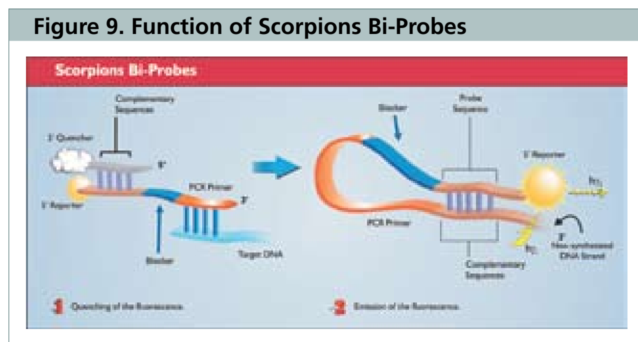
Figure 8. Function of Scorpions Probes



Scorpions bi-probe, or duplex Scorpions probes, were developed to increase the separation of the fluorophore and the quencher. The Scorpions bi-probe is a duplex of two complementary labeled oligonucleotides, where the specific primer, PCR blocker region, probe, and fluorophore make up one oligonucleotide, and the quencher is linked to the 3′-end of a second oligonucleotide that

is complementary to the probe sequence. The mechanism of action is essentially the same as the uni-probe format.[18] This arrangement retains the intramolecular probing mechanism resulting in improved signal intensity over the standard Scorpions uni-probe format. See Figure 9 for an illustration of how Scorpions bi-probes work.

Figure 9. Function of Scorpions Bi-Probes



Advantages: Scorpions probes combine the primer and probe in one molecule converting priming and probing into a unimolecular event. A unimolecular event is kinetically favorable and highly efficient due to covalent attachment of the probe to the target amplicon ensuring that each probe has a target in the vicinity.[19] Enzymatic cleavage is not required so the reaction is very rapid. This allows introduction of more rapid cycling conditions combined with a significantly stronger signal compared to both TaqMan probes and Molecular Beacons probes.[20] Another advantage over TaqMan assays is that the PCR reaction is carried out at the optimal temperature for the polymerase rather than at the reduced temperature required for the 5′-nuclease activity to displace and cleave the probe. The most important benefit is that there is a one-to-one relationship between the number of amplicons generated and the amount of fluorescence produced.

Uses: Scorpions probes are ideally suited to SNP/mutation detection and have been used to detect, type and quantitate human papillomaviruses.[21] SNP detection can be carried out either by allele specific hybridization or by allele specific extension. If the probe sequence is allele-specific, allelic variants of a SNP can be detected in a single reaction by labeling the two versions of the probe with different fluorophores. Alternatively, the PCR primer can be designed to selectively amplify only one allele of a SNP. Results with Scorpions probes compare favorably with the high signal/high background ratio of the TaqMan probes and low signal/low background ratio of Molecular Beacons probes.

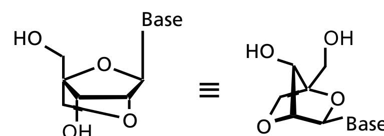
Modifications

A number of modifications can be incorporated when designing probes to provide enhanced performance. Locked Nucleic Acids and Minor Grove Binders are two of the most commonly used modifications.

Locked Nucleic Acid

A Locked Nucleic Acid (LNA) is a novel type of nucleic acid analog that contains a 2′-O, 4′-C methylene bridge. The bridge is locked in 3′-endo conformation restricting the flexibility of the ribofuranose ring and locking the structure into a rigid bicyclic formation. This structure confers enhanced hybridization performance and exceptional biological stability.

LNA Monomer



Advantages

- Increased thermal duplex stability[22]
- Improved specificity of probe hybridization to target sequence[23]
- Enhanced allelic discrimination
- Added flexibility in probe design
- Compatible with many systems

Increased Stability and Improved Specificity

The LNA monomer chemical structure enhances the stability of the hybridization of the probe to its target. As a result, the duplex melting temperature, Tm, may increase by up to 8 °C per LNA monomer substitution in medium salt conditions compared to a DNA fluorescent probe for the same target sequence, depending on the target nucleic acid.[24] This increase in hybridization creates a significant broadening in the scope of assay conditions and allows for more successful single-tube multiplexing.[25] Additionally, this benefit also makes it possible to optimize the Tm level and thus the hybridization specificity via placement of the LNA base(s) in the probe design.[26] By increasing the stability and the specificity, background fluorescence from spurious binding is reduced and the signal-to-noise ratio is increased.

Enhanced Allelic Discrimination

LNAs can also be used for allelic discrimination. They provide an extremely reliable and effective means for SNP-calling in genotyping applications. The presence of a single base mismatch has a greater destabilizing effect on the duplex formation between a LNA fluorescent probe and its target nucleic acid than with a conventional DNA fluorescent probe. Shorter probes incorporating LNA bases can be used at the same temperatures as longer conventional DNA probes.

Added Flexibility in Probe Design

Due to the enhanced hybridization characteristics and the Tm contribution, LNA containing qPCR probes can be synthesized to be shorter, allowing flexibility in design while still satisfying assay design guidelines. As such, certain design limitations that cannot be overcome with standard DNA chemistries can be reduced or eliminated. For instance, shorter probes can be designed to address traditionally problematic target sequences, such as AT- or GC-rich regions. LNAs also facilitate the querying of difficult or inaccessible SNPs, such as the relatively stable G:T mismatch.

LNAs can also be used when AT-rich qPCR probes need to be over 30 bases long to satisfy amplicon design guidelines. With LNA fluorescent probes, the selective placement of LNA base substitutions facilitates optimal design of highly specific, shorter probes that perform very well, even at lengths of 13-20 bases.

Compatible with Many Platforms

LNA probes are also compatible with real-time PCR platforms and end-point analytical detection instruments depending on the excitation/emission wavelengths of the dyes and of the equipment. This provides the ability to work with the instrumentation and reagents of choice under universal cycling conditions.

Minor Groove Binders

Minor Groove Binders (MGBs) are a group of naturally occurring antibiotics. They are long, flat molecules that can adopt a crescent shape allowing them to bind to duplex DNA in the minor groove. MGBs are stabilized in the minor groove by either hydrogen bonds or hydrophobic interactions. This results in the stabilization of the probe-DNA hybridization.

MGBs produce a "Tm leveling" effect as A/T content increases. Natural 8-mer probes exhibit a linear decrease in Tm as A/T content increases while MGB probes exhibit level Tm as A/T content increases. The increase in melting temperature allows for the use of shorter probes with improved mismatch discrimination.

Advantages

- Useful for functional assays for difficult targets
- Higher accuracy and confidence in results
- Compatible with any real-time PCR detection system

Functional Assays for Difficult Targets

There are many sequence features and applications that pose problems when trying to design probes and primers for specific targets. For example, sequential "Gs" result in secondary structure that can block efficient hybridization, weaker bonds within "A/T" rich regions result in lower melting temperatures (Tm) that inhibit the use of efficient PCR conditions, and limited target regions can restrict the length of optimal probe and primer sequences impacting their functionality. Since MGBs allow for design of shorter probes and for the design of specific Tms, they aid in the success of assays posing these difficulties.

Higher Accuracy and Confidence in Results

Since shorter probes can be used, mismatch sensitivity can be increased with the use of MGBs. Also, since MGB probes have more stable hybridization characteristics and remain intact after PCR, they are not cleaved by the 5′-nuclease activity of *Taq* DNA polymerase. As a result, more symmetric melting curves using the same probe and primer set in the same tube as the real-timer PCR experiments can be produced to confirm PCR and detection specificity.

Compatible with Any Real-Time PCR Instrument

MGB Eclipse probe systems will work on any manufacturer's real-time or end-point analytical instrumentation. The probes have been tested on most leading systems.

Template, Primer Design, Probe Design and Dye Choice

It is very important to ensure that the primers and probes are optimally designed in order to perform successful quantitative PCR. There are many points in the design process that must be considered: the template, primer design, probe design, dye choice in probe design, quenchers in probe design, and the software the system uses.

Template Considerations

1. Amplicons should ideally be between 50-150 bases in length.
Shorter amplicons tend to be more tolerant of less than ideal reaction conditions and allow probes to successfully compete with the complementary strand of the amplicon. This allows for faster and more efficient reactions and increased consistency of results.

2. For RNA targets, select primers spanning exon-exon junctions.
By choosing primers that span exon-exon junctions, amplification of contaminating genomic DNA in cDNA targets can be avoided.

3. Consideration of template secondary structure is important.
Close attention should be paid to template secondary structure. This is one area where qPCR primer and probe design differs slightly from traditional PCR design. In traditional PCR design, template secondary structure is not necessarily a critical factor. Templates containing homopolymeric stretches of greater than four consecutive bases should be avoided. Significant secondary structure may hinder primers from annealing and prevent complete product extension by the polymerase. This is a particular concern in cases of stretches of Gs. A useful tool for the assessment of template secondary structure in DNA targets is the Mfold server, developed by Dr. Michael Zuker and maintained at the Rensselaer and Wadsworth Bioinformatics Center. **(http://www.bioinfo.rpi.edu/applications/mfold/)**

Primer Design Considerations

1. Avoid secondary structure problems.
When feasible, one should attempt to pick regions of the template that are not apt to cause secondary structure problems. For more information, please refer to number 3 under "Template Design Considerations."

2. Avoid runs of Gs and Cs longer than three bases.
In the primer sequences, runs of Gs and Cs longer than three bases should be avoided. This is very important at the 3′-end of a primer where such runs may result in a phenomenon referred to as polymerase slippage, which can be a problem in standard PCR. Polymerase slippage occurs during replication when *Taq* polymerase slips from the DNA template strand at the repeat region and then reattaches at a distant site. This can cause a new DNA strand to contain an expanded section of DNA.

Primer and Probe Design

3. **Aim to design primers with a Tm higher than the Tm of any of the predicted template secondary structures.**
   To be sure that the majority of possible secondary structures have been unfolded before the primer-annealing step, one should also aim to design primers with a melting temperature, Tm, higher than the Tm of any of the predicted template secondary structures. Again, the Mfold server is a very useful resource in this phase of the design process.
4. **Design primers longer than 17 bases.**
   Shorter primers increase the chance of random, or non-specific, primer binding. Such random primer binding may be more marked in targets of higher complexity, such as genomic DNA, and should be guarded against even when using more predictable target sequences, such as plasmids. Even with a plasmid, it is not always the case that the sequence is completely known.
5. **Check the overall specificity of primers (and probes) by carrying out a BLAST search.**
   BLAST, or Basic Local Alignment Search Tool, searches can be performed using the resources of the publicly available site at NCBI at **http://www.ncbi.nlm.nih.gov/**. A BLAST search compares primer sequences against a library of genomic DNA sequences. Another method is to use electronic PCR, or e-PCR. e-PCR is used to identify sequence tagged sites (STS) within DNA sequences by searching for sub-sequences that closely match the PCR primers. STSs are short DNA segments occurring once in the human genome. Their exact location and sequence are known and, as a result, they can serve as landmarks in the human genome. e-PCR uses UniSTS, a database of STSs, to identify primers that have the proper order that could represent PCR primers used to generate the known STSs. This tool is available at **http://www.ncbi.nlm.nih.gov/sutils/e-pcr/**.
6. **Forward and reverse PCR primers should be analyzed for self-complementarity in their sequences.**
   In particular, 3′ self-complementarity primer-dimer formation should be avoided. A number of commercial primer analysis resources are available to aid in this process.
7. **Multiplexed primer pairs must all work efficiently at the same annealing temperature.**

Probe Design Considerations

1. **Probes may be anywhere from 9-40 bases in length.**
   In the case of the 5′-nuclease assay using dual-labeled probes, probes with a reporter and a quencher, the overall probe length tends to range up to 30 bases. Longer probes might compromise the efficiency of signal quenching. In the case of Molecular Beacons probes, where quenching is a function of the self-annealing hairpin design, probes may be a little longer, perhaps up to 35 bases.
2. **In most situations, the probe Tm should be approximately 10 °C higher than the Tm of the primers.**
   This allows for efficient probe-to-target annealing during the reaction. During the reaction, the probe should anneal before the primers. When probes anneal before the primers, shorter probes can be used. The use of shorter probes is important during mismatch detection. In such cases, the use of special modifying bases, or Locked Nucleic Acid® (LNA®) fluorescent probes may be beneficial. LNA fluorescent probes are modified RNA nucleotides that exhibit thermal stabilities towards complementary DNA and RNA. LNA probes serve to increase the thermal stability and hybridization stability, allowing for more accurate gene quantitation and allelic discrimination and providing easier and more flexible probe designs for problematic target sequences.
3. **Probe GC content may be anywhere from 30-80% preferably with a greater number of Cs than Gs.**
   As in the case of primers, there should not be homopolymeric runs of single bases, most definitely not of Gs, in probes.
4. **For optimal efficiency of dual-labeled probes, the 5′-terminal of the probe (carrying the reporter dye) should be as close as possible to the 3′-end of the forward primer.**
   For Molecular Beacons probes, the probe should be designed to anneal in the middle of the amplicon.
5. **A G placed at the extreme 5′ end of the probe adjacent to the reporter dye should be avoided.**
   This may lead to spontaneous fluorophore quenching.

Dye Choice in Probe Design

It is also important to consider the dye choice when designing probes.

1. **Make sure that the instrument being used can detect the dyes.**
2. **When designing fluorescent probes, it is important to be sure that the fluorophore and quencher are compatible.**
3. **When designing multiplexed reactions, spectral overlap should be minimized.**

Table 3 provides the excitation wavelength and the emission wavelength for several popular dyes used in probe design.

Table 3. Dye Choice in Probe Design

Filter	Excitation Wv	Emission Wv
Alexa 350	350	440
FAM/SYBR Green	492	516
TET	517	538
HEX/JOE/VIC	535	555
Cy3	545	568
TAMARA	556	580
ROX/Texas Red	585	610
Cy5	635	665

Quenchers in Probe Design

FRET occurs when donor and acceptor molecules are separated by about 100 Å. Since a helix occupies approximately 3.4 Å, the maximum distance between a reporter and its quencher on a linear probe should not exceed approximately 30 bases. The acceptor can be another fluorophore, in which case the transfer releases the energy from the quencher as fluorescence at a longer

wavelength. For instance, the combination of FAM (a fluorescein derivative) and TAMRA (a rhodamine derivative) will absorb at 492 nm (excitation peak for FAM) and emit at 580 nm (emission peak for TAMRA). The inherent fluorescence and broad emission spectrum of TAMRA result in a poor signal-to-noise ratio when this fluorophore is used as a quencher. This can make multiplexing difficult. To address this issue, dark quenchers were introduced.

Dark quenchers absorb the energy emitted by the reporter fluorophore and emit heat rather than fluorescence. Early dark quenchers, such as 4-(49-dimethylaminophenylazo) benzoic acid (DABCYL), had limited spectral overlap between the fluorescent dye and quencher molecule. Black Hole Quenchers (BHQ™-1 and BHQ™-2) from Biosearch Technologies have lower background fluorescence and a broad effective range of absorption. As a result, Black Hole Quenchers provide greater sensitivity and enable the simultaneous use of a wide range of reporter fluorophores thus expanding the options available for multiplex assays. It is important to ensure that the Black Hole Quencher is matched with the probe based on the excitation and emission spectra of the probe.

Table 4. Quencher Ranges

Quencher	Quenching Range
BHQ-1	480-580 nm
BHQ-2	550-650 nm
BHQ-3	620-730 nm
TAMARA	550-576 nm
DABCYL	453 nm

Primer and Probe Design Software and Web Sites

Several software programs can be used to design primers and probes, but software is only a tool to aid in the design process and cannot guarantee a perfect design.

Designing Primers

- Primer 3.0

Designing TaqMan Probes or Molecular Beacons Probes

- Beacon Designer from Premier Biosoft

Designing Scorpions Probes

- DNA Software at dnasoftware.com

Designing FRET Probes (LightCycler Probes)

- LightCycler Probe Design Software 2.0

Designing LNA Probes or Oligos

- A combination of different types of software must be used

Primer and Probe Design FAQs

What type of probe should I choose?

It depends on the experiment being performed and the machine being used.

Table 5 is intended to aid in the selection of probes based on the desired application.

Table 5. Probe Applications

Application	TaqMan/ Hydrolysis	Hybridization	Molecular Beacons	Scorpions
Quantification applications	X		X	
Multiplexing	X		X	
SNP/mutation detection		X	X	X
Generation of melt curves		X		
Monitoring intracellular mRNA hybridization, RNA processing, and transcription in living cells			X	
Analysis of G/C-rich sequences			X	
Detection of mRNA from single cells			X	

Why do I need to have a good design?

Well-designed primers and probes will provide ideal RT-qPCR data: high PCR efficiency, specific PCR products and the most sensitive results.

What should I do if no primers and/or probes are found for my sequence?

There are several parameters that can be adjusted to force the program to pick primers and/or probes without significantly sacrificing primer and/or probe quality.

I have a very short target sequence. Is it possible to design an optimal probe?

It depends on the sequence. To design the probe, it may be necessary to submit a longer target sequence (up to 160 bases). Additionally, LNA bases can be included in the probe sequence to reduce the probe length while retaining the optimal characteristic of the probe.

Instrumentation

Considerations

When Higuchi *et. al*[1] designed a qPCR system, a conventional PCR block, a UV light source and a camera for signal detection were the instruments used. Since the design of the first qPCR instrument, there have been many advances in qPCR instrumentation, but the requirements for a system have remained fundamentally the same. When choosing an instrument for qPCR, it is important to consider the following traits of the system: Components; Sensitivity; Specificity of Detection; Dynamic Range; Detection Linearity; Software.

Components

During instrument development, it became clear that certain components of the system required specific traits in order to be used for quantitative studies.

Conventional PCR thermal blocks were notorious for a lack of uniformity that resulted in the "edge effect." Lack of uniformity caused differences in PCR efficiency and product quantity. Since this situation is contrary to the needs of quantitative PCR, many manufacturers invested in unique thermal regulation mechanisms to reduce this effect.

Alternative excitation sources were also developed, with the first source being the laser. The major advantage, and also the disadvantage, of using lasers is that they emit at a single wavelength. A single wavelength allows for the specificity of excitation for a single dye but does not allow the flexibility to include additional dyes for multiplexing. The cost and application disadvantages were resolved by using broad wavelength excitation sources, tungsten bulbs or light emitting diodes (LEDs), in conjunction with specific light filters.

Many companies have continued to use silicon-based, charge-coupled device (CCD) cameras while others use photo multiplier tubes (PMTs) as photo detectors.

While the merits of one hardware design over another can be debated, the critical factors to consider when selecting a qPCR system are sensitivity, specificity of detection, dynamic range and detection linearity.

Sensitivity

Sensitivity refers to the minimum quantity of target that can be detected above the background noise of the system. Sensitivity is not only instrument dependent, but is also chemistry dependent. As a result, it is important to carry out comparisons between systems using exactly the same target and detection chemistry.

Specificity of Detection

Specificity of detection is important at the single dye level because it is essential that the signal detected is generated specifically from the detection chemistry and not from alternative sources or neighboring reactions on the plate. One significant example is termed "cross talk." Cross talk occurs in multiplex reactions when signal from one fluorescent dye is detected as signal from the other fluorescent dye in the reaction.

Dynamic Range

The dynamic range of detection is the difference between the minimum and maximum target concentrations that can be detected. The minimum detectable signal is determined by chemistry and signal-to-noise discrimination of the system. The maximum signal is restricted by chemistry and the range of signal detection is determined by the photo detector as well as by the software analyses capabilities.

Detection Linearity

Detection linearity is the concentration range that an instrument is able to linearly measure.

Software

For many scientists, the choice of the system will be largely dependant upon the capabilities of the integrated software. Some packages are targeted towards simplicity of analysis with little, if any, input from the end user. In contrast, other packages are fully flexible. In the case of full flexibility, every point of raw data is accessible and the scientist chooses among the extensive analysis choices. A second consideration is whether having raw fluorescence data would be useful, as in the case of using a fluorescent thermocycler as a thermostated fluorometer. An additional consideration is the presentation of data. The manner in which data is presented varies considerably among the current market offerings. Some manufacturers depend on commercial graphics packages while others simply list Ct values.

Comparing qPCR Instrumentation

As stated above, it is important to compare systems using exactly the same target and detection chemistry. When comparing systems, it is important to perform the following tests:

1. Uniformity–Thermal
   a. Run a calibrated end-point PCR and detect on a gel.
2. Uniformity–Optical
   a. Measure the fluorescence of identical concentrations of dye aliquoted to every well.
   b. Compare the starting fluorescence levels for identical qPCR probes allocated to every well of the plate.
3. Uniformity–Thermal and Optical
   a. Run identical qPCR experiments in every well and compare Ct values.
4. Dynamic Range–Optical
   a. Plot fluorescence measurements of a serial dye dilution. It is important to note that while optical dynamic range is significant, qPCR is based upon Ct measurement, which is approximated by the minimum above-background fluorescence. This specification is relevant to instruments that can also be used as fluorescent microplate readers.
5. Dynamic Range–Reaction
   a. Perform qPCR on a serially diluted template (1 copy to 10^{12} copies).
6. Wavelength Discrimination
   a. This is the ability to accurately measure fluorescence intensity from a fluorophore in a mixture of fluorophores.

On the following pages, qPCR instruments are categorized into two groups: high throughput and low throughput. Any device that processes fewer than 96 samples in a run will be called low throughput and anything that processes 96 samples or more will be considered high throughput. All qPCR instruments require a computer for operation and data management/analysis. Nearly all instruments are supplied with a computer and the appropriate software.

High-Throughput qPCR Instruments

Manufacturer System	Applied Biosystems ABI 7300 Real-Time PCR System	Applied Biosystems ABI 7500 Real-Time PCR System	Applied Biosystems ABI 7900HT Fast Real-Time PCR System	Roche Applied Science LightCycler® 480 Real-Time PCR System
Advantages	Affordable, 4 color multiplexing	5 color multiplexing. Rapid cycling upgrade available in the ABI 7500 Fast that allows run times of <40 min. TaqMan assays are available	96- and 384-well plate compatible. The system can handle TaqMan Low Density Arrays with fully automated robotic loading. Optional Fast real-time PCR capability. TaqMan assays are available	Interchangeable 96- and 384-well thermal blocks. The Therma-Base design provides added temperature uniformity
Excitation	Tungsten-halogen lamp, single-excitation filter	Tungsten-halogen lamp, 5 excitation filters	Extended-life 488 nm argon-ion laser is distributed to all wells by a dual-axis synchronous scanning head	Filtered (450, 483, 523, 558 and 615 nm) high-intensity Xenon lamp (430–630 nm)
Detection	4 emission filters and a CCD camera	5 emission filters and a CCD camera	CCD camera and a spectrograph	Filters (500, 533, 568, 610, 640, 670 nm) and a CCD camera
Heat block	Peltier	Peltier	Peltier	Peltier plus Therma-Base
Rxn volume(s):	20-100 µL	25-100 µL	25-100 µL/10-30 µL (fast) for 96-well and 5-20 µL for 384-wells	5-100 µL (96 well) or 5-20 µL (384 well)
Multiplexing	Up to 4 fluorophores	Up to 5 fluorophores	Multiplex number is limited by spectral overlap	The number is dependent on the excitation/emission combination
Sensitivity	10 starting copies of the RNase P gene from human genomic DNA	10 starting copies of the RNase P gene from human genomic DNA	10 starting copies of the RNase P gene from human genomic DNA	10 copies (plasmid)
Dynamic Range–Reaction – Orders of Magnitude	9	9	9	10
Software	System Software and Primer Express software included	System Software and Primer Express software included	System Software and Primer Express software included. Enterprise Edition Software with SNP Manager and RQ Manager are available	LightCycler Software

Instrumentation

Instrumentation

High-Throughput qPCR Instruments

Manufacturer System	Stratagene Mx3000P® Multiplex qPCR System	Stratagene Mx3005P® Multiplex qPCR System	Techne Quantica®
Advantages	A reference dye is unnecessary allowing the use of ROX or Texas Red probes. System can be used as a thermostated fluorescent microplate reader (e.g., to quantify fluorescently stained/labeled nucleic acids). An embedded computer safeguards data. The system can also be linked to a PC allowing multiple systems to be used at the same time for high-throughput applications from a single PC	A reference dye is unnecessary allowing the use of ROX or Texas Red probes. System can be used as a thermostated fluorescent microplate reader (e.g., to quantify fluorescently stained/labeled nucleic acids). An embedded computer safeguards data. The system can also be linked to a PC allowing multiple systems to be used at the same time for high-throughput applications from a single PC	Up to 4 instruments can be controlled from a single PC. The system has a robot-friendly CD-type block loading mechanism
Excitation	Quartz tungsten-halogen lamp	Quartz tungsten-halogen lamp	Filtered solid-state white light (470-650 nm)
Detection	Scanning fiber optics with four-position filter wheel and a photomultiplier tube	Scanning fiber optics with four-position filter wheel and a photomultiplier tube	Photomultiplier tube (500-710 nm)
Heat block	Peltier	Peltier	Gradient block option with a gradient range of up to 30 °C (between 20-70 °C)
Rxn volume(s):	10-60 µL	10-60 µL	15-50 µL
Multiplexing	Up to 4 fluorophores	Up to 5 fluorophores	Up to 4 colors
Sensitivity	To a single-copy equivalent	To a single-copy equivalent	Single copy of template and down to 1 nM fluorescein
Dynamic Range–Reaction – Orders of Magnitude	10	10	9
Software	MxPro™ included	MxPro™ included. Also includes Beacon Designer 4 software	Quansoft software included

High-Throughput qPCR Instruments

Manufacturer System	Bio-Rad Laboratories iQ5 Real-Time PCR Detection System	Bio-Rad Laboratories MyiQ™ Single-Color Real-Time PCR Detection System	Bio-Rad Laboratories DNA Engine Opticon 2® Real-Time PCR Detection System	Bio-Rad Laboratories Chromo4™ Real-Time Detector
Advantages	Optical upgrade to the iCycler® thermal cycler. Embedded tool for end-point fluorescence analysis	Optical upgrade to the iCycler thermal cycler. Limited to one color	Can perform plate reads	Has a 96-well Alpha unit. This allows it to be used on any DNA Engine® chassis
Excitation	Tungsten-halogen lamp (475-645 nm)	Tungsten-halogen lamp (475-645 nm)	96 LEDs (470-505 nm)	4 LEDs in photonics shuttle (450-650 nm)
Detection	5 color customizable filter wheel and CCD camera (515-700 nm)	12-bit CCD camera (515-545 nm)	Two photomultiplier tubes; one compatible with SYBR Green I or FAM; the other detects a variety of fluorophores	4 photodiodes (515-730 nm)
Heat block	Peltier and Joule (gradient: 25 °C max)	Peltier and Joule (gradient: 25 °C max)	Peltier (gradient: 24 °C max)	Peltier (gradient: 24 °C max)
Rxn volume(s):	15-100 µL	15-100 µL	10-100 µL	10-100 µL
Multiplexing	Up to 5 colors	No	Up to 2 colors	Up to 4 colors
Sensitivity	Linear to 10 copies of β-actin DNA	One copy of IL-1β in human genomic DNA	As little as one starting template copy	As little as one starting template copy
Dynamic Range–Reaction – Orders of Magnitude	9	8	10	10
Software	iQ5 Optical System Software Version 2.0	iCycler iQ Software Version 3.1	DNA Engine Opticon 2 Software Version 3.1	Chromo4 Software Version 3.1

Instrumentation

Low-Throughput qPCR Systems

Manufacturer System	Cepheid SmartCycler® TD System	Corbett Research Rotor-Gene™ 3000/3000A	Corbett Research Rotor-Gene™ 6000	Roche Applied Science LightCycler® 2.0
Advantages	Runs samples in blocks of 16, up to 96 samples total, and allows each reaction to be separately programmed for independent experimental protocols. Fast programs complete in as little as 20 minutes. Fast runs require proprietary reaction tubes	Centrifugal systems designed to work with any real-time chemistry. Samples are rotated in a changing thermal environment to produce a uniform temperature between reactions. Conventional or rapid cycling conditions possible using conventional reagents	A centrifugal system designed to work with any real-time chemistry. Samples are rotated in a changing thermal environment to produce a uniform temperature between reactions. Conventional or rapid cycling conditions possible using conventional reagents. Dedicated analysis software	The LightCycler series of instruments are rotor-based and work with a variety of chemistries. Rapid reactions take place in passivated glass capillaries
Excitation	LED	4 LEDs excite entire visible spectrum	Separate color high-intensity LED per channel	Blue LED (470 nm)
Detection	Silicon photo detector is used to collect filtered light	A series of filters and a photomultiplier tube	Separate emission filter per channel and a photomultiplier tube	Photodiodes (530, 560, 610, 640, 670 and 710 nm)
Heat block	Solid-state heater; forced-air cooling	Thermostated air	Thermostated air	Thermostated air
Rxn volume(s):	25 and 100 µL volumes	20-25 µl	5 µL to 100 µL, but 20 µL is typical	20 µL and 100 µL
Multiplexing	Up to 4 colors	Two-channel (3000A) or four channel (3000)	2, 5, or 6 color options	Provides multiplexing and measures fluorescence between 530-705 nm
Sensitivity	10 copies plasmid	Unpublished	Single-copy gene target amplification from a whole human genome	Single-copy gene in 3 pg of human genomic DNA. 1-10 copies of plasmid
Dynamic Range–Reaction – Orders of Magnitude	8	Unpublished	Unpublished	10
Maximum number of samples	Number of samples can be increased in increments of 16 – to a total number of 96 by linking additional units to a single PC	Up to 36 or 72 samples in specifically designed rotors	72 individual tubes or heat-sealed plates	32
Software	SmartCycler	Corbett system software	Corbett system software with analysis, graphing and statistical license	LightCycler® Software 4.05

qPCR Applications Guide

The popularity of quantitative PCR lies in the many applications for which it can be employed. Common applications include the detection of single nucleotide polymorphisms (SNPs), measurement of DNA copy number, determination of genomic allelic variation, diagnosis and quantitation of bacterial and viral infections and the validation of gene expression data from microarray experiments. In order to perform successful experiments using qPCR, the quality of the template and the level of the control must be considered.

Quality of the Template

Quantitative PCR can only be used reliably when the template of interest can be amplified and when the amplification method is without error. As a result, quality of the template is critical to success. PCR will only amplify DNA or cDNA with an intact phosphodiester backbone between the priming sites. In addition, DNA containing lesions that affect the efficiency of amplification, such as abasic sites and thymine dimers, will be either under-represented or may not be represented at all in qPCR. In order to achieve the best possible quantitation, it is of paramount importance that the highest quality nucleic acid template goes into the qPCR. One factor that may affect the quality of the template is the inclusion of additives that may degrade dNTPs, primers, and PCR substrates. Some additives can also directly inhibit the DNA polymerase thus affecting the results of the qPCR. In addition to using quality template, it is also important to include a set of rigorous controls with every experiment to ensure success.

Levels of Quantitative PCR Controls

The complexity and rigor of the controls required depends on the degree of quantitation demanded for the reaction. There are three levels of qPCR quantitation and therefore three levels of complexity for appropriately controlled reactions. The levels of quantitation are: absolute quantitation, relative quantitation and non-quantitative or qualitative.

Absolute Quantitation

Absolute quantitation is the most rigorous of all quantitation levels. Absolute quantitation requires the addition of external standards in every reaction to determine the absolute amount of the target nucleic acid of interest. To eliminate potential differences in quantitation due to annealing, the primer binding sites of the external standards must be the same as those in the target sequence. The ideal external standard contains sequences that are the same as the target sequence or which vary slightly from the target sequence. Equivalent amplification efficiencies between the target and the external standard are necessary for absolute quantitation. Once a suitable construct or amplicon is identified, a standard curve of external standard dilutions is generated and used to determine the concentrations of unknown target samples.

Relative Quantitation

Relative quantitation requires calculation of the ratio between the amount of target template and a reference template in a sample. The advantage of this technique is that using an internal standard can minimize potential variations in sample preparation and handling. The accuracy of relative quantitation depends on the appropriate choice of a reference template for standards. Variability of the standard will influence the results and so it is important that the appropriate standard is used.[27] Some researchers choose not to run a standard curve and to report target quantities as a fraction of the reference, a technique termed comparative quantitation. Alternatively, one may assume that the amplification efficiencies of the target and reference are negligible and quantify the target based solely on the standard curve determined for the reference sequence. Finally, in the most accurate of the relative quantitation techniques, the amplification efficiencies of both the reference and target are measured and a correction factor is determined. This process, termed normalization,[27] requires examining a sample containing known concentrations of both target and reference and the generation of two standard curves. Since this method measures the amount of target relative to a presumably invariant control, relative qRT-PCR is most often used to measure genetic polymorphism differences between tissues or between healthy and diseased samples. It is important to note that if using SYBR binding dye-based systems, the target and internal reference quantitation must be performed in separate reactions.

Qualitative Analysis

In contrast to quantitative analysis where each cycle (log-linear phase) of the PCR is analyzed, qualitative PCR is an end-point analysis after the PCR has reached plateau phase. Qualitative analysis is not as accurate as quantitative; however, it can provide rapid and adequate information for some experiments. Qualitative analysis is gel-based and provides a visual assessment of primer design, specificity and quality of the PCR amplification.

Summary

qPCR is a powerful and flexible technique, but the results achieved using this method are only valid if the appropriate controls have been included in the experiment. The level of required controls depends on the level of quantitiation desired: absolute, relative or qualitative. Once that level of control has been identified, employing it when performing qPCR will contribute to the success of the experiment.

Optimizing qPCR

No matter whether your quantitation is to be absolute or relative, the accuracy of qPCR depends on proper optimization of the PCR and appropriate setting of the threshold value. While some assays will not require complete optimization, there are a great many delicate qPCR assays which require maximal selectivity and sensitivity. For instance, pathogen detection or expression profiling of rare mRNAs require high sensitivity. Assays such as SNP detection or viral quantification require high specificity. Most challenging are multiplex reactions, as these often require both sensitivity and selectivity. By properly optimizing the conditions for the qPCR experiment, the researcher will be ensured of valid, reproducible results with maximal specificity and sensitivity.

Guidelines for Optimizing Both qPCR and qRT-PCR

Regardless of whether the target is DNA (qPCR) or RNA (qRT-PCR), the following preliminary steps will aid in the optimization of the reaction helping to ensure successful quantitation:

- Check primer design for primer-dimer potential
- Optimize primer concentrations
- Optimize probe concentration
- Validate performance with a standard curve
- Prepare a melt curve
- Set the threshold value

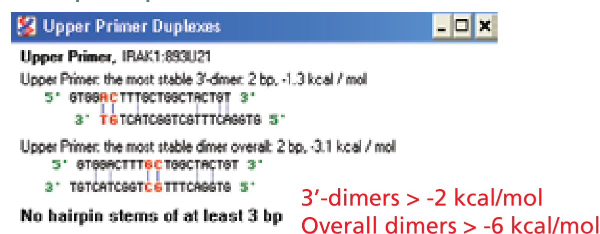
Check Primer Design for Primer-Dimer Potential

The propensity of primers to hybridize to one another may lead to primer extension during PCR and the formation of target-independent products known as primer-dimers. This is especially true for primers with complementarity at their 3′-ends. When primer-dimer products are produced and amplified, the reaction components are diverted from synthesis of the desired product, thereby reducing assay efficiency and sensitivity. Therefore, primer-dimers are an issue in both probe-based and SYBR Green dye-based detection. With SYBR Green dye-based detection, primer-dimers also affect assay specificity because the primers will be detected along with the desired product. As a result, primers that are likely to form primer-dimers should be avoided, most especially with SYBR Green dye-based detection.

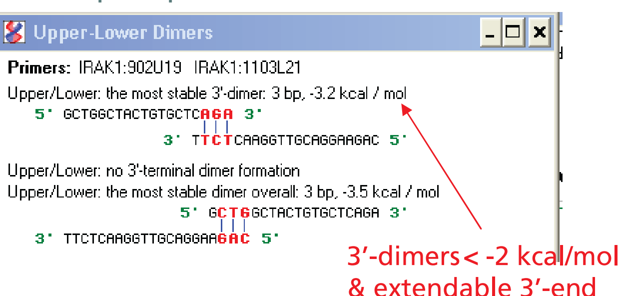
To check the potential for primer-dimer formation, use primer design software to analyze duplex formation. Any 3′-terminal dimers formed by either primer hybridizing with itself or with its partner must be non-existent or very weak ($\Delta G \geq -2.0$ kcal, Fig. 10A). Any primer with both a terminal $\Delta G \geq -2.0$ kcal and an extendable 3′-end (5′-overlap, Fig. 10B) should be avoided. The strongest overall dimer should be unstable as well ($\Delta G \geq -6.0$ kcal, Fig. 10C). To avoid strong 3′-terminal dimers while maintaining specificity, choose primers that have 2 G or C residues in the last 5 bases, 1 G or C in the last 3 bases, and an A or T at the 3′-end **(Fig. 10A)**.

Figure 10. Analysis of Primers for Primer-Dimer Potential

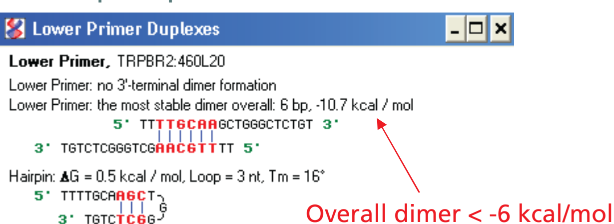
A. Acceptable primers:



B. Unacceptable primers:



C. Unacceptable primer:



Primer sequences were analyzed for their ability to form duplexes using Oligo 5.0 Primer Design Software. As described in the text, the primers shown in A are not expected to form significant amounts of primer-dimer because they only form weak duplexes. On the other hand, those in B hybridize too strongly at the 3′-end and those in C hybridize too strongly overall.

Optimize Primer Concentrations

Satisfactory results for probe-based qPCR are often obtained with final concentrations of both primers at 500 nM and the probe at 250 nM, especially if the PCR target is abundant and maximum sensitivity is not required. Somewhat lower primer levels, 200-400 nM, are usually better when using SYBR Green dye-based detection to minimize non-specific amplification. Conduct a standard curve analysis, as described in the next section. If detection is linear and efficiency is greater than 85% over the range of target expected in samples, it is not necessary to optimize primer and probe concentrations.

For maximum sensitivity, optimum primer concentrations must be determined empirically. Primer concentrations are most efficiently optimized by testing various combinations in qPCR, as shown in the example below. Regardless of the detection chemistry used in the final assay, the best assay sensitivity will be obtained if primer concentrations are optimized in the presence of SYBR Green. This allows detection of primer-dimer and other non-specific products, and helps the user to screen out reactions with multiple products (step 8, next section). Alternatively, if maximum sensitivity is not a concern, the corresponding probe may be included in reactions at 250 nM.

Primer Optimization Example

1. Prepare and dispense diluted primers
   a. Prepare 60 µL of 8 µM working solutions of both forward (fwd) and reverse (rev) primers in the first tubes of 2 separate 8-tube strips.
   b. Dispense 30 µL of water into tubes 2-5.
   c. Transfer 30 µL of the 8 µM primer solution from tube 1 into tube 2. Mix thoroughly by pipetting up and down at least 5 times.
   d. Repeat transfer and mixing from tube 2 to 3, 3 to 4, and 4 to 5.
   e. Using a multichannel pipettor, transfer 5 µL from the strip-tubes containing diluted fwd primer into the first 5 wells down columns 1-5 of a 96-well PCR plate. After adding reverse primer, PCR mix, and template (below), final concentrations of forward primer will be 1000, 500, 250, 125, and 62.5 nM.
   f. Similarly, transfer 5 µL from the strip-tubes containing diluted rev primer into the first 5 wells across rows A-E. After adding PCR mix and template (below), final concentrations of reverse primer will be 1000, 500, 250, 125, and 62.5 nM.
2. Prepare qPCR or qRT-PCR master mix (for 52 × 20 µL reactions):

qPCR

Reagent	Catalog Number	Volume
Water	**W1754**	155 µL
SYBR Green JumpStart™ *Taq* ReadyMix™	**S9939***	520 µL
Reference dye**	**R4526***	1.0 µL

qRT-PCR

Reagent	Catalog Number	Volume
Water	**W1754**	123.8 µL
SYBR Green JumpStart™ *Taq* ReadyMix™	**S9939***	520 µL
Reference dye**	**R4526***	1.0 µL
40 U/µL RNase inhibitor	**R2520**	26 µL
200 U/µL MMLV reverse transcriptase	**M1427**	5.2 µL

* S9939 and R4526 are components of Catalog Number S4438.
** Use 10 × more for ABI 7700; replace with FITC for BioRad iCycler.

3. Aliquot 26 µL master mix into all wells in the PCR plate that contain primers (A1-E5).
4. Mix thoroughly and transfer 18 µL from each of wells A1 through E5 to wells A8 through E12.
5. Add 2 µL template-containing DNA (10-50 ng genomic DNA or 0.1-1 ng plasmid) or RNA (10-100 ng total RNA or 0.5-10 ng mRNA) to one set of reactions (columns 1-5) and 2 µL water to the other (columns 8-12).
6. Perform thermal cycling:

	Number of Cycles	Temperature	Time for qPCR	Time for qRT-PCR
Reverse transcription	1	45 °C	0 min	15-30 min
Denature	1	94 °C	3 min	3 min
Denature	40	94 °C	15 sec	15 sec
Anneal, extend, and read fluorescence		60 °C	1 min	1 min
Dissociation/ melting curve	1	*	*	*

*See manufacturer's instructions for the real-time thermal cycler used.

7. Evaluate fluorescence plots (ΔRn) for reactions containing target nucleic acid (columns 1-5). Primer combinations with the lowest Ct and the highest fluorescence will give the most sensitive and reproducible assays.
8. Evaluate dissociation/melting curves. Primer combinations with single, sharp peaks in the presence of target nucleic acid (columns 1-5) and nothing detected in the corresponding no-template control (columns 8-12) will give the most sensitive and reproducible assays. If all primer combinations give some product in the absence of template, and this no-template product melts at a lower temperature than that with template, select the combination that gives the least amount of lower-melting no-template product. The latter is likely primer-dimer. Detection can be avoided, or at least minimized, by adding a 15 second melting step approximately 3 °C below the melting temperature of the desired PCR product during which fluorescence is measured after the annealing/extension step in each cycle.

Optimize Probe Concentration

For maximum sensitivity, 250 nM probe may be used in all assays. However, if maximum sensitivity is not required, lower levels of probe may suffice, thereby reducing the assay cost. To optimize probe concentration, test the probe at several levels from 50 to 250 nM final concentrations in PCR with optimized levels of primers and the lowest level of target nucleic acid expected. The lowest level of probe that allows acceptable detection (Ct ≤ 30 for best reproducibility) may be used.

Optimizing qPCR

Validate Performance with a Standard Curve

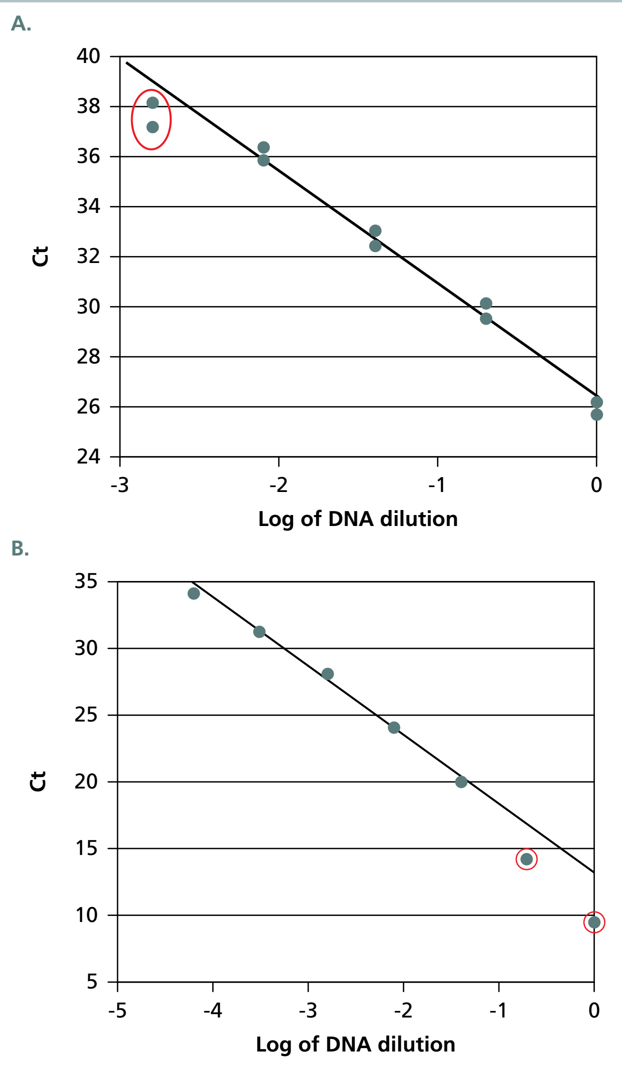
A standard curve, generated by performing qPCR with a serial dilution of template, is an excellent tool to test assay efficiency, precision, sensitivity, and working range. Prepare at least three, but preferably five or more, 4- to 10-fold serial dilutions from a DNA or RNA sample that contains the PCR target. Plan this dilution series to extend past both the highest and lowest levels of target expected in test samples. Conduct qPCR with all dilutions and with a no-template control, using previously optimized primer and probe concentrations. With SYBR Green dye-based detection, also include a melt curve test at the end of thermocycling. The software for most real-time qPCR instruments can be set up to prepare a standard curve and to calculate efficiency (see the user guide for the instrument being used). If this feature is not available, prepare a plot of Ct versus the log of nucleic acid input level and perform a linear regression. Calculate the reaction efficiency from the slope of the line using the equation:

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

The correlation coefficient of the line, R^2, is a measure of how well the data fits the model and how well the data fits on a straight line, and is influenced by pipetting accuracy and by the range of the assay. If R^2 is ≤ 0.985, the assay may not give reliable results. If one or more points at the lowest levels of input nucleic acid are shifted away from the linear region of the plot, it is likely that the level exceeds assay sensitivity **(Fig. 11A)**. To improve sensitivity, optimize primer concentrations or design different primers. Similarly, if one or more points at the highest levels of input nucleic acid are shifted away from the linear region of the plot, it is likely that the reaction is saturated and that the level of target exceeds the useful assay range **(Fig. 11B)**. To address this situation, add less or dilute the sample nucleic acid. Alternatively, if several random points are above or below the line, pipetting accuracy may be a problem. Verify that the pipette tips fit the pipettor properly and that the volume dispensed is reproducible.

If the PCR is 100% efficient, the amount of PCR product will double with each cycle and the slope of the standard curve will be –3.33 ($100 = 100\% = 10^{(-1/-3.33)}-1$). A slope between –3.9 and –3.0 (80-110% efficiency) is generally acceptable. Calculated levels of target input may not be accurate if the reaction is not efficient. To improve efficiency, optimize primer concentrations or design alternative primers.

Figure 11. Use of Standard Curves to Evaluate qPCR Optimization



A. Assay not linear at low levels of input nucleic acid. **B.** Assay not linear at high levels of input nucleic acid.

Prepare a Melt Curve

Since SYBR Green binding dye is a non-specific dye that will detect any double-stranded DNA, it is important to verify that the PCR produces only the desired product. This can often be detected when PCR efficiencies are larger than 120%. Melt, or dissociation, curve analysis can also be used to determine the number and approximate size of products. An assay with high specificity will give a single peak at a high temperature (> 80 °C) in all reactions and nothing, or very little, detected in the no-template controls **(Fig. 12A)**. If the melting curve has more than one major peak, as in Figures 12B and 12C, the identities of the products should be determined by fractionating them on an ethidium bromide-stained agarose gel. As shown in Figures 12E and 12F, reactions B and C contain excessive amounts of primer-dimer or other non-specific products. Lowering the primer concentrations will often reduce the amount of non-specific products. If non-specific products are still detected in significant amounts with low primer levels, redesign the primers.

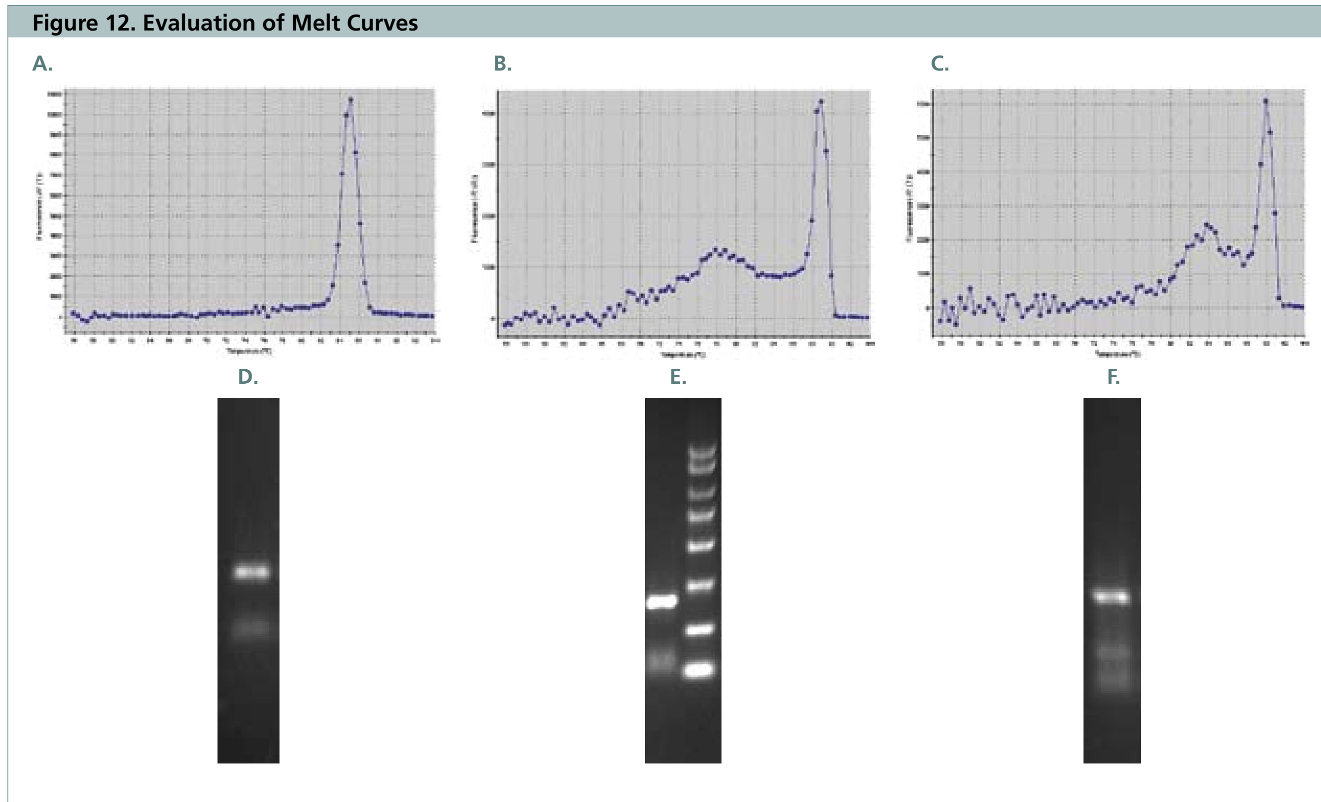
Setting the Threshold Value

There are several methods that can be used to calculate the threshold value. The critical factors for determining the level of the threshold are: (a) the fluorescence value must be statistically higher than the background signal, (b) the samples must all be measured in the exponential phase of amplification and (c) the efficiency of amplification must be identical for all samples.

The fluorescence value must be statistically higher than the background signal to ensure that real data are collected. Most instruments automatically calculate a threshold level of fluorescence signal by determining the baseline (background) average signal and setting a threshold 10-fold higher than the baseline average signal.

Setting a manual threshold is best accomplished using a log signal plot, as the exponential part of the curve shows clearly as a linear trace.

Figure 12. Evaluation of Melt Curves



Melt, or dissociation, curves showing a sharp peak of specific product at > 80°C, very little non-specific product at lower temperatures **(A)**, or significant amounts of non-specific, lower melting product (**B&C**). **D-F** show PCR products from **A-C**, respectively, fractionated on ethidium bromide-stained 2% agarose gels.

Optimizing qPCR

Additional Guidelines for Quantitative Reverse Transcription PCR (qRT-PCR)

When performing qRT-PCR, it is not only important to consider the guidelines for standard qPCR, but for optimum qRT-PCR results, the following points should be addressed as well:

- Verify RNA quality
- Confirm that primers span or flank long introns
- Conduct no-reverse transcriptase (no-RT) controls
- Optimize reverse transcription

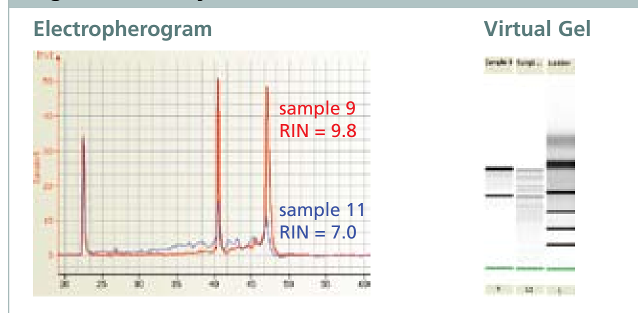
For additional information, please see Appendix 2: How to Optimize your Quantitative Real-Time RT-PCR (qRT-PCR).

Verify RNA Quality

The quality of total RNA is most readily assessed by capillary electrophoresis with an Agilent 2100 bioanalyzer. The instrument software evaluates the proportion of RNA detected before, between, and after the rRNA peaks to determine a relative integrity number (RIN) for the RNA sample analyzed. Perfectly intact RNA has a RIN of 10 whereas completely degraded RNA has a RIN of 1. Whether or not partially degraded RNA (RIN < 9) will give satisfactory results in qRT-PCR depends upon the level of sensitivity required as well as the RT-PCR strategy. For example, an RT-PCR strategy that uses a two-step Oligo-dT to prime reverse transcription and PCR primers near the 5'-end of a long cDNA will require much higher integrity than a strategy that uses one-step RT-PCR with gene-specific primers. In addition, higher integrity will be required to detect a rare mRNA compared to an abundant mRNA. Therefore, the correlation between RIN and qRT-PCR success must be determined empirically for each assay.

By way of example, the RNA samples with Bioanalyzer traces shown in Figure 13, gave quantifiable difference for several rare mRNAs when using a one-step qRT-PCR using gene-specific primers. This same RNA template was quantified to have relative mRNA amounts varying by four-fold when using two-step qRT-PCR with Oligo-dT to prime RT. The latter difference was shown to correlate to RIN, as mRNAs were detected up to 2 cycles later in sample 11 (RIN = 9.8) than in sample 9 (RIN = 7.0).

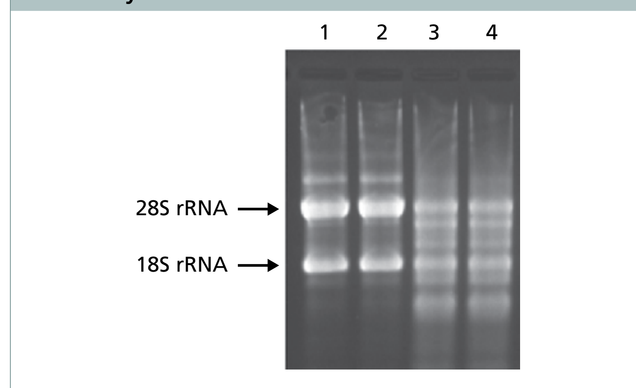
Figure 13. Evaluation of RNA Integrity with the Agilent Bioanalyzer 2100



Samples of total RNA preparations (~150 ng) were fractionated on a RNA 6000 Nanochip and integrity was evaluated before use in qRT-PCR.

If a Bioanalyzer is not available, 1-2 µg of total RNA may be evaluated by ethidium bromide-stained agarose gel electrophoresis to verify that the RNA appears reasonably intact. For good quality total RNA, the two largest rRNAs should appear as discrete bands at approximately 5 kb and 2 kb and the upper band should have approximately twice the intensity of the lower. The mRNA should appear as a light smear, mostly between the two rRNA bands, as in lanes 1 and 2 in Figure 14. The RNA in lanes 3 and 4 of Figure 14 are partially degraded.

Figure 14. Evaluation of RNA Integrity by Agarose Gel Analysis



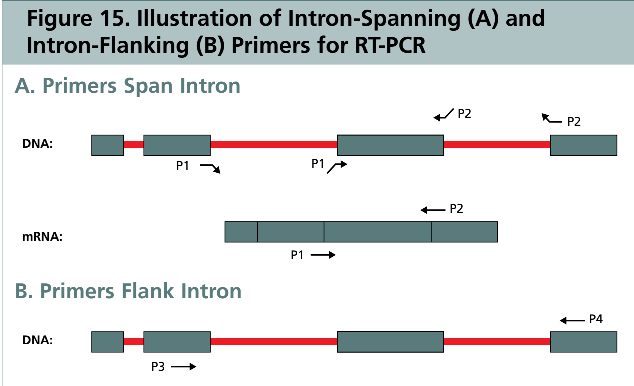
Samples of total RNA (2 µg) were fractionated on a 1% agarose gel in TBE buffer and stained with ethidium bromide.

Confirm that Primers Span or Flank Long Introns

While most DNA is eliminated during RNA purification, no procedure removes 100% of the DNA. Because PCR can amplify even a single molecule of DNA, RT-PCR can amplify contaminating DNA as well as RNA. If the target mRNA is fairly abundant (hundreds or thousands of copies per cell), DNA amplification will be negligible in comparison to the products from the RNA. If, however, the target mRNA is less than 100 copies/cell, DNA amplification can lead to erroneously high estimates of mRNA levels. To avoid DNA amplification during RT-PCR, use primers that either flank an intron that is not present in the mRNA sequence or that span an exon-exon junction **(Fig. 15)**. If both genomic and cDNA sequences for the target mRNA are publicly available, intron positions can be identified by performing a BLAST search with the cDNA sequence against the genomic database for the target organism **(Fig. 16)**. DNA sequences with short intervening sequences (~1 kb) may be amplified in RT-PCR **(e.g. Fig. 17A)**. For example, Intron 1 in Figure 16 is long enough (~ 6.5 kb) to preclude amplification of the genomic DNA, while all other introns are short (< 1 kb) and likely will be amplified during RT-PCR. The example in these two figures illustrates that, if possible, primers should either span exon-exon junctions, flank a long (several kb) intron, or flank multiple small introns.

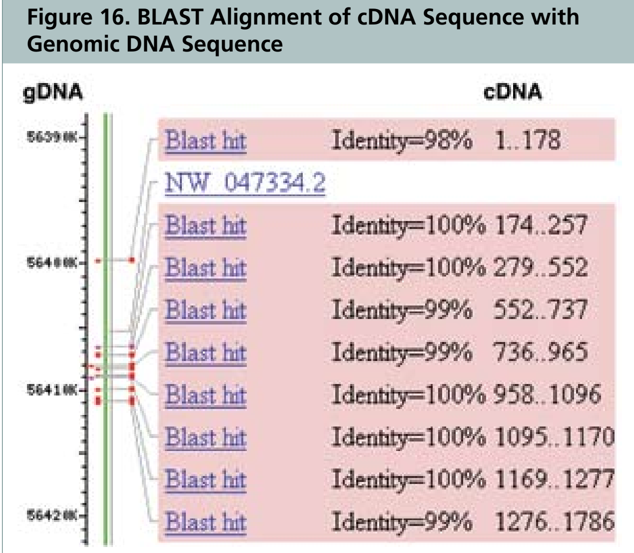
If the gene of interest has no introns, if the intron positions are unknown, or if there are no suitable primers that span or flank introns, it may be necessary to digest input RNA with an RNase-free or amplification-grade DNase I. Conduct no-RT controls to determine whether or not digestion with DNase I is needed.

Figure 15. Illustration of Intron-Spanning (A) and Intron-Flanking (B) Primers for RT-PCR



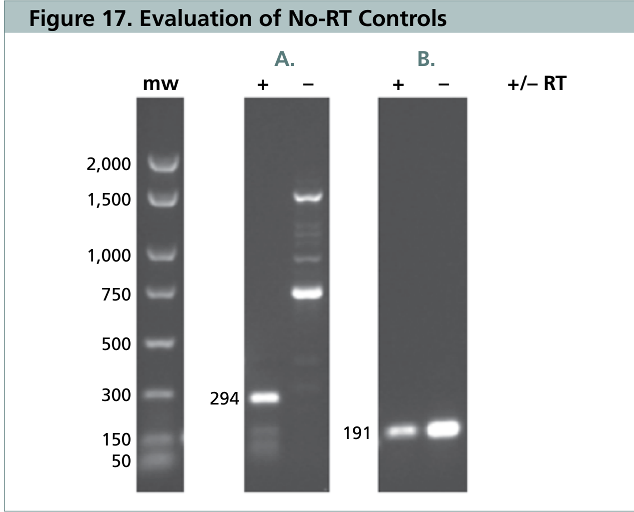
Introns are in red, exons are in green. Primers P1 & 2 span an intron and primers P3 & 4 flank an intron. Note that primers P1 & 2 are only partly complimentary to the gDNA strand and will not generate a PCR product from DNA unless the annealing temperature is extremely low. P3 & 4 may generate a longer PCR product from DNA if the intron is short (~1 kb), but not if it is sufficiently long (several kb).

Figure 16. BLAST Alignment of cDNA Sequence with Genomic DNA Sequence



The complete cDNA sequence for rat p53 from Genbank (accession number NM_030989) was used in a megaBLAST search for identical sequences in the rat genome (http://www.ncbi.nlm.nih.gov/genome/seq/RnBlast.html). The alignment on chromosome 10 is shown; each tick mark on the scale represents 1 kb.

Figure 17. Evaluation of No-RT Controls



RT-PCR products produced in the presence (+) or absence (-) of RT enzyme were fractionated on an ethidium bromide-stained 2% agarose gel in TBE. Primers for the mRNA target in **(A)** flank a 1 kb intron. Note the 1.5 kb band in the no RT control. The mRNA target in **(B)** aligns with several genes, at least one of which is a pseudogene that lacks the intron between the primers used for RT-PCR. As such, the no RT control gives a larger yield than when reverse transcriptase is added.

Conduct No-Reverse Transcriptase (No-RT) Controls

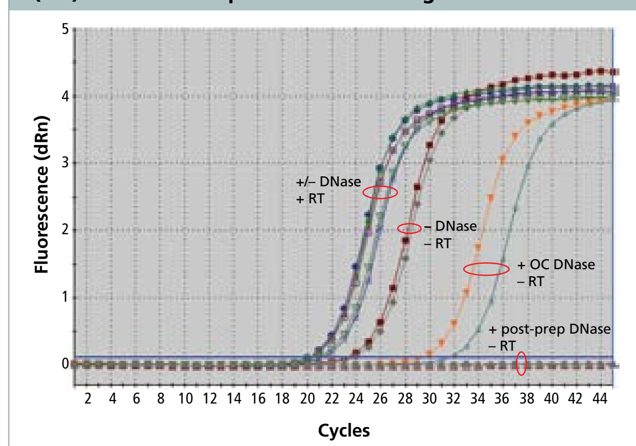
Regardless of whether primers span or flank introns, the specificity of qRT-PCR assays should be tested in reactions without reverse transcriptase (no-RT control) to evaluate the specificity of DNA amplification. As mentioned above, DNA sequences with short introns (≤ 1 kb) may be amplified in RT-PCR. Many genes have additional copies, or pseudogenes, that lack one or more introns **(Fig. 17B)**. As a result, qRT-PCR assays should be tested for potential DNA-only amplicons by performing reactions that contain RT, the same RNA, but no RT enzyme. DNA amplification is not a problem if the Ct values for no-RT reactions are at least 5 cycles greater (32-fold less) than those for reactions with RT. However, if there are fewer than 5 cycles between Ct values for reactions with and without RT, DNA amplification may skew attempts at mRNA quantitation.

In cases where DNA amplicons contribute significantly, one should digest the RNA with an RNase-free or amplification grade DNase I before qRT-PCR to allow reliable mRNA quantitation. Note that on-column DNase digestion, which is commercially available in several RNA purification kits, is less effective at eliminating DNA than digestion in solution after eluting RNA from the column. As a result, on-column (OC) DNase digestion may or may not be sufficient for qRT-PCR (Fig. 18, see +OC DNAse -RT samples versus post prep DNAse -RT). No-RT controls should be conducted with DNase-digested RNA to verify that the digestion was successful and sufficient. In the example shown in Figure 17, OC DNase digestion is sufficient to reliably detect the target mRNA. It would not be sufficient to reliably quantitate a less abundant mRNA, if the samples contained less of the mRNA shown, or if greater sensitivity was required.

Optimizing qPCR

Note that different types of cells and tissues, as well as different growth conditions, produce significantly different levels of specific mRNAs. In addition, different RNA purification methods give different levels of contaminating DNA. As a result, reactions with and without RT should be performed at least once with each new starting material and RNA preparation method.

Figure 18. Comparison of On-Column DNase Digestion (OC) with Post-Preparation DNase Digestion



Total RNA was prepared from 30 mg pieces of mouse liver with either the Sigma GenElute™ Total RNA Kit or the Qiagen RNeasy Mini Kit according to the manufacturers' instructions. Two RNA samples were prepared with the respective manufacturer's on-column DNase product and two were prepared without DNase digestion. After purification, aliquots of the four RNA samples prepared without on-column DNase were digested with Sigma's Amplification Grade DNase I according to the manufacturer's instructions. Equal proportions of all were used in one-step qRT-PCR. Fluorescence plots for two of the RNA samples are shown. Similar results were obtained with both manufacturers' products.

Optimize Reverse Transcription

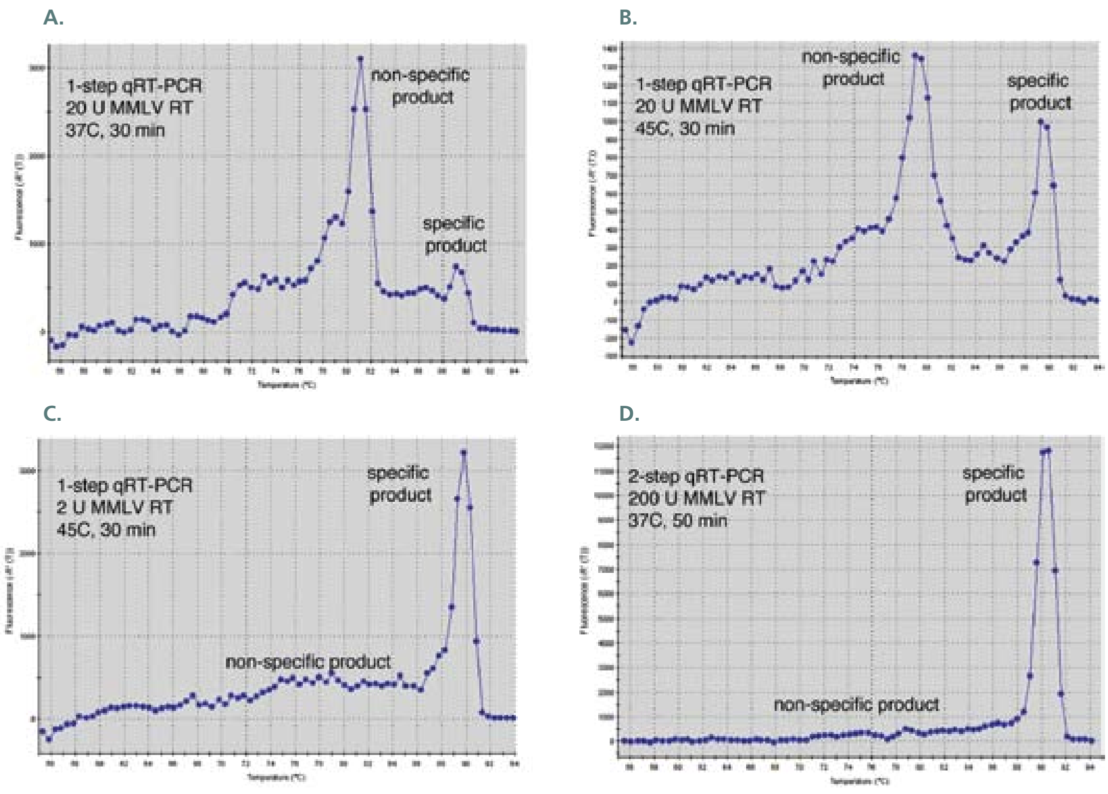
The choice of primers used to initiate reverse transcription can greatly affect qRT-PCR results. For one-step qRT-PCR, gene-specific primers must be used. When performing a two-step assay, a reverse gene-specific primer, Oligo-dT, random hexamers, nonamers, decamers, or dodecamers may be used. Gene-specific primers require separate reactions for each target RNA. These separate reactions may have very different efficiencies, thus complicating comparisons between RNA levels. On the other hand, with a gene-specific primer, all of the RT product will encode the gene of interest and may allow quantitation of very low abundance mRNAs not detected using non-specific RT primers.

Give these complications, the choice of RT priming should be carefully considered. To avoid the potentially high inter-assay variations in RT that can occur with gene-specific primers, non-specific primers may be used to generate a pool of cDNA. This would be followed by separate qPCR assays for each target performed with aliquots from the cDNA pool. If all qPCR targets are near the 3′-end of polyadenylated mRNAs, oligo-dT is a good choice for primer. On the other hand, if the qPCR targets are more than a few kilobases from the 3′-end or if the RNA is not polyadenylated, random hexamers, octamers, nonamers, or decamers will give better detection. If the location of qPCR targets or the polyadenylation level of RNAs varies, a mixture of Oligo-dT and random oligomers will give the best results.

The temperature used for RT reactions may affect specificity, especially with gene-specific primers. Primers that can form a strong 3′-duplex will hybridize more readily at lower temperatures. Since RT enzymes can extend a DNA primer on a DNA template, primer-dimer formation may start during the RT step. Increasing RT incubation temperature to the highest temperature at which the enzyme is fully active or using a high-temperature enzyme may reduce the amount of primer-dimer. For example, the primers used in Figure 18 gave significantly less non-specific product in one-step qRT-PCR when RT was performed with MMLV-RT (Moloney Murine Leukemia Virus-Reverse Transcriptase) at 45 °C **(Fig. 19B)** than when the reaction was performed at 37 °C **(Fig. 19A)**. Similarly, performing two-step RT-PCR with a non-specific primer for RT and hot-start *Taq* polymerase for qPCR may give less primer-dimer **(Fig. 19D)** than one-step qRT-PCR with gene specific primers that can form a 3′-duplex **(Fig. 19B)**.

The amount of RT enzyme per reaction can also affect qRT-PCR results. As shown in Figure 18, one-step reactions with 2 units of MMLV-RT **(Fig. 19C)** gave better specificity than reactions with 20 units **(Fig. 19B)**. Superscript™ III, an RNaseH– deletion of MMLV-RT, and Omniscript from Qiagen gave results similar to those shown in Figure 18 (data not shown). Two-step RT-PCR with Oligo-dT or random primers for RT often gives greater specificity than does one-step RT-PCR **(Fig. 19D)**. This could be attributed to the fact that the gene-specific primers are not present to form non-specific products during the low temperature RT reaction. Higher levels of RT may give better results in two-step reactions, but because the RT enzyme can interfere with *Taq* activity, the amount of RT product transferred to qPCR should be limited to no more than 10% of the final reaction volume.

Figure 19. Optimization of RT



Melt curves of RT-PCR products produced with one-step **(A-C)** or two-step **(D)** qRT-PCR. Reactions **(A-C)** each contained 10 µL of SYBR Green JumpStart Taq ReadyMix, 0.02 µL of Reference Dye, both gene-specific primers at 0.4 µM, and 10 ng human total RNA in a final volume of 20 µL. Gene-specific primers were 5′-CGGGCTTCAACGCAGACTA-3′ and 5′-CTGGTCGAGATGGCAGTGA-3′ for c-fos (Accession NM_005252). Reactions **(A&B)** also contained 20 units of MMLV- RT, whereas reaction **(C)** contained 2 units. Reaction A was incubated at 37 °C for 30 min before qPCR, whereas **(B&C)** were incubated at 45 °C for 30 min before qPCR. In **(D)**, the RT reaction contained 1× MMLV buffer, 0.5 mM dNTPs, 1 µM Oligo-dT, 0.8 units/µL RNase inhibitor, 200 units MMLV-RT, and 10 ng human total RNA in a final volume of 20 µL. The reaction was incubated at 25 °C for 10 min, 37 °C for 50 min, and 80 °C for 10 min. 2 µL of the RT reaction product was added to qPCR containing 10 µL of SYBR Green JumpStart Taq ReadyMix, 0.02 µL of Reference Dye, and both gene-specific primers at 0.4 µM as for the one-step reactions **(A-C)**. All qPCR reactions were incubated at 94 °C for 3 min to denature, then for 40 cycles of 94 °C for 15 sec and 60 °C for 1 min.

Optimizing qPCR

Additional Optimization for Multiplex Reactions

Successful multiplex qPCR, in which more than one target is quantitated in a single reaction, often require additional optimization. One simple consideration is to minimize the spectral separation of the multiple emissions. This facilitates signal isolation and data analysis. As a result, fluorophores with narrow, well-resolved bandwidths are useful for multiplex applications. Appendix 1 contains Traits of Common Fluorophores to aid in the selection of fluorophores. For multiplex reactions, it is also recommended to optimize the following:

- Check primer design
- Optimize primer concentartions
- Optimize Mg^{2+} concentration

Check Primer Design

As for single-target reactions, multiplex qPCR will give the best results if all primers in the reaction have similar melting temperatures (Tm difference ≤ 2 °C) and none can form strong 3′-duplexes (ΔG ≥ –2.0 kcal). For more information, see the section *Check Primer Design for Primer Potential* on page 18. Individual reaction optimizations should be performed as well as optimization with several or all primer combinations. It is very often the case that individual primers work singly, but when combined in multiplex the primers cross-react or otherwise alter reaction specificity and efficiency.

Optimize Primer Concentrations

If one target in a multiplex reaction is significantly more abundant than the other(s) or if one primer pair gives a much lower Ct or higher ΔR (the amount of fluorescence in the no-template control) than the other(s), amplification of that target may dominate the reaction, using up reactants before other targets are detectable. Adjusting the levels of primers may allow a more balanced amplification of all targets. To determine if such adjustments will be beneficial, prepare standard curves that cover the range of targets expected for each primer pair alone (singleplex) and with all primers combined (multiplex). There is no need to modify primer levels if multiplex and singleplex reactions give similar results. On the other hand, optimizing primer concentrations will likely improve results if sensitivity is unacceptable in multiplex reactions. Decrease primer concentrations for those primer pairs that give low Ct values and/or increase concentrations for those that give high Ct values, within the range of 50-500 nM.

Optimize Mg^{2+} Concentration

Magnesium plays several roles in PCR. It is a required divalent cationic counter-ion for dNTPs and a co-factor for all polymerases. Divalent cations strongly affect DNA double-strand hybridization, and increasing magnesium raises the stability, or melting temperature, of a DNA duplex. It follows that high magnesium levels increase the affinity of primers toward hybridization, including mis-priming events and primer-primer interactions. The mis-primed DNA duplexes become substrates for the DNA polymerase, in effect creating side products and sapping PCR efficiency. Salts, such as KCl, will also change DNA duplex Tm, but the effect is less drastic for these monovalent cations.

PCR requires a minimal amount of magnesium, and both efficiency and product Tm change as the cation concentration increases. These effects are magnified when one attempts to perform multiplex PCR. Running multiple reactions concurrently introduces competition for reagents and exacerbates any non-optimal conditions creating major changes in PCR efficiency. Figure 20 demonstrates this point. The efficiency curves for two primer/probe targets were performed individually and then in multiplex. The graph shows that while the individual reactions (dark blue and green lines) give relatively similar efficiencies and sensitivities (y-axis values) running the reactions together dramatically changes the sensitivity and efficiency of the later reaction.

Figure 20. Singleplex Reaction vs. Duplex Reaction

qPCR Reagent Selection Table

Use the following table to choose the most appropriate reagent for your needs. The table is read horizontally to select the thermal cycler format. Products are compatible with tube, plate, or capillary-based instruments. The table is read vertically to narrow the reagent to SYBR Green dye or probe-based formulations.

High-throughput mixes include the reference dye in the ReadyMix to eliminate an extra pipetting step. The Quantitative RT-PCR reagents are noted in gray. These are for use when RNA is used as the starting template.

qPCR Reagent Selection Table

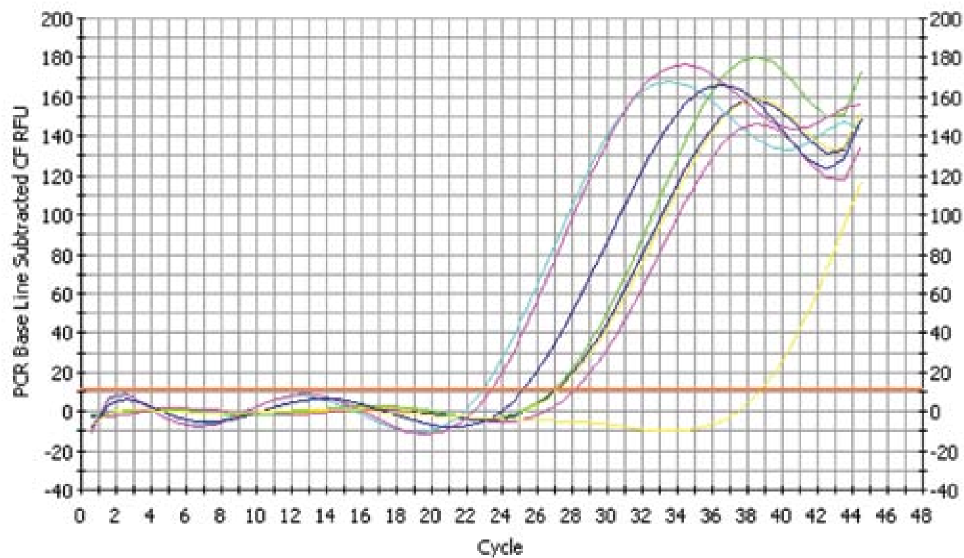
	Plate/Tube Instruments			**Glass Capillary Instruments**		
	Catalog Number	**Product Name**	**Package Size**	**Catalog Number**	**Product Name**	**Package Size**
SYBR Green based qPCR	**S4438**	SYBR Green JumpStart Taq ReadyMix for Quantitative PCR	100 Reactions 500 Reactions	**S1816**	SYBR Green JumpStart Taq ReadyMix for Quantitative PCR, Capillary Formulation	20 Reactions 100 Reactions 400 Reactions
SYBR Green based qPCR	**S5193**	SYBR Green JumpStart Taq ReadyMix without $MgCl_2$ (with separate tube of $MgCl_2$)	20 Reactions 100 Reactions 400 Reactions	**S5193**	SYBR Green JumpStart Taq ReadyMix without $MgCl_2$ (with separate tube of $MgCl_2$)	20 Reactions 100 Reactions 400 Reactions
SYBR Green based qPCR	**S9194**	SYBR Green JumpStart Taq ReadyMix for High-Throughput Quantitative PCR (with internal reference dye)	20 Reactions 400 Reactions 2000 Reactions			
SYBR Green based qPCR	**QR0100**	SYBR Green Quantitative RT-PCR Kit	1 Kit (100 Reactions)	**QR0100**	SYBR Green Quantitative RT-PCR Kit	1 Kit (100 Reactions)
	Catalog Number	**Product Name**	**Package Size**	**Catalog Number**	**Product Name**	**Package Size**
Probe based qPCR	**D7440**	JumpStart Taq ReadyMix for Quantitative PCR	100 Reactions 400 Reactions	**D9191**	JumpStart Taq ReadyMix with dUTP	20 Reactions 100 Reactions 400 Reactions
Probe based qPCR	**D9191**	JumpStart Taq ReadyMix with dUTP	20 Reactions 100 Reactions 400 Reactions			
Probe based qPCR	**D6442**	JumpStart Taq ReadyMix for High-Throughput Quantitative PCR (with internal reference dye)	20 Reactions 400 Reactions 2000 Reactions			
Probe based qPCR	**QR0200**	Quantitive RT-PCR ReadyMix	1 Kit (100 Reactions)	**QR0200**	Quantitive RT-PCR ReadyMix	1 Kit (100 Reactions)

Package sizes based on 50 µL reactions

Troubleshooting

Fluorescence Issues			
Number 1	**No, or low, fluorescence in both the test sample and in the positive control with the correct PCR product on the gel**		
	SYBR Green Dye-Based Detection	**Probe Detection**	
Possible cause	Bad SYBR Green binding dye	High-background fluorescence	Degraded probe
Diagnostic test	Compare fluorescence of the SYBR Green binding dye mix ± 1 µg DNA. Fluorescence should be at least 10,000 units higher with DNA added than without	Check the raw fluorescence (multicomponent plot). Fluorescence should increase at least 10,000 units between cycles 1 & 40	Digest 5 fmoles of probe with DNase I. Fluorescence should be at least 10,000 units greater than without DNase digestion
Solution	Purchase new SYBR Green binding dye or a new qPCR mix with SYBR Green binding dye	Purchase a new probe	Purchase a new probe
Number 2	**Low fluorescence from test sample, but the positive control has good fluorescence**		
Possible cause	Fluorescence quenching		
Diagnostic test	Positive control gives good fluorescence		
Solution	Purify the input nucleic acid		
Number 3	**Declining or hooked fluorescence plots**		
Possible cause	As PCR product accumulates, the complimentary strand competes with the primer and/or probe for annealing to template		
Diagnostic test	See figure		
Solution	Ignore it if the Ct is not affected		

Figure 21. Hooked Fluorescence Plots



Troubleshooting

Troubleshooting

Fluorescence Issues	
Number 4	**The fluorescence plots suddenly spike upwards**
Possible cause	Bad reference dye
Diagnostic test	Disable reference dye normalization or look at the results for ΔR (level of fluorescence in the no-template control) instead of ΔRn (the difference of the reporter fluorescence in the sample and that in the no template control). The plots should become smooth if fluorescence is not normalized to an inactive reference dye
Solution	Purchase a new reference dye. Always protect dye from light during storage
Number 5	**No amplification results from a sample known to contain target and the positive control does amplify**
Possible cause	Inhibition in the test sample is likely
Diagnostic test	Test amplification with a diluted sample. Spike the reaction with a low level of exogenous target and test for amplification of the exogenous target
Solution	Try adding BSA to 0.3% in the PCR or purify the input nucleic acid

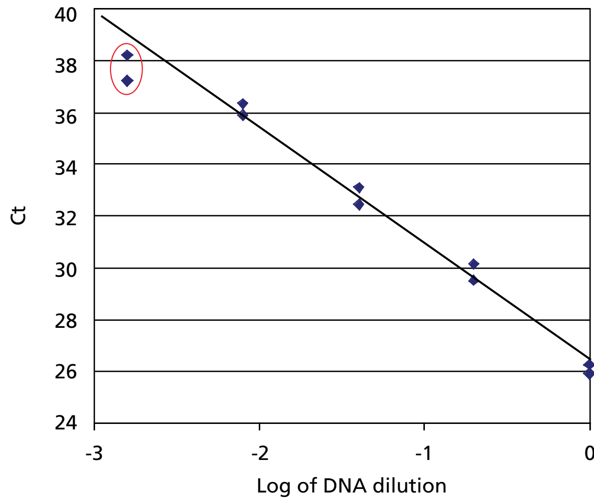
Dissociation/Melting Curves		
Number 6	**There are multiple peaks in the dissociation plot/melt curve**	
Possible cause	The Mg^{2+} concentration in the reaction is too high or the annealing temperature is too low	
Diagnostic test	Fractionate the PCR product on an ethidium bromide-stained agarose gel or use the Bioanalyzer to verify multiple products	
Solution	Titrate the Mg^{2+} to determine the optimum concentration. Perform annealing temperature gradient to select the optimum annealing temperature	
Number 7	**There is a broad peak at a lower Tm than the desired product, especially in low or no-template reactions**	
Possible cause	Primer-dimer, generated from primers that anneal at their 3′-ends, extend, and then amplify	
Diagnostic test	Fractionate the PCR product on an ethidium bromide-stained agarose gel or use the Bioanalyzer. The primer-dimer will appear as a diffuse band at ≤ 50 bp	
Solution	Try lower primer concentrations. With RT-PCR, try using less RT enzyme and use a 2-step and/or a higher incubation temperature for the RT step	
Number 8	**Multiple Tm peaks**	
	Single product on gel	**Multiple products on gel**
Possible cause	Localized AT or GC-rich regions or short repeats in PCR product	Multiple targets in the source material
Diagnostic test	Check sequence of amplicon for AT or GC-rich regions or repeats	BLAST primer sequences against the sequence of the source organism to verify single target
Solution	This is not a problem if the gel analysis shows that all product is specific. Continue to use the primers	Design unique primers

<table>
<tr><th colspan="4">Standard Curve</th></tr>
<tr><td>**Number 9**</td><td colspan="3">**PCR efficiency < 80%**</td></tr>
<tr><td>**Possible cause**</td><td>Suboptimal PCR conditions (See example. This illustrates low *Taq* activity)</td><td colspan="2">Poorly designed primers</td></tr>
<tr><td>**Diagnostic test**</td><td>Prepare or purchase fresh PCR mix</td><td colspan="2">Check the primer design. Test the PCR mix with a set of primers known to work well and a positive control template</td></tr>
<tr><td>**Solution**</td><td>Prepare or purchase fresh PCR mix</td><td colspan="2">Design new primers</td></tr>
<tr><td>**Number 10**</td><td colspan="2">**PCR efficiency is greater than 120%**</td><td></td></tr>
<tr><td>**Possible cause**</td><td>Excessive primer-dimer</td><td>Pipetting is inaccurate</td><td>Inhibition</td></tr>
<tr><td>**Diagnostic test**</td><td>Evaluate dissociation plot as shown in the figure below</td><td>Test pipette calibration</td><td>See Problem Number 4: The fluorescence plots suddenly spike upward</td></tr>
<tr><td>**Solution**</td><td>Try lower primer concentrations. With RT-PCR, try using less RT enzyme, doing 2-step and/or using a higher incubation temperature for the RT step</td><td>If the pipettes are inaccurate, get them re-calibrated. If the pipettes are properly calibrated, practice pipetting accurately</td><td>See Problem #4: The fluorescence plots suddenly spike upward</td></tr>
</table>

Troubleshooting

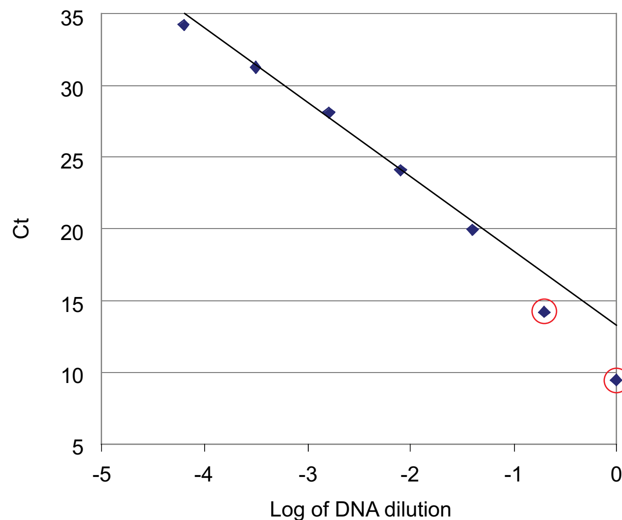
Standard Curve	
Number 11	The standard curve is not linear with low levels of sample input
Possible cause	Exceeded assay sensitivity
Diagnostic test	Ct > 30; qPCR replicates highly variable
Solution	Optimize primer concentrations

Figure 22. Standard Curve that is not Linear with Low Levels of Sample Input



Number 12	The standard curve is not linear with high levels of sample input
Possible cause	Exceeded assay capacity
Diagnostic test	Low Ct (< 12-15, depending on instrument)
Solution	Use less input nucleic acid

Figure 23. Standard Curve that is not Linear with High Levels of Sample Input



qRT-PCR Specific		
Number 13	**Product is detected in the no-RT reaction with primers that flank an intron**	
Possible cause	The intron is short enough that the primers amplify (~1.4 kb in example)	Pseudogenes are present that lack introns
Diagnostic test	Fractionate the PCR product on an ethidium bromide-stained agarose gel. No-RT product will migrate more slowly than +RT product as in gel photograph shown	Fractionate PCR product on ethidium bromide-stained agarose gel. No-RT product will migrate the same as +RT on gel
Solution	Re-design the primers to flank or span a longer intron or multiple introns	

Multiplex	
Number 14	**One, or more, primer/probe set does not work well in a multiplex reaction**
Possible cause	The target is less abundant or the primers are less efficient
Diagnostic test	Conduct reactions with individual primer/probe sets to see if they work adequately in the absence of competition
Solution	Limit the level of primers that dominate the reaction and give higher signal. Determine the level that reduces ΔRn without increasing Ct significantly

Sigma Product Specific	
Number 15	**Sigma's JumpStart Taq doesn't work as well as Qiagen's HotStarTaq**
Possible cause	Reactions were denatured at 95 °C for 15 min before cycling
Diagnostic test	Check the thermocycling profile
Solution	3 min at 94 °C is sufficient to activate JumpStart Taq; longer incubations will partially inactivate
Number 16	**Sigma's ReadyMix doesn't work, but competitors do**
Possible cause	REDTaq ReadyMix was used for real-time qPCR
Diagnostic test	Check color of qPCR mix
Solution	Use clear mixes for real-time qPCR. Red dye will interfere with fluorescence detection for most instruments

Appendix 1

Traits of Commonly Used Fluorophores

Dye	Ab_{max} (nm)	Extinction Coefficient (l mole^{-1} cm^{-1})	Em_{max} (nm)
Acridin	362	11,000	462
AMCA	353	19,000	442
BODIPY® FL-Br2	531	75,000	545
BODIPY® 530/550	534	77,000	554
BODIPY® TMR	544	56,000	570
BODIPY® 558/568	558	97,000	569
BODIPY® 564/570	563	142,000	569
BODIPY® 576/589	575	83,000	588
BODIPY® 581/591	581	136,000	591
BODIPY® TR	588	68,000	616
BODIPY® 630/650	625	101,000	640
BODIPY® 650/665	646	102,000	660
Cascade Blue®	396	29,000	410
Cy®2	489	150,000	506
Cy®3	552	150,000	570
Cy®3.5	581	150,000	596
Cy®5	643	250,000	667
Cy®5.5	675	250,000	694
Cy®7	743	250,000	767
DABCYL	453	32,000	None
EDANS	335	5,900	493
Eosin	521	95,000	544
Erythrosin	529	90,000	553
Fluorescein	492	78,000	520
6-FAM™	494	83,000	518
TET™	521	—	536
JOE™	520	71,000	548
HEX™	535	—	556
LightCycler® 640	625	110,000	640
LightCycler® 705	685	—	705
NBD	466	22,000	535
Oregon Green® 488	492	88,000	517
Oregon Green® 500	499	78,000	519
Oregon Green® 514	506	85,000	526
Rhodamine 6G™	524	102,000	550
Rhodamine Green™	504	78,000	532
Rhodamine Red™	560	129,000	580
Rhodol Green™	496	63,000	523
TAMRA™	565	91,000	580
ROX™	585	82,000	605
Texas Red™	583	116,000	603
NED™	546	not available	575
VIC®	538	not available	554
Yakima Yellow™	526	84,000	448

Appendix 2

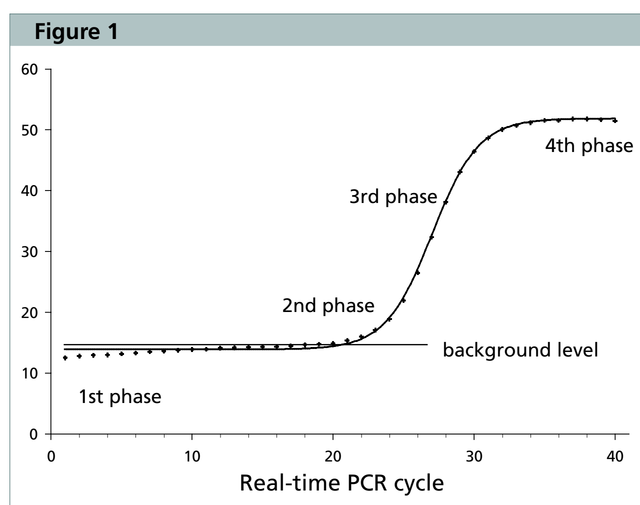
How to Optimise Your Quantitative Real-Time RT-PCR (qRT-PCR)

Michael W. Pfaffl, Physiology Weihenstephan, Technical University of Munich (TUM), Weihenstephaner Berg 3, 85354 Freising Weihenstephan, Germany
http://optimisation.gene-quantification.info

Introduction

To get very reliable and quantitative real-time RT-PCR results, the applied mRNA quantification assay and working procedure should be highly optimised. The efficacy of kinetic RT-PCR is measured by its specificity, low background fluorescence, steep fluorescence increase and high amplification efficiency, and constant high level plateau. Therefore the typical reaction history can be divided in four characteristic phases, Figure 1. The 1st phase is hidden under the background fluorescence, where an exponential amplification is expected; 2nd phase with exponential amplification that can be detected and above the background; 3rd phase with linear amplification efficiency and a steep increase of fluorescence; and finally 4th phase, or plateau phase, defined as the attenuation in the rate of exponential product accumulation, normally seen in later cycles.

Figure 1



The four characteristic phases of PCR.

The following information addresses optimisation strategies in quantitative real-time RT-PCR. Special focus is laid on the pre-analytical steps, sampling techniques, RNA extraction, and reverse transcription (RT), primer usage, and post-analytical steps, especially on crossing point evaluation and crossing point measuring at elevated temperatures.

Tissue Sampling and RNA Extraction

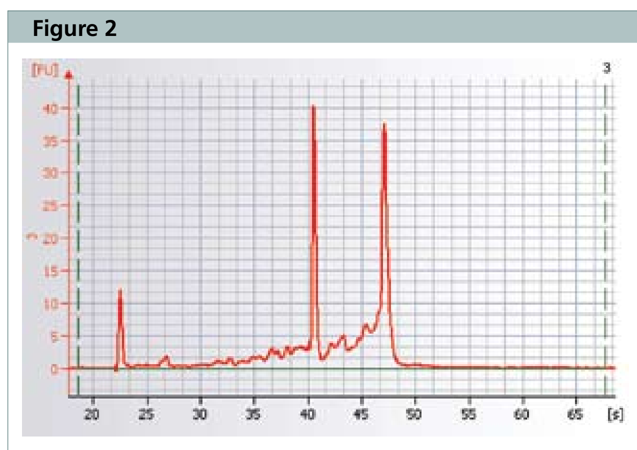
The sampling and preparation of intact cellular total RNA or mRNA is critical to all gene expression analysis techniques. A successful and reliable experiment needs high quality, DNA free, undegraded RNA. The source of tissue sampling techniques and the subsequent storage of the tissue material often varies significantly between processing laboratories (e.g. biopsy material, single cell sampling, laser micro-dissection, slaughtering samples). Some researchers store the tissue sample in RNA*later*® or in the first extraction buffer containing guanidine isocyanate, provided in the extraction kits, and others freeze it in liquid nitrogen. The extracted analyte (either total RNA or poly-adenylated mRNA) as well as the RNA isolated using columns or a liquid-liquid extraction, could result in varying RNA qualities and quantities. For example RNA extracted from collagen rich or adipose tissues often has a lower total RNA yield, is of lesser quality, and contains partly degraded RNA sub-fractions. Particular RNA extraction techniques can work more effectively in one specific tissue type compared with another one and result in up to 10-fold variations in total RNA yield per extracted tissue mass and as a result, on the following real-time RT-PCR gene expression analysis as well. A lot of total RNA preparations are contaminated with genomic DNA fragments and protein at very low levels. The enormous amplification power of kinetic PCR may result in even the smallest amount of DNA contamination interfering with the desired specific RT-PCR product. To confirm the absence of residual DNA, either a negative control (minus-RT or water) should be included in each experimental setup. It may be necessary to treat the RNA sample with commercially available RNase-free DNase, to get rid of any DNA. However, unspecific side reactions of the DNase often result in RNA degradation. However, it is always recommended to remove the DNase prior to any RT or PCR step. Furthermore, the design of the PCR product should incorporate at least one exon-exon splice junction to allow a product obtained from the cDNA to be distinguished on electrophoresis from genomic DNA contamination.

RNA Quantity and Integrity

Accurate quantification and quality assessment of the starting RNA sample is particularly important for an absolute quantification strategy that normalises specific mRNA expression levels against a given calibration curve measured in molecules or concentrations/grams RNA. The RNA quality assessment requires accurate quantification of the isolated total RNA or mRNA fraction by optical density at 260 nm, (OD_{260}), and determination of the RNA quality calculated by the OD_{260}/OD_{280} ratio or by the RiboGreen RNA Quantification Kit from Molecular Probes. Furthermore, the RNA quality can be verified by capillary electrophoresis with the Bioanalyzer 2100 on a microchip lab-on-chip system from Agilent Technologies. The recently developed RNA integrity number (RIN) determines the level of intact total RNA on the basis of an electropherogram, Figure 2. The RIN value can range between 1-10: RIN 1 for totally degraded RNA, and RIN 10 for a perfect and intact total RNA. RIN numbers under 5 are at least partly degraded and result in low 18S rRNA and 28S rRNA peaks in the electropherogram. In a broader sense, poor RNA quality and low RIN numbers influence the qRT-PCR performance significantly and lead to an inhibition of the PCR performance in general and to a later crossing point. Therefore, the extracted total RNA quality and quantity must be verified prior to qRT-PCR experiments to end in reliable mRNA quantification results.

Appendix 2

Figure 2



An electropherogram of total RNA is shown (178 ng/µl total RNA; RIN = 7.9; : ratio 28s/18s = 1.4). The characteristic total RNA profile represent an internal reference peak (22 s), a small 5S RNA peak (27 s), a dominant 18S RNA peak (41 s) and a dominant 28S RNA peak (43 s).

Reverse Transcription

An optimal reverse transcription (RT) is essential for a reliable and successful qRT-PCR assay. The RT step is the source of high variability in a kinetic RT-PCR experiment and for each enzyme specific reaction, conditions have to be optimised. Buffer salt contamination, pH, fatty acids, alcohol, phenol and other chemical or enzyme inhibitors carried over from the RNA isolation process can affect the apparent RT efficiency. The extracted total RNA may contain chemical or enzymatic tissue inhibitors that result in reduced RT and PCR reaction efficiencies and generate unreliable quantification results. For many quantitative applications, MMLV H minus RT is the enzyme of choice, as the cDNA synthesis rate can be up to 10-fold greater than that of AMV. In numbers, the MMLV H minus RT has the ability to reverse transcribe between 50 and 80% of the total RNA and the AMV only around 5-20%. Newly available thermo-stable RNAse H minus RT maintains its activity up to 70 °C, thus permitting increased specificity and efficiency of first primer annealing.

Another source of variability is the choice of priming method used to initiate cDNA synthesis, which can be either target gene-specific or non-specific. Target gene specific primers work well in conjunction with elevated RT reaction temperatures to eliminate spurious transcripts. The same reverse primer is used for the subsequent PCR assay in conjunction with the corresponding gene-specific sense primer (forward primer); however, the use of gene-specific primers necessitates a separate RT reaction for each gene of interest. It cannot be assumed that different reactions have the same cDNA synthesis efficiencies. The result can be high variability during multiple RT reactions.

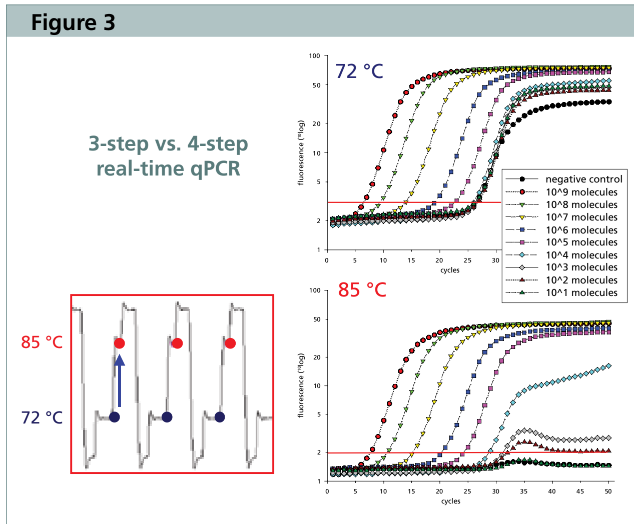
To circumvent these high inter-assay variations in RT, target gene unspecific primers, e.g. random-hexamer, -octamer or -decamer primers, can be used and a cDNA pool can be synthesised. Similarly, poly-T oligo-nucleotides (consisting solely of 16-25 desoxythymidine residues) can anneal to the poly-adenylated 3-' (poly-A) tail found on most mRNAs. cDNA pools synthesised with unspecific primers can be split into a number of different target-specific kinetic PCR assays. This maximises the number of genes that can be assayed from a single cDNA pool derived from one small RNA sample. Therefore, the gene expression results are directly comparable between the applied assays, at least within one and the same RT pool. In conclusion, a rank order of RT efficiency can be shown for the applied different primers for one specific gene: random hexamer primers > poly-dT primer > gene specific primer (M. Pfaffl, unpublished results).

Elevated Fluorescence Acquisition

Real-time assays using SYBR Green I binding dye can easily reveal the presence of primer dimers, which are the product of non-specific annealing and primer elongation events. These events take place as soon as PCR reagents are combined in the tube. During PCR, formation of primer dimers competes with formation of specific PCR product, leading to reduced amplification efficiency and a less successful specific RT PCR product. To distinguish primer dimers from the specific amplicons, a melting curve analysis can be performed in all available quantification software. The pure and homogeneous RT-PCR product produces a single, sharply defined melting curve with a narrow peak. In contrast, the primer dimers melt at lower temperatures (< 80 °C) and have diffuse and broader peaks. To get rid of primer dimers, an intensive primer optimisation is needed by testing multiple primer pair by cross-wise combinations. Multiples of such primer optimisation strategies have been developed.

The easiest and most effective way to get rid of any dimer structures, at least during the quantification procedure, is to add an additional 4th segment to the classical three segmented PCR procedure, Figure 3: 1st segment with denaturation at 95 °C; 2nd segment with primer annealing at 60 °C; 3rd segment with elongation at 72 °C; 4th segment with fluorescence acquisition at elevated temperatures (herein 85 °C). The fluorescence acquisition in the 4th segment eliminates the non-specific fluorescence signals derived by primer dimers or unspecific minor products and ensures accurate quantification of the desired qRT-PCR product. High temperature quantification keeps the background fluorescence and the 'no-template control' fluorescence under 10% of maximal fluorescence at plateau and ensures an optimal dynamic fluorescence range.

Figure 3



Advantage of elevated real-time PCR fluorescence acquisition temperature.

Crossing Point Data Evaluation

The amount of amplified target is directly proportional to the input amount of target only during the exponential phase of PCR amplification. Hence the key factor in the quantitative ability of kinetic RT-PCR is that it measures the product of the target gene within that phase. The data evaluation, or crossing points (CP) or threshold cycle (Ct) determination is very critical to the users. For CP determination, various fluorescence acquisition methodologies are possible. The *"fit point method"* (e.g. in LightCycler software) and the *"threshold cycle method"* (used in most quantification software) measure the CP at a constant fluorescence level. These constant threshold methods assume that all samples have the identical synthesised DNA concentration at the point where the fluorescence signal significantly increases over the background fluorescence, 2nd to 3rd phase in Figure 1. Measuring the level of background fluorescence can be a challenge in real-time PCR reactions with significant background fluorescence variations, caused by drift-ups and drift-downs over the course of the reaction. Averaging over a drifting background will give an overestimation of variance and thus increase the threshold level. The threshold level can be calculated by fitting the intersecting line upon the ten-times value of ground fluorescence standard deviation, e.g., Applied Biosystems software. This acquisition mode can be easily automated and is very robust. In the *"fit point method"* the user has to discard the uninformative background points, exclude the plateau values by entering the number of log-linear points, and then fit a log-line to the linear portion of the amplification curves. These log lines are extrapolated back to a common threshold line and the intersection of the two lines provides the CP value. The strength of this method is that it is extremely robust. The weakness is that it is not easily automated and so requires a lot of user input. The problems of defining a constant background for all samples within one run, sample-to-sample differences in variance, and absolute fluorescence values led to the development of new acquisition modus according to mathematical algorithms. In the LightCycler software the *"second derivative maximum method"* is performed in which CP is automatically identified and measured at the maximum acceleration of fluorescence. The amplification reaction and the kinetic fluorescence history over various cycles is obviously not a smooth and easy function. The mathematical algorithm on which the *"second derivative maximum method"* is unpublished, but it is possible to fit sigmoidal and polynomial curve models, with high significance, $p<0.001$, and coefficient of correlation, $r>0.99$.

This increase in the rate of fluorescence increase, or better called the acceleration of the fluorescence signal, slows down at the beginning of the 3rd linear phase. Therefore the cycle where the 2nd derivative is at its maximum is always between 2nd exponential and 3rd linear phase. Here, above the background level within the real exponential phase and very close to the background line, the optimal CP should be placed.

References

1. Higuchi, R., *et al.,* Kinetic PCR analysis: Real-time monitoring of DNA amplification reactions. *Biotechnology,* **11,** 1026 (1993).
2. Pals, G., *et al.,* A rapid and sensitive approach to mutation detection using real-time polymerase chain reaction and melting curve analyses, using BRCA1 as an example. *Mol Diagn.,* **4,** 241-246 (1999).
3. Selvin, P.R., Fluorescence resonance energy transfer. *Meth. Enzymol.,* **246,** 300-334 (1995).
4. Pals, G., *et al.,* Detection of a single base substitution in a single cell using the LightCycler. *J. Biochem. Biophys. Methods,* **47,** 121-129 (2001).
5. Bonnet, G., *et al.,* Thermodynamic basis of the enhanced specificity of structured DNA probes. *Proc. Natl. Acad. Sci. USA,* **96,** 6171-6176 (1999).
6. Broude, N.E., Stem-loop oligonucleotides: A robust tool for molecular biology and biotechnology. *Trends Biotechnol.,* **20,** 249-256 (2002).
7. Tyagi, S. and Kramer, F.R., Molecular Beacons: probes that fluoresce upon hybridization. *Nat. Biotechnol.,* **14,** 303-308 (1996).
8. Antony, T. and Subramaniam, V., Molecular Beacons: nucleic acid hybridization and emerging applications. *J. Biomol. Struct. Dyn.,* **19,** 497-504 (2001).
9. Perlette, J. and Tan, W., Real-time monitoring of intracellular mRNA hybridization inside single living cells. *Anal. Chem.,* **73,** 5544-5550 (2001).
10. Dirks, R.W., *et al.,* Methods for visualizing RNA processing and transport pathways in living cells. *Histochem. Cell Biol.,* **115,** 3-11 (2001).
11. Liu, J., *et al.,* Real-time monitoring *in vitro* transcription using Molecular Beacons. *Anal. Biochem.,* **300,** 40-45 (2002).
12. Mhlanga, M.M. and Malmberg, L., Using Molecular Beacons to detect single-nucleotide polymorphisms with real-time PCR. *Methods,* **25,** 463-471 (2001).
13. Giesendorf, B.A., *et al.,* Molecular Beacons: a new approach for semi-automated mutation analysis. *Clin. Chem.,* **44,** 482-486 (1998).
14. Marras, S.A., *et al.,* Multiplex detection of single-nucleotide variations using Molecular Beacons. *Genet. Anal.,* **14,** 151-156 (1999).
15. Tapp, I., *et al.,* Scoring of single-nucleotide polymorphisms: comparison of the 5'-nuclease TaqMan assay and Molecular Beacon probes. *Biotechniques,* **28,** 732-738 (2000).
16. Steuerwald, N., *et al.,* Analysis of gene expression in single oocytes and embryos by real-time rapid cycle fluorescence monitored RT-PCR. *Mol. Hum. Reprod.,* **5,** 1034-1039 (1999).
17. Vet, J.A., *et al.,* Multiplex detection of four pathogenic retroviruses using Molecular Beacons. *Proc. Natl. Acad. Sci. USA,* **96,** 6394-6399 (1999).
18. Solinas, A., *et al.,* Duplex Scorpion primers in SNP analysis and FRET applications. *Nucleic Acids Res.,* **29,** E96 (2001).
19. Whitcombe, D., *et al.,* Detection of PCR products using self-probing amplicons and fluorescence. *Nat. Biotechnol.,* **17,** 804-807 (1999).
20. Thelwell, N., *et al.,* Mode of action and application of Scorpion primers to mutation detection. *Nucleic Acids Res.,* **28,** 3752-3761 (2000).
21. Hart, K.W., *et al.,* Novel method for detection, typing, and quantification of human papillomaviruses in clinical samples. *J. Clin. Microbiol.,* **39,** 3204-3212 (2001).
22. Braasch, D., *et al.,* RNA interference in mammalian cells by chemically-modified RNA. *Biochem.,* **42,** 7967-7975 (2003).
23. Latorra, D., *et al.,* Design considerations and effects of LNA in PCR primers. *Molecular and Cellular Probes,* **17,** 253-259 (2003).
24. Christensen, U., *et al.,* Stopped-flow kinetics of Locked Nucleic Acid oligonucleotide duplex formation: studies of LNA-DNA and DNA-DNA interactions. *Biochem. J.,* **354,** 481-484, (2001).
25. Latorra, D., *et al.,* Multiplex allele-specific PCR with optimized locked nucleic acid primers. *Biotechniques,* **34,** 150-152, 1154, 1158 (2003).
26. Braasch, D. and Corey, D., Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA. *Chem. Biol.,* **8,** 1-7 (2001).
27. Bustin, S.A., Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J. Molecular Endocrinology,* **29,** 23-29 (2002).

Trademarks

JumpStart, ReadyMix, and GenElute are trademarks of Sigma-Aldrich Biotechnology, LP and Sigma-Aldrich Co.

SYBR, YOYO, YO-PRO, BODIPY, Cascade Blue, Oregon Green, and Texas Red are registered trademarks of Molecular Probes, Inc.

Rhodamine Green, Rhodamine Red, and Rhodol Green, are trademarks of Molecular Probes, Inc.

Amplifluor is a trademark of Intergen Co.

Scorpion is a trademark of DxS Limited.

TaqMan and LightCycler are registered trademarks of Roche Molecular Systems, Inc.

Rotor-Gene is a trademark of Corbett Research.

SmartCycler is registered trademark of Cepheid, Inc.

MX3000P and MX3005P are registered trademarks of Stratagene, Inc.

MxPro is a trademark of Stratagene, Inc.

LUX is a trademark of Invitrogen Corporation.

LNA is a registered trademark of Exiqon A/S.

MGB and Yakima Yellow are registered trademarks of Epoch Biosciences, Inc.

Quantica is a registered trademark of Techne Ltd. and Techne, Inc.

RNA*later* is a registered trademark of Ambion.

FAM, HEX, JOE, NED, ROX, TAMRA, and TET are trademarks of Applera Corporation or its subsidiaries in the United States and certain other countries.

VIC is a registered trademark of Applera Corporation or its subsidiaries in the United States and certain other countries.

Chromo4 and MyiQ are trademarks of Bio-Rad Laboratories, Inc.

DNA Engine and DNA Engine Opticon, iCycler are registered trademarks of Bio-Rad Laboratories, Inc.

Cy is a registered trademark of GE Healthcare.

Argentina
SIGMA-ALDRICH DE ARGENTINA S.A.
Free Tel: 0810 888 7446
Tel: (+54) 11 4556 1472
Fax: (+54) 11 4552 1698

Australia
SIGMA-ALDRICH PTY LTD.
Free Tel: 1800 800 097
Free Fax: 1800 800 096
Tel: (+61) 2 9841 0555
Fax: (+61) 2 9841 0500

Austria
SIGMA-ALDRICH HANDELS GmbH
Tel: (+43) 1 605 81 10
Fax: (+43) 1 605 81 20

Belgium
SIGMA-ALDRICH NV/SA.
Free Tel: 0800 14747
Free Fax: 0800 14745
Tel: (+32) 3 899 13 01
Fax: (+32) 3 899 13 11

Brazil
SIGMA-ALDRICH BRASIL LTDA.
Free Tel: 0800 701 7425
Tel: (+55) 11 3732 3100
Fax: (+55) 11 5522 9895

Canada
SIGMA-ALDRICH CANADA LTD.
Free Tel: 1800 565 1400
Free Fax: 1800 265 3858
Tel: (+1) 905 829 9500
Fax: (+1) 905 829 9292

China
SIGMA-ALDRICH (SHANGHAI)
TRADING CO. LTD.
Free Tel: 800 819 3336
Tel: (+86) 21 6141 5566
Fax: (+86) 21 6141 5567

Czech Republic
SIGMA-ALDRICH spol. s r. o.
Tel: (+420) 246 003 200
Fax: (+420) 246 003 291

Denmark
SIGMA-ALDRICH DENMARK A/S
Tel: (+45) 43 56 59 10
Fax: (+45) 43 56 59 05

Finland
SIGMA-ALDRICH FINLAND OY
Tel: (+358) 9 350 9250
Fax: (+358) 9 350 92555

France
SIGMA-ALDRICH CHIMIE S.à.r.l.
Free Tel: 0800 211 408
Free Fax: 0800 031 052
Tel: (+33) 474 82 28 00
Fax: (+33) 474 95 68 08

Germany
SIGMA-ALDRICH CHEMIE GmbH
Free Tel: 0800 51 55 000
Free Fax: 0800 64 90 000
Tel: (+49) 89 6513 0
Fax: (+49) 89 6513 1160

Greece
SIGMA-ALDRICH (O.M.) LTD.
Tel: (+30) 210 994 8010
Fax: (+30) 210 994 3831

Hungary
SIGMA-ALDRICH Kft
Ingyenes zöld telefon: 06 80 355 355
Ingyenes zöld fax: 06 80 344 344
Tel: (+36) 1 235 9055
Fax: (+36) 1 235 9050

India
SIGMA-ALDRICH CHEMICALS
PRIVATE LIMITED
Telephone
Bangalore: (+91) 80 6621 9600
New Delhi: (+91) 11 4165 4255
Mumbai: (+91) 22 2570 2364
Hyderabad: (+91) 40 4015 5488
Fax
Bangalore: (+91) 80 6621 9650
New Delhi: (+91) 11 4165 4266
Mumbai: (+91) 22 2579 7589
Hyderabad: (+91) 40 4015 5466

Ireland
SIGMA-ALDRICH IRELAND LTD.
Free Tel: 1800 200 888
Free Fax: 1800 600 222
Tel: (+353) 1 404 1900
Fax: (+353) 1 404 1910

Israel
SIGMA-ALDRICH ISRAEL LTD.
Free Tel: 1 800 70 2222
Tel: (+972) 8 948 4100
Fax: (+972) 8 948 4200

Italy
SIGMA-ALDRICH S.r.l.
Numero Verde: 800 827018
Tel: (+39) 02 3341 7310
Fax: (+39) 02 3801 0737

Japan
SIGMA-ALDRICH JAPAN K.K.
Tel: (+81) 3 5796 7300
Fax: (+81) 3 5796 7315

Korea
SIGMA-ALDRICH KOREA
Free Tel: (+82) 80 023 7111
Free Fax: (+82) 80 023 8111
Tel: (+82) 31 329 9000
Fax: (+82) 31 329 9090

Malaysia
SIGMA-ALDRICH (M) SDN. BHD
Tel: (+60) 3 5635 3321
Fax: (+60) 3 5635 4116

Mexico
SIGMA-ALDRICH QUÍMICA, S.A. de C.V.
Free Tel: 01 800 007 5300
Free Fax: 01 800 712 9920
Tel: 52 722 276 1600
Fax: 52 722 276 1601

The Netherlands
SIGMA-ALDRICH CHEMIE BV
Free Tel: 0800 022 9088
Free Fax: 0800 022 9089
Tel: (+31) 78 620 5411
Fax: (+31) 78 620 5421

New Zealand
SIGMA-ALDRICH NEW ZEALAND LTD.
Free Tel: 0800 936 666
Free Fax: 0800 937 777
Tel: (+61) 2 9841 0555
Fax: (+61) 2 9841 0500

Norway
SIGMA-ALDRICH NORWAY AS
Tel: (+47) 23 17 60 60
Fax: (+47) 23 17 60 50

Poland
SIGMA-ALDRICH Sp. z o.o.
Tel: (+48) 61 829 01 00
Fax: (+48) 61 829 01 20

Portugal
SIGMA-ALDRICH QUÍMICA, S.A.
Free Tel: 800 202 180
Free Fax: 800 202 178
Tel: (+351) 21 924 2555
Fax: (+351) 21 924 2610

Russia
SIGMA-ALDRICH RUS, LLC
Tel: +7 (495) 621 6037
+7 (495) 621 5828
Fax: +7 (495) 621 5923

Singapore
SIGMA-ALDRICH PTE. LTD.
Tel: (+65) 6779 1200
Fax: (+65) 6779 1822

South Africa
SIGMA-ALDRICH
SOUTH AFRICA (PTY) LTD.
Free Tel: 0800 1100 75
Free Fax: 0800 1100 79
Tel: (+27) 11 979 1188
Fax: (+27) 11 979 1119

Spain
SIGMA-ALDRICH QUÍMICA, S.A.
Free Tel: 900 101 376
Free Fax: 900 102 028
Tel: (+34) 91 661 99 77
Fax: (+34) 91 661 96 42

Sweden
SIGMA-ALDRICH SWEDEN AB
Tel: (+46) 8 742 4200
Fax: (+46) 8 742 4243

Switzerland
SIGMA-ALDRICH CHEMIE GmbH
Free Tel: 0800 80 00 80
Free Fax: 0800 80 00 81
Tel: (+41) 81 755 2828
Fax: (+41) 81 755 2815

United Kingdom
SIGMA-ALDRICH COMPANY LTD.
Free Tel: 0800 717 181
Free Fax: 0800 378 785
Tel: (+44) 1747 833 000
Fax: (+44) 1747 833 313
SAFC (UK) Free Tel: 01202 712305

United States
SIGMA-ALDRICH
P.O. Box 14508
St. Louis, Missouri 63178
Toll-Free: 800 325 3010
Toll-Free Fax: 800 325 5052
Call Collect: (+1) 314 771 5750
Tel: (+1) 314 771 5765
Fax: (+1) 314 771 5757

Internet
sigma-aldrich.com

World Headquarters
3050 Spruce St., St. Louis, MO 63103
(314) 771-5765
sigma-aldrich.com

Order/Customer Service (800) 325-3010 • Fax (800) 325-5052
Technical Service (800) 325-5832 • sigma-aldrich.com/techservice
Development/Bulk Manufacturing Inquiries SAFC™ (800) 244-1173

*Accelerating Customers' Success through Leadership in **Life Science, High Technology** and **Service***

©2008 Sigma-Aldrich Co. All rights reserved. SIGMA, , SAFC, SAFC™, SIGMA-ALDRICH, ALDRICH, , FLUKA, , and SUPELCO, are trademarks belonging to Sigma-Aldrich Co. and its affiliate Sigma-Aldrich Biotechnology, L.P. Sigma brand products are sold through Sigma-Aldrich, Inc. Sigma-Aldrich, Inc. warrants that its products conform to the information contained in this and other Sigma-Aldrich publications. Purchaser must determine the suitability of the product(s) for their particular use. Additional terms and conditions may apply. Please see reverse side of the invoice or packing slip.

02503-504204
0038

SIGMA-ALDRICH®