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).

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**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Catalog Number</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>W1754</td>
<td>155 µL</td>
</tr>
<tr>
<td>SYBR Green JumpStart™ Taq ReadyMix™</td>
<td>S9939*</td>
<td>520 µL</td>
</tr>
<tr>
<td>Reference dye**</td>
<td>R4526*</td>
<td>1.0 µL</td>
</tr>
</tbody>
</table>

**qRT-PCR**

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

* 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:

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

*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\(^{-1/(-3.33)}\)). 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).

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.
Intron-Spanning (A) and Intron-Flanking (B) Primers for RT-PCR

**A. Primers Span Intron**

DNA: ![DNA Structure]

mRNA: ![mRNA Structure]

**B. Primers Flank Intron**

DNA: ![DNA Structure]

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**

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.

**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.
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 concentrations
- 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 mispriming 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

Singleplex vs duplex

- **CDC multiplex**
  - $y = -3.1601x + 45.67$
  - $R^2 = 0.9736$

- **CDC single**
  - $y = -2.3993x + 31.829$
  - $R^2 = 0.9648$

- **cmyc duplex**
  - $y = -3.4387x + 30.482$
  - $R^2 = 0.9863$

- **cmyc single**
  - $y = -3.2114x + 29.872$
  - $R^2 = 0.9993$