Scientific article

A systematic guideline for developing the best real-time PCR primers

Lessons learned from designing assays for more than 14,000 genes

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Abstract: Primer design is the most important factor affecting the quality of SYBR® Green real-time PCR analyses. Although they seem to generate acceptable results at first, many homemade or "do-it-yourself" primers often come up short in their specificity, PCR amplification efficiency, reproducibility, and sensitivity. This paper aims to summarize the important principles that we have learned after designing real-time RT-PCR assays for over 14,000 genes. Our conclusion is that the primer design algorithm and the buffer conditions must work together to provide the best results. Here, we outline the steps that we see as being crucial in designing and verifying real-time RT-PCR assays. Moreover, the solutions to potential problems in primer design are described.

Introduction

Quantitative or real-time RT-PCR has become routine in many of today's research laboratories to monitor relative changes in gene expression under different experimental conditions. However, many researchers design their own real-time PCR primers without complete knowledge about the important aspects affecting good primer design. As a result, gene expression results obtained by PCR are often unknowingly compromised. For the real-time RT-PCR technique to give consistent and reliable results, the assays must meet specific performance requirements to address typical technical concerns, such as:

1. **High sensitivity**
   How many copies can I detect?

2. **Single-amplicon specificity**
   Am I measuring the right gene?

3. **High degrees of accuracy and reliability**
   Is this the real fold change?

4. **Wide linear dynamic ranges**
   Can I simultaneously detect genes expressed at levels that vary by up to 10,000-fold?

5. **Reproducibility**
   Will my assay work each time? How close are my replicates?

The time and resources necessary to design and verify SYBR Green real-time PCR assays that pass all of these performance requirements is often underestimated. Simple experiments drawn up on paper to measure a few genes can quickly turn into frustration as the first assay results in primer dimers, off-target amplification, or suboptimal amplification curves with poor efficiencies.

Each failed real-time PCR assay means time and resources wasted. When it comes to real-time PCR primer design, however, there is no need to reinvent the wheel.

Contents

This white paper summarizes our knowledge about high-quality real-time PCR primer design from our collective experience of designing real-time PCR assays for more than 14,000 genes. It highlights the steps needed to design and verify fully optimized real-time RT-PCR assays for gene expression analysis. It also outlines specific experiments that can be performed to test each performance parameter.

Design algorithm

The primer design algorithm is key to generating successful primers. Each design must meet several important thermodynamic and sequence criteria (Table 1). In order to avoid missing any gene expression, the primers must detect every alternative transcript and splicing variant of the queried gene. To do so, all known entries in the public databases should be found and aligned to reveal a common gene-specific region for primer design. If GC content, primer length, and primer melting temperature range are controlled, each assay can use a standard set of PCR cycling conditions. Uniform cycling conditions, in turn, allow researchers to scale up from a single assay to multiple assays on an entire 96- or even 384-well plate. Through a comparison of the primer sequences with the single nucleotide polymorphism (SNP) database, sequences containing known SNP locations can be eliminated so that any individual source of total RNA may be analyzed with the same assay. A BLAST analysis further insures that the chosen primer sequences are sufficiently different from the rest of the transcriptome in the species of interest. Often, a BLAST analysis against the *E. coli* genome is also warranted because many *Taq* polymerases are contaminated with DNA from the organism used to over-express and purify the enzyme. Finally, stability at the 3′-end of the primers controls the start position for the DNA polymerase, further enhancing specificity.

Table 1. The design algorithm for the RT² qPCR Assays utilizes more than ten thermodynamic and sequence alignment criteria.

Amplicon length	50–210 bp
Primer length	19–23 nucleotides
GC content	35–65%
Tm	60–68°C
3′-end stability	Composition of last 3 base pairs
Complementarities	Avoid primer self- or cross-annealing stretches greater than 4 bp
Specificity	BLAST versus entire mRNA RefSeq database
SNP database	Primer sequences do no include known SNP

Any algorithm's primer design must also be experimentally verified for high performance with wet-bench quality control protocols, starting with two major success criteria. First, a melt curve analysis must verify that a single gene-specific product is produced. Following the melt curve, an agarose gel can also be run to further verify a single product of the predicted size, based on the amplicon design, without primer dimers or off-target amplifications. Second, the amplification efficiency must be greater than 90 percent for accurate and reliable results. If a real-time RT-PCR assay does not meet all of the above requirements, then the quality control fails, and the assay must be re-designed.

Specificity

All real-time PCR assays must generate a single band of the correct size for the results to accurately represent the expression of the queried gene. Secondary products confound the analysis. Detecting other genes at the same time as the gene of interest returns a weighted sum of their relative expression levels. Amplification of primer dimers or other secondary products causes artificially high and/or false positive signals. The assay could report the presence of product when there is none, or a greater amount of product than is really there.

How can you tell if your real-time PCR assays are specific enough? If you are using SYBR Green detection, just routinely run the default melting program on your instrument immediately after the completion of the cycling program. Use your instrument software to generate the dissociation curve (the first derivative of the melt curve). A single peak indicates a single melting event, and therefore a single product. If SYBR Green detection is not used, this analysis is not possible. Instead (or to be completely rigorous with SYBR Green detection), characterize a portion of the reaction by agarose gel electrophoresis. Not only should you see a single band, but that band should be of the correct size based on your primer design and amplicon size. See Figure 1 for representative results.

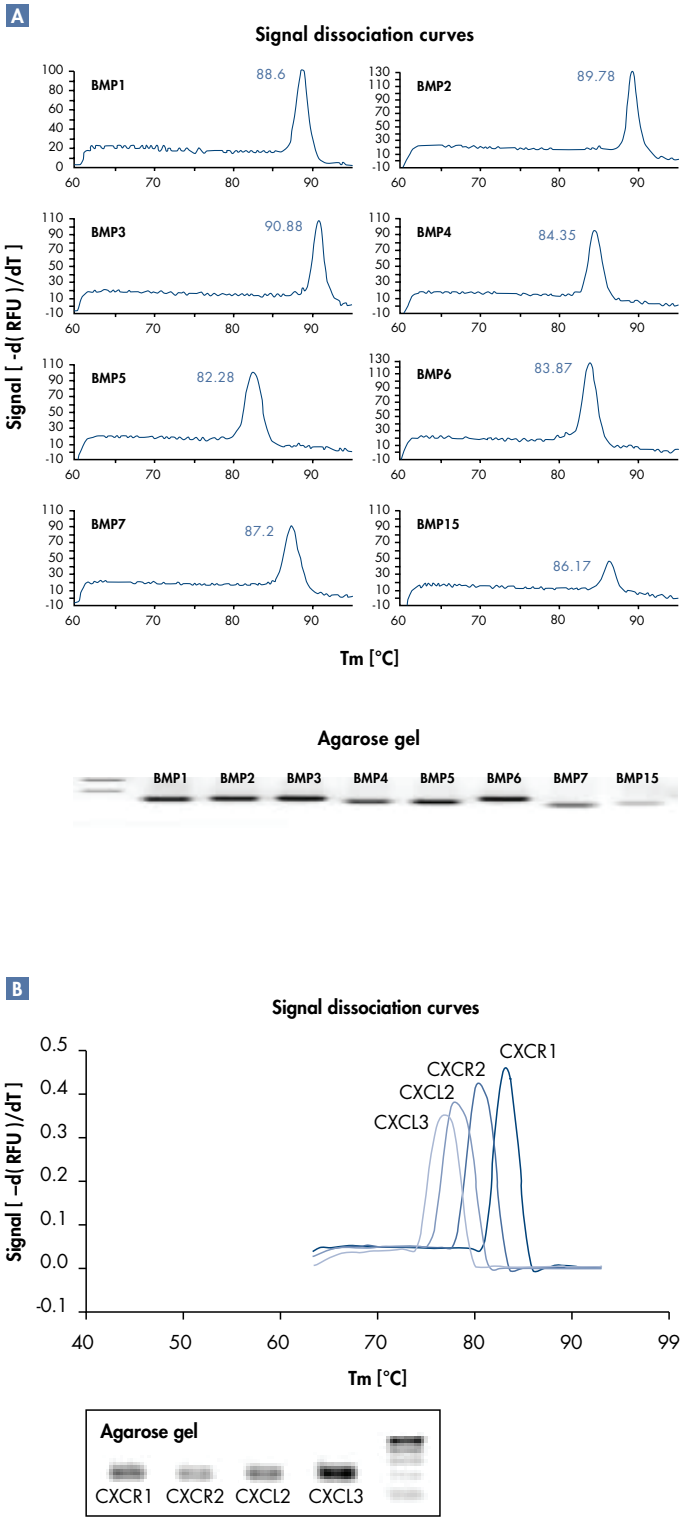


Figure 1. RT² Profiler PCR Arrays & RT² qPCR Assays amplify a single gene-specific product in every reaction. Human XpressRef Universal Total RNA was characterized on both the Human TGFβ/BMP Signaling Pathway A and the Human Common Cytokines B RT² Profiler™ PCR Arrays, followed by dissociation (melt) curve and gel electrophoretic analyses. Each RT² qPCR™ Assay specifically detects an individual gene, in particular for the displayed BMP and cytokine genes, whose specific assays tend to be notoriously difficult to design.

Accuracy and reliability

The most common way to analyze real-time RT-PCR data is the $\Delta\Delta C_T$ method. Its mathematics assumes that the real-time PCR assay has 100 percent amplification efficiency; that is, that the amount of template product doubles with every cycle. With increasing assay deviation from this ideal, the error in the fold difference or fold change calculation increases exponentially. Only with consistently high amplification efficiencies can all real-time RT-PCR assays accurately analyze multiple genes at the same time using the $\Delta\Delta C_T$ method.

Several methods for determining real-time PCR amplification efficiency

1. From calibration curve slope as determined by:
   - ☐ Fit-point method
   - ☐ Second derivative maximum for the 4 parametric logistical model
2. From single amplification plots using algorithms like:
   - ☐ Mid-value point regression —also known as data analysis for real-time PCR or DART-PCR[1]
   - ☐ Window-of linearity algorithm or LinREG PCR[2]
   - ☐ Noise-resistant iterative nonlinear regression or Real-Time PCR Miner[3]

How can you tell if you are achieving amplification efficiencies that are high enough in all of your assays? There are various methods of determining amplification efficiency as summarized in the bullets above. The most rigorous and classical method examines the slope of a calibration curve, much like those used to assess dynamic range as described later. An assay with 100 percent efficiency yields a –3.33 calibration curve slope. Newer methods use algorithms to analyze the amplification curve shapes. All of these methods act as effective and accurate surrogates for the calibration curve method. By whichever method you choose, the amplification efficiencies of all of your assays should average around 100 percent with a relatively narrow standard deviation about that mean. For example, see Figure 2.

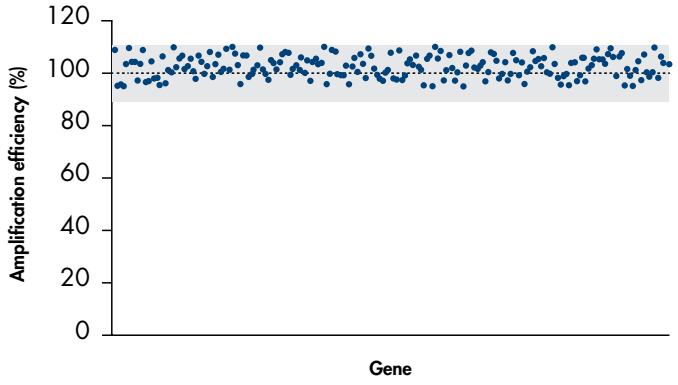


Figure 2. RT² Profiler PCR Arrays and RT² qPCR Assays yield the most accurate results. A representative set of assays for 4,000 genes used in the RT² PCR Arrays demonstrate their average amplification efficiency of 99 percent and their 95 percent confidence interval about the mean from 90 to 110 percent. Consistently high amplification efficiencies enable PCR Arrays to accurately analyze multiple genes simultaneously using the $\Delta\Delta C_t$ method.

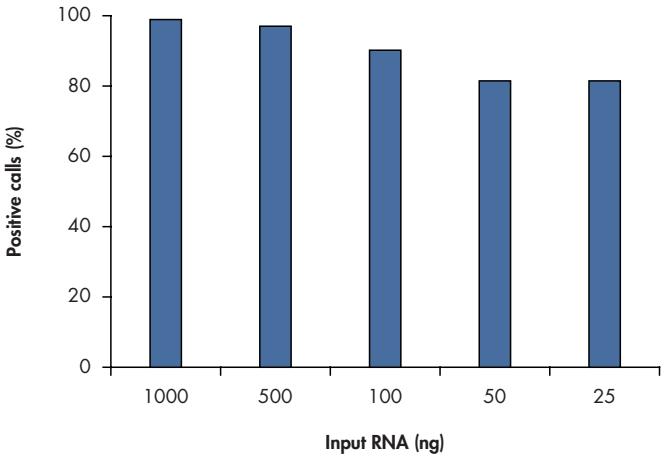


Figure 3. RT² Profiler PCR Arrays and RT² qPCR Primer Assays have the sensitivity to see more genes, like un-induced cytokines, with less RNA. Different amounts of Human XpressRef™ Universal Total RNA (pooled from more than 20 different human cell lines) were characterized using Human Inflammatory Cytokines & Receptors RT² Profiler PCR Arrays. The percentage of detectable genes (those yielding threshold cycle values less than 35) was calculated and plotted against each RNA amount. As little as 25 ng total RNA yields greater than 80 percent positive calls —even for cytokine genes in un-induced cells.

Sensitivity

Beyond the minimal technical requirements for optimal real-time RT-PCR assays, research is beginning to require more genes with less RNA, as well as increasingly rarer and rarer transcripts, either from genes expressed at very low levels or genes only expressed in a small fraction of a biological sample. Minimizing the amount of sample used in the experiment is also extremely important, as samples may be very precious, or you may want the flexibility to perform multiple experiments.

How can you tell whether your assays are sensitive enough? To rigorously test real-time PCR assay sensitivity, screen a panel of genes in a biological sample where you know that they are expressed at very low levels. For example, look for inflammatory cytokine expression in RNA from un-induced cells, such as the experiment shown in Figure 3. See how many of those genes can be detected with the amounts of total RNA that you typically get from the numbers of cells or amounts of tissues that you work with.

Dynamic range

Sensitivity may also be judged by how many copies of cDNA can be detected. When analyzing the expression of multiple genes at once in the same cycling run, however some genes may be expressed at very low copy numbers while other may be expressed at much higher copy numbers. Real-time PCR assays, in general, have the unique potential capability of detecting transcripts down to one individual copy and up to several orders of magnitude more. Researchers have come to expect or even assume that real-time PCR assays indeed detect a wide variety of transcripts expressed at very different levels.

However, how can you tell if your assays actually have a dynamic range that is wide enough? Generate a calibration curve like you may have done determine amplification efficiency. Start with an artificial template of known concentration —for example, the purified product of a reaction from the same assay or a pool of genomic DNA. Perform five- or ten-fold serial dilutions of that nucleic acid, and use each dilution as template for different reactions assaying the same gene or set of genes.

Plot the C_T values versus the initial amounts of input material on a semi-log10 plot, and fit the data to a straight line. The length of the linear phase tells you the dynamic range. The lower end of the dynamic range provides another rigorous test of assay sensitivity.

Typically, assays should have a linear dynamic range from ten copies up to 10^9 copies. Detecting fewer than ten copies becomes problematic because the concentration is low enough that equal-volume aliquots may or may not even contain a copy. Detecting more that 10^9 copies becomes difficult because either assay components become limiting or the initial amount of template introduces too much background in the instrument readout. To detect a wide variety of transcripts expressed at such different levels, all real-time PCR assays for each gene must have a similarly broad linear dynamic range.

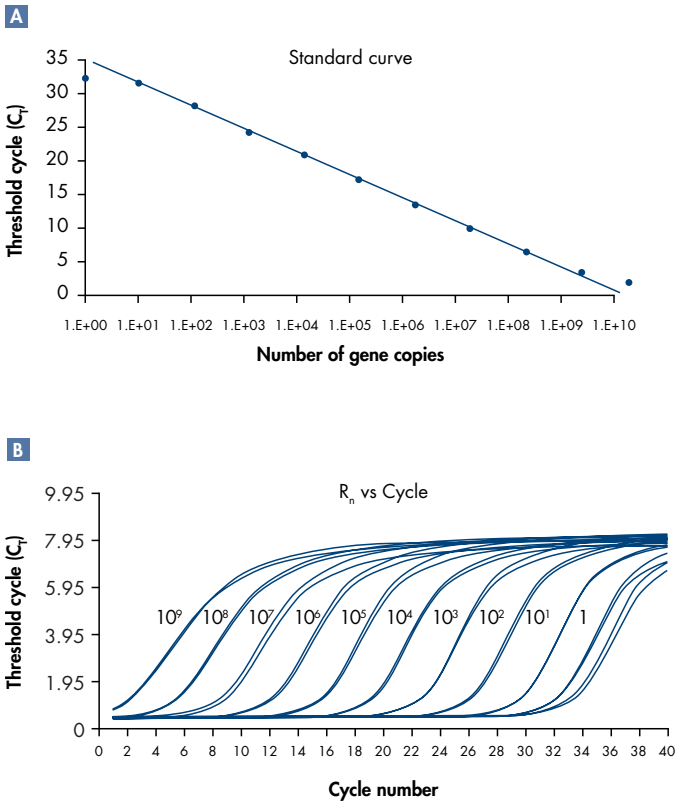
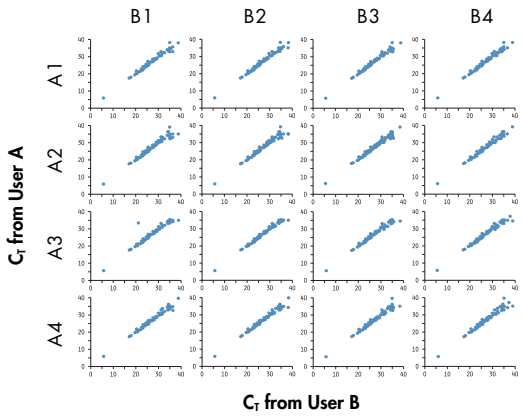


Figure 4. RT² Profiler PCR Arrays and RT² qPCR Primer Assays have sufficiently wide dynamic ranges. A standard curve A was generated using duplicate ten-fold serial dilutions of purified template and the RT² qPCR Primer Assay for the human nicotinic acetylcholine receptor alpha 5 (CHRNA5) B. Our realtime RT-PCR assays have an eight-log linear dynamic range, from 10 to 10^9 copies of template.

Reproducibility

Reproducibility is a key component of real-time PCR assay reliability. A high degree of reproducibility not only allows confidence in the accuracy of your results, enhancing publication quality, but also enables profiling of multiple genes in the same sample. When analyzing a large number of genes on a 96-well or even 384-well plate, the assays must be reproducible enough so that the data can be legitimately compared between separate and individual runs, plates, and samples. Furthermore, technical reproducibility ensures that changes seen in the results are due to the biology under study and not the technology itself or sample handling. In this way, the variability seen across the replicates of your experiment represents the biological variation. More practically, a high degree of reproducibility ensures that new lab members get the same results as previous members and that other labs get the same results that your lab does.

A



B

Correlation coefficients (R values)

	A1	A2	A3	A4
B1	0.993	0.989	0.995	0.992
B2	0.994	0.990	0.995	0.992
B3	0.992	0.990	0.993	0.992
B4	0.993	0.992	0.994	0.992

Figure 5. RT² Profiler PCR Arrays & RT² qPCR Primer Assays are reproducible enough for different technicians to produce the same raw threshold cycle data. The MAQC brain reference RNA sample (4,5) was reverse transcribed and run on four replicate Human Drug Metabolism RT² Profiler PCR Arrays three months apart by two different investigators, each using a different production lot. A The raw data from each end-user's four replicates were plotted with all four of the other enduser's replicates in a scatter plot, and fit to a straight line with a slope of one. B The average C_T value correlation coefficient between the replicate runs by the respective end-users was 0.995 ± 0.001 and 0.998 ± 0.000.

So, how can you tell if your real-time PCR assays are reproducible enough? Directly ask whether a new technician or graduate student or post-doc in the lab can get the same results as the previous or outgoing end-user. Figure 5 shows an example of this test. How else can you tell if your real-time PCR assays are reproducible enough? Ask another lab whether they can reproduce your results, as in the experimental results in Figure 6.

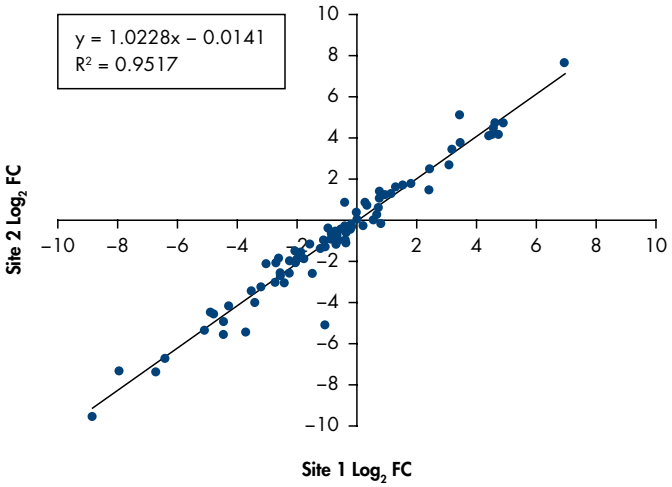


Figure 6. RT² Profiler PCR Arrays & RT² qPCR Primer Assays are reproducible enough for two different laboratories to get the same fold-difference results. Fold-difference results were obtained using the two MAQC reference RNA samples (4,5) analyzed on five replicate Human Drug Metabolism RT² Profiler™ PCR Arrays using two different real-time PCR thermal cyclers at two different sites. The fold-difference (or fold-change or FC) results between the two RNA samples were calculated from the average C_T value for each assay on the arrays. The results from each site were plotted against one another and fit to a straight line with a slope of one. The inter-site comparison of fold-difference results obtained from the two sites has a correlation coefficient of 0.976.

Summary

Whether verifying DNA microarray results, looking at the effects of specific experimental treatments or biological conditions, or verifying an RNA interference-based gene knockdown experiment, gene expression analysis by real-time PCR is the technique of choice. Developing the best possible primer pairs is critical. Good real-time PCR as says are characterized by their high levels of accuracy, dynamic range, reliability, reproducibility, sensitivity, and specificity. This paper has described the "diagnostic" techniques needed to identify potential problems with primers and has offered a systematic guideline to solve the problems.

Often, this rigorous level of verification is more time-consuming and complex than the actual experiment itself. Whether starting with one gene or a set of genes, many labs do not have the time or resources to optimize each new real-time RT-PCR assay in their laboratory in the same fashion so that every assay can be performed together. How can you possibly achieve this level of quality control just with the assays that you need today, not to mention the assays that you may need to perform in the future?

To help researchers like you, QIAGEN is pleased to offer a genome-wide approach, providing the best primer pairs with these characteristics for every gene. Read on to learn more about these SYBR Green optimized real-time RT-PCR assays.

Appendix

RT² qPCR Primer Assays from QIAGEN

QIAGEN is the leader in SYBR Green real-time PCR gene expression. In 2002, SABiosciences, now a QIAGEN company, began extensive efforts to systematically develop solutions to overcome difficulties associated with SYBR Green real-time RT-PCR. Over the intervening years, our R & D and Bioinformatics teams have worked together on many rounds of bioinformatics algorithm upgrades, experimental verification, and master mix formulations. We have trained our computer algorithms by designing and experimentally verifying more than 14,000 real-time PCR assays for the key performance criteria discussed in detail throughout this white paper. We have experimentally developed a unique master mix containing proprietary reagents that maximize gene-specific detection while minimizing primer dimers and mis-priming artifacts. Through this process, we have gained great insight into how to achieve high-performance SYBR Green PCR. Our combination of an advanced primer design algorithm, a proprietary master mix, and extensive experimental verification makes the RT² Profiler PCR Arrays and RT² qPCR Primer Assays accurate, reliable, reproducible, sensitive, and specific.

Our goal has been to provide SYBR Green qPCR with more uniform performance, with greater flexibility, and at a lower cost than TaqMan assays. We have also aimed to develop simple-to-use protocols and reagents that work on any real-time PCR instrument. These benefits would enable the research community to spend their precious time and resources investigating questions related to their unique biological system rather than optimizing qPCR assays. Now, RT² qPCR Primer Assays using SYBR Green are available for analyzing every gene in human, mouse and rat genomes. And, our RT² SYBR Green qPCR Mastermixes are also optimized for any available real-time instrument, including ABI, Bio-Rad, Stratagene, Roche, Eppendorf, and others. There is no longer any need to reinvent the wheel with homemade primer assays, when you can instead go from an RNA sample to relative fold-change results in as little as two hours using QIAGEN's RT² qPCR Primer Assays.

The optimal design triad for real-time PCR assays

There are four important components to any real time PCR assay:

1. Gene-specific primer sequence and concentration
2. Master mix chemistry (buffer conditions and *Taq* polymerase)
3. PCR cycling conditions
4. High-quality DNase-treated intact RNA

Often, during the design and wet-bench testing of an individual assay, each component will need to be optimized multiple times, because adjusting one variable will affect the other variables as well. QIAGEN's RT² qPCR Primer Assays have already optimized three of the crucial components. A researcher like you only needs to provide the fourth item, high-quality RNA samples.

In silico primer design algorithm

There are many free PCR primer design algorithms available, like PRIMER3 and its derivatives. (Even your oligo synthesis company can provide one to you.) Their performance is generally fine for many easy-to-work-with genes, but their designs for more-difficult-to- amplify genes can be problematic. When a gene is expressed at a low level or is highly homologous with other genes, you will often encounter non-specific amplification, primer dimers, low amplification efficiencies, and low sensitivity.

QIAGEN designs all of the RT² qPCR Primer Assays on site. Using the results from our large number of experimental verification assays, we have implemented 16 new filters into our primer design algorithm in addition to the more commonly known filters in the free software packages. We use advanced nearest neighbor and salt-corrected primer design algorithms specifically tailored to our proprietary master mixes. It is important to thoroughly understand the complexity of the chemistry that you are using, because target sequence selection must account for each ingredient for optimal primer design. We also include all of the genome BLAST filters and SNP filters discussed in this white paper to improve the specificity of the primer sequences.

Experimental wet-bench verification

Once the primers are designed, they are passed from the bioinformatics group to the production group for wet-bench quality control and verification to guarantee high-performance in our customers' hands. QIAGEN experimentally verifies every primer pair before it is shipped. Each RT² qPCR Primer Assay is certified to generate a single melting curve peak and single gel electrophoresis band with high amplification efficiency. If a RT² qPCR Primer Assay does not meet all of the above requirements, then it fails quality control and is sent back to our bioinformatics scientists for re-design.

Optimized master mix formulation

Master mix formulation also plays a very important role in SYBR Green real-time PCR. The major challenge in designing a master mix is nonspecific amplification, such as primer dimers and other secondary products, which compromise the specificity of the signal. A good primer design algorithm can significantly reduce this problem. In addition to primer design, a tightly controlled hot-start *Taq* enzyme and several chemical additives can also significantly minimize or eliminate non-specific amplification during SYBR Green qPCR. Through our verification of assays for over 14,000 genes, we have also optimized the formulation of our RT² SYBR Green qPCR Mastermixes to achieve this level of performance.

Thousands of researchers have now successfully used our RT² qPCR Primer Assays and RT² SYBR Green qPCR Mastermixes in their research. Are you ready to join them? Here's how:

References

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Ordering Information

Product	Contents	Cat. no.
RT² Profiler PCR Arrays	Pathway, disease, or custom* panels of gene assays	330231
RT² SYBR Green qPCR Mastermixes	Reagents for real-time PCR reactions (available with ROX, fluorescein, or no internal reference dye)	Varies
RT² First Strand Kit (12)	Reagents for cDNA synthesis reactions for 12 samples	330401
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RNase-free DNase Set (50)	Reagents for 50 preps	79254

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* For custom arrays, please visit www.sabiosciences.com/custompcrplate.php

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