A Systematic Guideline for Developing the Best Real-Time PCR Primers
What we have 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 home-made 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 validating 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 for 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 see genes expressed at 10,000 fold different levels at the same time?
5. Reproducibility
   Will my assay work each time? How close are my replicates?

The time and resources necessary to design and validate SYBR Green-based real-time PCR assays that pass all of these performance requirements is often misleading. 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 time a real-time PCR assay fails, there is another set of primers to throw away, not to mention the wasted reagents, machine-time, and most importantly, your research time.

You don’t have to reinvent the wheel!

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 validate 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

Primer design algorithm is key. It 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. By controlling the GC content, primer length, and the primer melting temperature range, 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 from SABiosciences

<table>
<thead>
<tr>
<th>Amplicon Length</th>
<th>50 - 210 bp</th>
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<tbody>
<tr>
<td>Primer Length</td>
<td>19-23 Nucleotides</td>
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<tr>
<td>GC Content</td>
<td>35 - 65%</td>
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<tr>
<td>Tm</td>
<td>60 - 68 °C</td>
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<tr>
<td>3'-End Stability</td>
<td>Composition of last 3 base pairs</td>
</tr>
<tr>
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<td>Specificity</td>
<td>BLAST versus entire mRNA RefSeq database</td>
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<td>SNP Database</td>
<td>Primer sequences do no include known SNP</td>
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Any algorithm’s primer design must also be experimentally validated 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. You could be detecting product when it really isn’t there, or the results tell you that there is more product than the actual amount.

How can you tell if your real-time PCR assays are specific enough? If you are using SYBR Green-based 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 you are not using SYBR Green-based detection, this analysis is not possible. Instead (or to be completely rigorous), a single product. If you are not using SYBR Green-based detection, also 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 & 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. The more that assays deviate 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.

Table 2: Several Methods for Determining Real-Time PCR Amplification Efficiency Have Been Described in the Literature and Tested by SABiosciences.

<table>
<thead>
<tr>
<th>1. From Calibration Curve Slope as determined by:</th>
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<tr>
<td>a. Fit-Point Method</td>
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<td>b. Second Derivative Maximum for the 4 Parametric Logistical Model</td>
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</table>

<table>
<thead>
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<th>2. From Single Amplification Plots Using Algorithms like:</th>
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<tr>
<td>a. Mid-Value Point Regression - AKA Data Analysis for Real-Time PCR or DART-PCR¹</td>
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<tr>
<td>b. Window-of-Linearity Algorithm or LinREG PCR²</td>
</tr>
<tr>
<td>c. Noise-Resistant Iterative Nonlinear Regression or Real-Time PCR Miner³</td>
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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 Table 2. The most rigorous and classical method examines the slope of a calibration curve, much like those used to assess dynamic range as described here 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.

Sensitivity

Beyond the minimal technical requirements for optimal real-time RT-PCR assays, researchers also want to see more genes with less RNA. They are looking for increasingly rarer and rarer transcripts, either genes expressed at very low levels or genes only expressed in a small fraction of a biological sample. Researchers are also always looking for ways to minimize the amount of sample used in the experiment. The samples may be very precious, or researchers 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.

Figure 3: RT² Profiler PCR Arrays and RT² qPCR 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 and Receptors RT² Profiler PCR Arrays. The percentage of detectable genes (those yielding threshold cycle values less than 35) was calculated for and plotted against each RNA amount. As little as 25 ng total RNA yields greater than an 80 percent positive call – even for cytokine genes in un-induced cells.

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 98 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.
**Dynamic Range**

Sensitivity may also be judged by how many copies of cDNA can be detected. But when analyzing the expression of multiple genes at once in the same cycling run, 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 might have to 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-log$_{10}$ plot, and fit the data to 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 than $10^9$ 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 have a similarly broad linear dynamic range.

![Figure 4: RT2 Profiler PCR Arrays and RT2 qPCR Assays Have Sufficiently Wide Dynamic Ranges.](image)

A standard curve (A) was generated using duplicate ten-fold serial dilutions of purified template and the RT2 qPCR Assay for the Human Nicotinic Acetylcholine Receptor Alpha 5 (CHRNA5, B). Our real-time RT-PCR assays have an eight-log linear dynamic range, from $10^0$ to $10^8$ copies of template.

**Reproducibility**

Researchers are also understandably concerned about the reproducibility of their real-time PCR assay results, if only to help make sure that reviewers will let their results be published. In addition, profiling multiple genes in the same sample also requires a high degree of reproducibility. When looking at enough genes to fill an entire 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 insures 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 insures that new lab members get the same results as previous members and insures that other labs get the same results that your lab does.

![Figure 5: RT2 Profiler PCR Arrays & RT2 qPCR Assays Are Reproducible Enough for Different Technicians to Get the Same Raw Threshold Cycle Data.](image)

The MAQC brain reference RNA sample was reverse transcribed and run on four replicate Human Drug Metabolism PCR Arrays three months apart by two different investigators each using a different production lot. The raw data from each end-user’s four replicates with all four of the other end-user’s replicates in a scatter plot, and fit to a straight line with a slope of one. The average $R^2$ 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 and example of this idea. How else can you tell if your real-time PCR assays are reproducible enough? Ask another lab whether they can reproduce your results, just as the experimental results in Figure 6 do.
Figure 6: RT² Profiler PCR Arrays & RT² qPCR 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 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 intersite comparison of fold-difference results obtained from the two sites has a correlation coefficient of 0.976.

Summary

Whether validating DNA microarray results, looking at the effects of specific experimental treatments or biological conditions, or validating an RNA interference-based gene knock down 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 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 validation 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, SABiosciences 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 Assays from SABiosciences

SABiosciences is the leader in SYBR Green based real time PCR gene expression. In 2002, SABiosciences began its extensive efforts to systematically develop solutions to overcome difficulties and even problems associated with SYBR Green-based 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 validation, and master mix formulations. We have trained our computer algorithms by designing and experimentally validating 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. Only through this process, have we finally gained great insights on how to achieve high-performance SYBR Green PCR. Our combination of an advanced primer design algorithm, a proprietary master mix, and extensive experimental validation makes the RT² Profiler PCR Arrays and RT² qPCR Assays accurate, reliable, reproducible, sensitive, and specific.

Our goal has been to provide SYBR Green based qPCR with more uniform performance, with greater flexibility, and at a lower cost than TaqMan-based 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 SYBR Green-based qPCR Assays are available for analyzing every gene in human, mouse and rat genomes. And, our RT² SYBR Green qPCR Master Mixes are also optimized for any available real-time instrument, like ABI, Bio-Rad, Stratagene, Roche, Eppendorf, and others. Why should you reinvent the wheel, when you can instead go from an RNA sample to relative fold-change results in as little as two hours using SuperArray’s RT² qPCR Assays?

Perfect Real-Time RT-PCR Assay Design Triad

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

1.) Gene-Specific Primer Sequence and Concentration
2.) Master Mix Chemistry (Buffer Conditions & 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. SABiosciences’ RT² qPCR 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 have a lot of problems. 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.

SABiosciences designs all of the RT² qPCR Assays on site. Using the results from our large number experimental validation 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 Validation**

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

**Optimized Master Mix Formulation**

Master Mix formulation also plays a very important role in SYBR Green-based real-time PCR. The major challenge is non-specific 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 validation of our assays for over 14,000 genes, we have also optimized the formulation of our RT² qPCR Master Mixes™ to achieve this level of performance.

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

**RT² qPCR Assays**

Search for your genes of interest at:
http://www.SABiosciences.com/QRTsearch.php

**RT² Profiler PCR Arrays**

Search for your pathways of interest at:
http://www.SABiosciences.com/ArrayList.php?pline=PCRArray

**RT² qPCR Master Mixes**

- RT² SYBR Green / ROX qPCR Master Mix PA-012
- RT² SYBR Green / Fluorescein qPCR Master Mix PA-011
- RT² SYBR Green / ROX qPCR Master Mix PA-010

**RT² First Strand Kit**

C-03

**References**


PCR Array & Primer Assay Buyer's Guide

Step 1: Find your pathway in the list below. For complete PCR Array gene lists, see our web site at: www.SABiosciences.com/ArrayList.php

Step 2: Determine which PCR Array format fits the instrument in your lab using the Real-Time PCR Systems table.

Step 3: Select your pack sizes and reagents. Place your order by phone, fax, or e-mail:
Phone: 888.503.3187 Fax: 888.465.9859 E-mail: order@SABiosciences.com

Pathway / Topic Focus  PCR Array Catalog Number

Angiogenesis  PAXX-024Y
Angiogenic Growth Factors & Angiogenesis Inhibitors  PAXX-072Y
Apoptosis  PAXX-012Y
Atherosclerosis  PAXX-038Y
Breast Cancer and Estrogen Receptor Signaling  PAXX-005Y
cAMP and Calcium Signaling Pathway  PAXX-066Y
Cancer Drug Resistance and Metabolism  PAXX-004Y
Cancer PathwayFinder™  PAXX-033Y
Cell Cycle  PAXX-020Y
Chemokines and Receptors  PAXX-022Y
Common Cytokines  PAXX-021Y
Diabetes  PAXX-023Y
DNA Damage Signaling Pathway  PAXX-029Y
Drug Metabolism  PAXX-002Y
Drug Metabolism: Phase I Enzymes  PAXX-068Y
Drug Transporters  PAXX-070Y
Endothelial Cell Biology  PAXX-015Y
Extracellular Matrix and Adhesion Molecules  PAXX-013Y
Growth Factors  PAXX-041Y
Hypoxia Signaling Pathway  PAXX-032Y
Inflammatory Cytokines and Receptors  PAXX-011Y
Insulin Signaling Pathway  PAXX-030Y
Interferons (IFN) and Receptors  PAXX-064Y
JAK / STAT Signaling Pathway  PAXX-039Y
MAP Kinase Signaling Pathway  PAXX-061Y
Neuroscience Ion Channels and Transporters  PAXX-036Y
Neurotransmitter Receptors and Regulators  PAXX-060Y
Neurotrophins and Receptors  PAXX-031Y
NFκB Signaling Pathway  PAXX-038Y
Nitric Oxide Signaling Pathway  PAXX-032Y
Notch Signaling Pathway  PAXX-059Y
Obesity  PAXX-017Y
Osteogenesis  PAXX-026Y
Oxidative Stress and Antioxidant Defense  PAXX-065Y
p32 Signaling Pathway  PAXX-027Y
Signal Transduction PathwayFinder™  PAXX-014Y
Stem Cell  PAXX-040Y
Smad Signaling Pathway  PAXX-034Y
Th1-Th2-Th3  PAXX-034Y
Toll-Like Receptor Signaling Pathway  PAXX-012Y
Tumor Metastasis  PAXX-028Y
Tumor Necrosis Factor (TNF) Ligands and Receptors  PAXX-035Y
Wnt Signaling Pathway  PAXX-043Y
Housekeeping Genes  PAXX-000Y
RT2™ PCR Arrays - quality control plates  PAXX-999Y
Custom Options - Inquire

**XX**= HS, MM, RN (Human, Mouse, Rat) see web site for availability

Real-Time PCR Systems

Determine the plate type and master mix that fits your real-time PCR system.

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<th>Instrument Make and Model</th>
<th>PCR Array Plate Format</th>
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Pack Sizes and Required Reagents

Volume discounts are built into the PCR Array 12-pack and 24-pack sizes.

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<th>PCR Array Pack Sizes</th>
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<td>Twelve (12) 96-well PCR Arrays</td>
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<tr>
<td>Twenty-Four (24) 96-well PCR Arrays</td>
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<td>Four (4) 384-well PCR Arrays (Format “E”)</td>
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RT<sup>®</sup> SYBR Green qPCR Master Mixes are required for use with PCR Arrays.

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Catalog Number

- Two-Pack (2) PA-01#
- Twelve-Pack (12) PA-01#-12
- Twenty-Four-Pack (24) PA-01#-24

The RT<sup>®</sup> First Strand Kit is required for use with PCR Arrays. For best results, we also recommend the RT<sup>®</sup> qPCR-Grade RNA Isolation Kit.

**RT First Strand Kit**
(Cat. No. C-03, enough for 12 reactions)

**RT qPCR-Grade RNA Isolation Kit**
(Cat. No. PA-001, enough for 12 RNA isolations)