Introduction

Whether you are beginning to develop a QPCR assay, have a QPCR assay you want to optimize, or are getting questionable results and don’t know why, this guide is for you.

Simply bringing together all the necessary components for QPCR is often not enough to obtain accurate and consistent results. Following a protocol that instructs you how to combine your template with reagents before running them on your chosen instrument only covers the basics of real-time PCR. The science of real-time PCR comes from understanding how the nuances of this technique affect your results.

This quick reference guide is intended to educate you to gain a better understanding of these finer details, empowering you to optimize or troubleshoot your assay in order to achieve the best results possible on a consistent basis. It captures the knowledge that we have accumulated over 20 years manufacturing and optimizing PCR reagents and plastics for the life science industry.
Refer to this table before you begin your qPCR assay. Use the information to ensure that you plan your protocol using best practice methodologies.

<table>
<thead>
<tr>
<th>Optimization Parameter</th>
<th>Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>QPCR Plate</td>
<td>It is recommended that opaque white PCR plates are used for QPCR analysis. The white color virtually eliminates cross talk and improves the efficiency of fluorescent detection thereby increasing assay sensitivity and well-to-well consistency.</td>
</tr>
<tr>
<td>Template Quality</td>
<td>It is essential that the nucleic acid is sufficiently pure for QPCR analysis. Template contamination (i.e., genomic DNA, protein, carbohydrates or organic solvents) can have a huge impact on assay reliability and reproducibility. Template quality should be determined by spectrophotometry (i.e., Nanodrop), microfluidics or PAGE.</td>
</tr>
<tr>
<td>Amplicon Size</td>
<td>Ideally, the amplicon should always be between 100bp and 150bp to ensure the QPCR reaction efficiency is as close to 100% as possible. Good QPCR efficiency promotes assay reproducibility and sensitivity.</td>
</tr>
<tr>
<td>Primer Design</td>
<td>Given that PCR primers are a relatively cheap component of a QPCR assay, it is good practice to order and test at least 2 primer pairs for every new QPCR assay. This will maximize the chance of establishing a reliable, reproducible and sensitive assay.</td>
</tr>
<tr>
<td>Test Primers</td>
<td>Measure the reproducibility, specificity, sensitivity and dynamic range of your QPCR assay using SYBR Green chemistry across a template dilution series. Ideally, the efficiency of the QPCR reaction should be at least 90% and below 105%, while the assay reproducibility should be higher than r=0.998.</td>
</tr>
<tr>
<td>Efficient RT</td>
<td>Initially, the RT step should be performed as specified in the supplier protocol. However, the length and the temperature of the RT step can be optimized to increase the efficiency of the reverse transcriptase. The reverse transcriptase should be tested across a range of RNA concentrations to ensure assay linearity.</td>
</tr>
<tr>
<td>Hot-Start</td>
<td>Standard Thermo Scientific QPCR master mixes require a 15 minute heating step at 95°C to ensure the hot-start Taq is fully activated. This step cannot be shortened under any circumstances. A shorter heating step will impact assay reproducibility and sensitivity.</td>
</tr>
<tr>
<td>Thermal Protocol</td>
<td>Even if your assay has been optimized using an alternative supplier’s mix, once the Thermo-Start mix is used it is advisable to start using the protocol recommended in the Thermo Scientific QPCR master mix. If assay optimization is required, the annealing temperature should be examined first.</td>
</tr>
<tr>
<td>Annealing Temperature</td>
<td>Test a range of annealing temperatures. Depending on the QPCR results, the annealing temperature should be increased or decreased in 2-3°C increments. This can be done in a single experiment using a thermal gradient. Alternatively, a range of annealing temperatures should be tested using multiple QPCR experiments.</td>
</tr>
<tr>
<td>Primer Concentration</td>
<td>Always start by using the primer concentration recommended in the master mix protocol. If optimization is required, try stepping the primer concentration up and down in 25mM increments. Optimizing primer concentration using a titration matrix can give improved results in rare circumstances but this is time-consuming.</td>
</tr>
</tbody>
</table>
# Troubleshooting Guide

Refer to this table if you have performed a QPCR assay that resulted in sub-optimal results. Use this information to tweak the components, conditions, and reaction parameters in order to overcome erroneous results.

<table>
<thead>
<tr>
<th>PROBLEM</th>
<th>POSSIBLE CAUSES</th>
<th>ACTIONS (Based on Thermo Scientific QPCR Master Mix Protocol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No amplification or very high Ct</td>
<td>Enzyme not fully activated</td>
<td>Ensure the initial 95ºC activation step is carried out for the full 15 minutes (or 10 minutes in the Fast Protocol).</td>
</tr>
<tr>
<td></td>
<td>Poor primer design</td>
<td>Check the PCR product by melt curve analysis or on an agarose gel. It is good practice to try at least 2 primer pairs.</td>
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<tr>
<td></td>
<td>RT step too short</td>
<td>Extend the RT step in 5 minute increments up to 60 minutes.</td>
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<td></td>
<td>RT temperature too low</td>
<td>Increase RT reaction temperature in 5ºC increments up to 57ºC.</td>
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<tr>
<td></td>
<td>Annealing step too short</td>
<td>Increase annealing step in 3s increments up to 30s.</td>
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<tr>
<td></td>
<td>Annealing temperature too high</td>
<td>Decrease the annealing temperature in 2ºC increments.</td>
</tr>
<tr>
<td></td>
<td>Extension time too short</td>
<td>Increase the extension time in 5s increments, up to 30s for amplicons of up to 500bp.</td>
</tr>
<tr>
<td></td>
<td>Amplicon too long</td>
<td>Amplicons should ideally be 100-150bp long and should not exceed 500bp.</td>
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<tr>
<td></td>
<td>Poor template quality</td>
<td>Check the quality of the template preparation using spectrophotometry, microfluidics or PAGE.</td>
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<tr>
<td></td>
<td>Insufficient template</td>
<td>Increase amount of template to ensure enough copies of target included.</td>
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<tr>
<td></td>
<td>Template contains inhibitors</td>
<td>Purify template or repeat the assay using a 1:10 or a 1:100 dilution of the template.</td>
</tr>
<tr>
<td></td>
<td>Insufficient cycles</td>
<td>Increase the number of PCR cycles to 40.</td>
</tr>
<tr>
<td></td>
<td>Wrong dye/channels used</td>
<td>Check machine settings correspond with the intercalating dye (eg. SYBR Green) or the dye conjugated to the fluorescent probe and that the ROX / fluorescein levels are correct.</td>
</tr>
<tr>
<td></td>
<td>Error in setup</td>
<td>Check concentrations and storage conditions of the reaction components and then repeat the reaction.</td>
</tr>
<tr>
<td></td>
<td>Reaction components not mixed thoroughly</td>
<td>Repeat assay ensuring serial dilutions are vortexed for at least 15s and that the reaction components are mixed together thoroughly.</td>
</tr>
<tr>
<td></td>
<td>Primers degraded</td>
<td>Check the integrity of the PCR primers by denaturing polyacrylamide gel electrophoresis.</td>
</tr>
<tr>
<td></td>
<td>Primer concentration not optimal</td>
<td>Start with primer concentration recommended in protocol and increase in 25mM increments if necessary.</td>
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<tr>
<td></td>
<td>Fluorescent data collected at wrong step</td>
<td>Ensure fluorescent data is collected at the appropriate step and in the correct channel.</td>
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<tr>
<td></td>
<td>Fluorescent reporter not being released from probe</td>
<td>Validate performance of PCR primers using SYBR Green. Redesign probe or optimize probe binding step if primers are performing well.</td>
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<tr>
<td></td>
<td>Probe exposed to light and been bleached</td>
<td>Once reaction set up is complete return your probe to the product packaging as soon as possible to minimize exposure to light.</td>
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</tbody>
</table>
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<tr>
<td>Non-specific amplification and / or primer-dimers</td>
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<tr>
<td>Annealing temperature too low</td>
<td>Increase annealing temperature in 2°C increments - use a thermal gradient if possible.</td>
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<tr>
<td>Poor primer design</td>
<td>Re-design primers using Primer3 or another QPCR primer design tool. It is good practice to try at least 2 primer pairs.</td>
<td></td>
</tr>
<tr>
<td>RNA template contaminated with genomic DNA</td>
<td>Remove genomic DNA from RNA template with DNase I or use RT Enhancer with Verso QRT-PCR kits. Design primers to span exon-exon boundaries.</td>
<td></td>
</tr>
<tr>
<td>RT reaction setup at room temperature</td>
<td>Setup the RT reactions on ice and transfer the RT reactions from ice to the reaction block, starting the RT protocol immediately.</td>
<td></td>
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<tr>
<td>Primers degraded</td>
<td>Check the integrity of the PCR primers by denaturing polyacrylamide gel electrophoresis.</td>
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<tr>
<td>Fluorescence in the 'no template control' (NTC)</td>
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<tr>
<td>Reagents contaminated</td>
<td>Discard reagents and repeat assay with fresh reaction components.</td>
<td></td>
</tr>
<tr>
<td>Contamination occurred during reaction setup</td>
<td>Use barrier tips, screw-cap tubes and setup QPCR reaction in a DNA-free zone before adding the template in a separate location.</td>
<td></td>
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<tr>
<td>Fluorescence in 'no RT control'</td>
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<tr>
<td>RNA template contaminated with genomic DNA</td>
<td>Remove genomic DNA from RNA template with DNase I or use RT Enhancer with Verso QRT-PCR kits. Design primers to span exon-exon boundaries.</td>
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<tr>
<td>Poor linearity of Ct values across dilution series (R value ≤ 0.998)](<a href="https://example.com">https://example.com</a>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Too much nucleic acid in 'high copy number' assays</td>
<td>Use less than 500ng of template in each QPCR or QRT-PCR reaction.</td>
<td></td>
</tr>
<tr>
<td>Too little nucleic acid present in 'low copy number' assays</td>
<td>Increase the amount of template or increase PCR reaction efficiency by optimizing thermal protocol / re-designing primers.</td>
<td></td>
</tr>
<tr>
<td>Annealing temperature too low</td>
<td>Increase the annealing temperature in 2°C increments - use a thermal gradient if possible.</td>
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<td>Reaction components not mixed thoroughly</td>
<td>Repeat assay ensuring all serial dilutions are vortexed for 15s and reaction components are mixed properly.</td>
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<td>Poor template quality</td>
<td>Check the quality of the template preparation using spectrophotometry, microfluidics or PAGE.</td>
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<td>PCR efficiency is too high (&gt;105%)</td>
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<td></td>
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<tr>
<td>Primer-dimers bound to SYBR Green</td>
<td>Optimize thermal protocol i.e. increase the annealing temperature in 2°C increments - use a thermal gradient if possible.</td>
<td></td>
</tr>
<tr>
<td>Serial dilutions not calculated properly</td>
<td>Repeat serial dilution using fresh sample material and calculate concentrations accurately.</td>
<td></td>
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<tr>
<td>Reaction not reproducible</td>
<td>Improve reproducibility by improving technique / optimizing thermal protocol / re-designing primers.</td>
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<td>PCR efficiency is too low (&lt;90%)</td>
<td>Poor primer design</td>
<td>Re-design primers using primer design software. It is good practice to try at least 2 primer pairs.</td>
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<td>Template contains inhibitors</td>
<td>Purify template or use different template extraction method and repeat the assay.</td>
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<td>Amplicon should ideally be 100-150bp long and should not exceed 500bp.</td>
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<td>Fluorescent signal climbs and then falls sharply</td>
<td>Fluorescence increased so rapidly that baseline correction tilted curve forwards</td>
<td>Adjust baseline correction (ie from cycles 3-15 to 3-10 or dilute template between 1:100 and 1:1000 and repeat).</td>
</tr>
<tr>
<td>Amplification plot goes up, down and all around</td>
<td>Baseline has been set so when software applies data correction curves are distorted</td>
<td>Adjust baseline correction (ie from cycles 3-15 to 3-10 or dilute template between 1:100 and 1:1000 and repeat).</td>
</tr>
<tr>
<td>Amplification plot doesn’t reach threshold</td>
<td>Baseline fluorescence is very high in ‘high template’ reactions</td>
<td>Manually adjust threshold so it crosses log-linear phase of each amplification plot or dilute template between 1:100 and 1:1000 and repeat.</td>
</tr>
<tr>
<td>Amplification plot not exponential</td>
<td>Template contains inhibitors</td>
<td>Purify template or repeat assay using a 1:10 or a 1:100 dilution of template.</td>
</tr>
<tr>
<td>Data plots are very jagged</td>
<td>Data being collected at lowest detection limits of cycler</td>
<td>Smooth data by applying a moving average data correction algorithm or re-design assay.</td>
</tr>
</tbody>
</table>
Innovative QPCR Solutions

Refer to our table of products for a start-to-finish solution of reagents, plates, and seals designed specifically to optimize QPCR results.

This system of products can dramatically improve the quality and reliability of your QPCR data.

<table>
<thead>
<tr>
<th>ABI</th>
<th>Bio-Rad</th>
<th>Stratagene</th>
<th>Roche</th>
<th>Eppendorf</th>
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<tbody>
<tr>
<td>7000/7300</td>
<td>7500</td>
<td>7900HT</td>
<td>7500 FAST</td>
<td>7900HT FAST</td>
</tr>
</tbody>
</table>

QPCR Plastics

**Thermo Scientific ABgene White QPCR Plates, 25 Plates (50 Plates with 384-well)**

- 96-Well: AB2400W, AB2400W, AB1900W, AB1900W, AB1900W, AB1900W, AB1900W, AB1900W, AB2400W, AB2400W, AB28000W - AB28000W

**Thermo Scientific EasyStrip Snap White QPCR Strip Tubes, 250 Strips with Attached QPCR Caps**


QPCR Reagents

**Thermo Scientific Absolute Blue QPCR Master Mixes, 400 x 25uL (1 x 5mL)**


**Thermo Scientific Absolute Blue Fast QPCR Master Mixes, 400 x 25uL (1 x 5mL)**

- Probe: AB4328A, AB4330A, AB4330A, AB4330A, AB4330A, AB4330A, AB4330A, AB4330A, AB4330A, AB4330A, AB4330A, AB4330A

QRT-PCR Reagents

**Thermo Scientific Verso 1-Step QRT-PCR Mixes, 200 x 25uL (2 x 1.25mL)**

- Probe: AB4101A, AB4102A, AB4102A, AB4102A, AB4102A, AB4102A, AB4102A, AB4102A, AB4102A, AB4102A, AB4102A, AB4102A

**Thermo Scientific Verso 2-Step QRT-PCR Kits, 200 x 25uL (2 x 1.25mL)**

- Probe: AB4110A, AB4117A, AB4110A, AB4117A, AB4110A, AB4117A, AB4110A, AB4117A, AB4110A, AB4117A, AB4110A, AB4117A

QPCR Plate Seal

**Thermo Scientific Absolute QPCR Seal Ultra-Clear Adhesive Plate Seal, 50 Sheets**

- AB-1170 (Compatible with all PCR plates) + Free Applicator AB-1391

Larger pack sizes available. Please enquire.

Need help? Contact your local technical support team

| United States | 800-235-9880, option #4 | abgeneus.techservice@thermofisher.com |
| United Kingdom | 01732 840 410 | abgene.techsupport@thermofisher.com |
| France | 01 60 92 48 68 | abgene.techsupport@thermofisher.com |
| Germany | 040 23 51 36 79 | abgene.techsupport@thermofisher.com |
| Rest of the World | +44 1732 840 410 | abgene.techsupport@thermofisher.com |
# How to order

<table>
<thead>
<tr>
<th>United States</th>
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<tbody>
<tr>
<td><strong>Direct:</strong> (PCR Reagents Only)</td>
<td><strong>Fisher Scientific:</strong> (All Products)</td>
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<tr>
<td>Telephone</td>
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<tr>
<td>(800) 874-3723</td>
<td>(800) 766-7000</td>
<td>(800) 926-1166</td>
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<tr>
<td>To fax an order</td>
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<tr>
<td>(800) 842-6007</td>
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<tr>
<td>Order online: <a href="http://www.thermo.com/abgene">www.thermo.com/abgene</a></td>
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<td>Order online: <a href="http://www.fishersci.com">www.fishersci.com</a></td>
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<tr>
<td><strong>Direct:</strong> (PCR Reagents Only)</td>
<td><strong>Dominique Dutscher:</strong> (All Products)</td>
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<td>03 88 59 33 90</td>
<td>03 88 67 53 23</td>
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<td>03 88 67 85 11</td>
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<td>Order online: <a href="http://www.dutscher.com">www.dutscher.com</a></td>
<td>Order online: <a href="http://www.fr.fishersci.com">www.fr.fishersci.com</a></td>
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<tr>
<th>Rest of World</th>
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<tr>
<td><strong>Please refer to our Distributors’ list by visiting <a href="http://www.thermo.com/PCRcontact">www.thermo.com/PCRcontact</a></strong></td>
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