Troubleshooting

Problem
Possible Cause
Solution
Signs are present in no-template controls, and/or multiple peaks are present in the melting curve graph.
Template or reagents are contaminated by nucleic acids (DNA, cDNA).

Use melting curve analysis if possible, and/or run the PCR products on a 4% agarose gel after the reaction to identify contaminants.

To reduce the risk of contamination, take standard precautions when preparing your PCR reactions.

Primers or other primer artifacts are present.

Use melting curve analysis to identify primer dimers by their lower melting temperature if possible. Use validated primer sets or design primers/probes using dedicated software programs or primer databases.

Check the purity of your primers by gel electrophoresis. If agarose gels are used, we recommend coiling the gels before visualization with intercalating dyes.

No amplification curve appears on the qPCR graph.

There is no PCR product or reagents are contaminated by nucleic acids (DNA, cDNA)

Run the reaction on a gel to determine whether PCR worked. Then proceed to the troubleshooting steps below.

No amplification curve appears on the qPCR graph.

No amplification curve on the qPCR graph.

There was no protocol followed not recognized.

Verify that all steps have been followed and the correct reagents, dilutions, volumes, and cycling parameters have been used.

Template or reagents are contaminated by nucleic acids (DNA, cDNA)

Check the purity of your primers by gel electrophoresis. If agarose gels are used, we recommend coiling the gels before visualization with intercalating dyes.

Template contains inhibitors, nucleases, or proteins, or has otherwise been degraded.

Purify or re-purify your template.

Verify your primer selection. Use validated primer sets or design primers/probes using dedicated software programs or primer databases.

qPCR instrument settings are incorrect.

Verify that you are using the correct instrument settings (dye selection, reference dye, filters, acquisition points, etc.) for your application.

Confirn that you are using the correct instrument settings (dye selection, reference dye, filters, acquisition points, etc.) for your application.

For instrument-specific tips and troubleshooting, visit www.invitrogen.com/qpcr

Troubleshooting

qPCR efficiency is above 110%.

Template contains inhibitors, nucleases, or proteins, or has otherwise been degraded.

Purify or re-purify your template. Inhibitors in the template may result in changes in qPCR efficiency between dilutions.

PCR efficiency is below 80%.

The PCR efficiency is suboptimal.

Perform a singleplex reaction using the same primers and template to check efficiency. Then determine which primer set should be in limiting concentration.

Typically, you should limit the amount of primer for the most abundant gene(s). For additional troubleshooting tips see www.invitrogen.com/qpcr

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General Protocol for ABI Instruments

Follow the protocol below for qPCR using either LUX™ Primers or TaqMan® Probes on ABI real-time instruments. Note the separate cycling conditions for the ABI 7500 in Fast Mode, and the lower amount of ROX Reference Dye required for the ABI Mx3000P®, Mx3005P™, and Mx4000®; the Corbett Research Rotor-Gene™; the MJ Research DNA Engine Opticon™, Opticon® 2, volumes can be scaled as desired (e.g., scaled down to a 20-μl reaction volume for 384-well plates).

1. Program your real-time instrument as shown below. Optimal temperatures and incubation times may vary.

**Standard Cycling Program for ABI Instruments**

For two-step qRT-PCR, use 5 μl of undiluted or 10 μl of diluted cDNA generated from 10 pg to 1 μg of total RNA. For cDNA synthesis, we recommend SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR (see Additional Products, page 1). Note that detecting high-abundance genes in undiluted cDNA may result in very low CTs in qPCR, leading to reduced quantification accuracy. Prepare a dilution series of the cDNA template for the most accurate results.

**Optimal cycling conditions will vary with different instruments.**

**Plasmid and Genomic DNA**

Use 100 pg to 1 μg of genomic DNA or 10–107 copies of plasmid DNA in a 10-μl volume. Note that 1 μg of plasmid DNA contains 9.1 × 1010 copies divided by the plasmid size in kilobases.

**Magnesium Concentration**

Magnesium chloride is included in the SuperMix at a final concentration of 3 mM. This works well for most targets; however, the optimal concentration may range from 3 to 6 mM. If necessary, use the 50 mM magnesium chloride provided in the kit to increase the magnesium concentration, as shown below (the table assumes a 50-μl reaction containing 25 μl of SuperMix):

<table>
<thead>
<tr>
<th>Instrument</th>
<th>ROX per 50-μl reaction</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABI 7000, 7200, 7700, 7900HT, and 7900HT Fast</td>
<td>1 μl</td>
<td>500 nM</td>
</tr>
<tr>
<td>ABI 7500, Stratagene Mx3000P™, Mx3005P™, and Mx4000P™</td>
<td>0.1 μl*</td>
<td>50 nM</td>
</tr>
</tbody>
</table>

Decrease the amount of water in the reaction accordingly.

**ROX Reference Dye**

ROX Reference Dye can be included in the reaction to normalize the fluorescent reporter signal, for instruments that are compatible with that option. ROX is supplied at a 25 μM concentration, and is composed of a glycine conjugate of 5-carboxy-X-rhodamine succinimidyl ester in 20 mM Tris-HCl (pH 8.4), 0.1 mM EDTA, and 0.01% Tween® 20. Use the following table to determine the amount of ROX to use with a particular instrument (per 50-μl reaction volume):

<table>
<thead>
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<th>Final Concentration</th>
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</thead>
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</tr>
<tr>
<td>ABI 7500, Stratagene Mx3000P™, Mx3005P™, and Mx4000P™</td>
<td>0.1 μl*</td>
<td>50 nM</td>
</tr>
</tbody>
</table>

*To accurately pipet 0.1 μl per reaction, we recommend diluting ROX 1:10 immediately before use and use 1 μl of the dilution.

**Multiplexing**

In multiplex applications, different reporter dyes are used to label separate primers or probes targeting different genes. For relative expression studies using multiplex PCR, the amount of primer for the reference gene (e.g., β-actin or GAPDH) should be limited to avoid competition with the sample gene. In general, the final concentration of the reference gene primer should be between 25 and 100 nM. A primer titration is recommended for optimal results. For additional optimization guidelines, visit www.invitrogen.com/qpcr.

**Detection Methods**

For best results using the following detection systems, the amplicon size should be 80–200 bp.

**LUX™ Primers**

LUX™ Primers are fluorogenic primers for qPCR that provide high sensitivity, high specificity, multiplexing capability, and melting analysis. LUX™ Primers are available separately from Invitrogen (www.invitrogen.com/lux), and may be designed for specific targets using the D-LUX™ Designer at www.invitrogen.com/dtlxdesigner.

A final concentration of 200 nM per primer is effective for most reactions. Optimal results may require a primer titration between 100 and 500 nM.

**Dual-Labeled Probes**

A final probe concentration of 100 nM is effective for most reactions. The optimal concentration may vary between 50 and 500 nM. PCR primers used with probes should be designed according to standard PCR guidelines. A final concentration of 200 nM per primer is effective for most reactions.
Guidelines and Parameters

Instruments Compatibility

This kit can be used with a variety of real-time instruments, including all instruments that can perform qPCR, such as the ABI PRISM® 7000 and 7700 Fast, the MJ Research DNA Engine Opticon® 1, 2, and 3, and the Corbett Research Rotor-Gene™. For instrument-specific protocols, go to www.invitrogen.com/qpcr. Optimal cycling conditions will vary with different instruments.

Template cDNA

For two-step qRT-PCR, use 5 µl of undiluted or 10 µl of diluted cDNA generated from 10 pg to 1 µg of total RNA. For cDNA synthesis, we recommend SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR (see Additional Products, page 1). Note that detecting high-abundance genes in undiluted cDNA may result in very low CTS in qPCR, leading to reduced quantification accuracy. Prepare a dilution series of the cDNA template for the most accurate results.

Plasmid and Genomic DNA

Use 100 pg to 1 µg of genomic DNA or 10–10–1 copies of plasmid DNA in a 10-µl volume. Note that 1 µg of plasmid DNA contains 9.1 × 1010 copies divided by the plasmid size in kilobases.

Magnesium Concentration

Magnesium chloride is included in the SuperMix at a final concentration of 3 mM. This works well for most targets; however, the optimal concentration may range from 2.5 to 5.0 mM. If necessary, use the 5.0-mM magnesium chloride provided in the kit to increase the magnesium concentration, as shown below (the table assumes a 50-µl reaction containing 25 µl of SuperMix).

For a Final MgCl2 Concentration of

<table>
<thead>
<tr>
<th>Add this Volume of 50-mM MgCl2 (per 50-µl Rxn)</th>
<th>4.0 mM</th>
<th>5.0 mM</th>
<th>6.0 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original SuperMix</td>
<td>1 µl</td>
<td>2 µl</td>
<td>3 µl</td>
</tr>
</tbody>
</table>
| Decrease the amount of water in the reaction accordingly.

ROX Reference Dye

ROX Reference Dye can be included in the reaction to normalize the fluorescent reporter signal, for instruments that are compatible with this option. ROX is supplied at a 25 µM concentration, and is comprised of a glycosylated dye, 5-carboxy-|5(6)-carboxyfluorescein [carboxymethoxy]luciferin, in 20 mM Tris-HCl (pH 8.4), 0.1 mM EDTA, and 0.01% Tween® 20. Use the following table to determine the amount of ROX to use with a particular instrument (per 50-µl reaction volume):

<table>
<thead>
<tr>
<th>Instrument</th>
<th>ROX per 50-µl reaction</th>
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<tbody>
<tr>
<td>ABI 7000, 7700 Fast, and 7900HT Fast</td>
<td>1.0 µl</td>
<td>500 nM</td>
</tr>
<tr>
<td>ABI 7500, Stratagene Mx3000™, Mx3005™, and Mx4000™</td>
<td>0.1 µl</td>
<td>50 nM</td>
</tr>
</tbody>
</table>

*To accurately pipet 0.1 µl per reaction, we recommend diluting ROX 1:10 immediately before use and use 1 µl of the dilution.

Note: Platinum® Quantitative PCR SuperMix-UDG with ROX (Catalog nos. 11743-100 and 11743-500) includes ROX in the SuperMix at a 500-nM final concentration (see Additional Products, page 1).

Multiplexing

In multiplex applications, different reporter dyes are used to label separate primers or probes targeting different genes. For relative expression studies using multiplex PCR, the amount of primer for the reference gene (e.g., β-actin or GAPDH) should be limited to avoid competition with the sample gene. In general, the final concentration of the reference gene primer should be between 25 and 100 nM. A primer titration is recommended for optimal results. For additional optimization guidelines, visit www.invitrogen.com/qpcr.

Detection Methods

For best results using the following detection systems, the amplicon size should be 80–200 bp.

**LUX™ Primers**

LUX Primers are fluorogenic primers for qPCR that provide high sensitivity, high specificity, multiplexing capability, and melting analysis. LUX Primers are available separately from Invitrogen (www.invitrogen.com/lux) and may be designed for specific targets using the D-LUX® Designer at www.invitrogen.com/dltuxdesigner.

A final concentration of 200 nM per primer is effective for most reactions. Optimal results may require a primer titration between 100 and 500 nM.

**Dual-Labeled Probes**

A final probe concentration of 100 nM is effective for most reactions. The optimal concentration may vary between 50 and 500 nM. PCR primers used with probes should be designed according to standard PCR guidelines. A final concentration of 200 nM per primer is effective for most reactions.

General Protocol for ABI Instruments

Follow the protocol below for qPCR using either LUX™ Primers or TaqMan® Probes on ABI real-time instruments. Note the separate cycling conditions for the ABI 7500 in Fast Mode, and the lower amount of ROX Reference Dye required for the ABI 7500 and 7900 Fast systems. This generic protocol may also be used for other real-time instruments.

For protocols for specific instruments, visit www.invitrogen.com/qpcr. A standard 50-µl reaction size is provided; component volumes can be scaled as desired (e.g., scaled down to a 20-µl reaction volume for 384-well plates).

1. Program your real-time instrument as shown below. Optimal temperatures and incubation times may vary.

**Standard Cycling Program for ABI Instruments**

- 50°C for 2 minutes hold (UDG incubation)
- 95°C for 2 minutes hold
- 40 cycles of:
  - 95°C, 15 seconds
  - 60°C, 30 seconds

Melting curve analysis (LUX™ Primers only): Refer to instrument documentation

**Fast Cycling Program (for the ABI 7500 in Fast Mode)**

- 50°C for 2 minutes hold (UDG incubation)
- 95°C for 2 minutes hold
- 40 cycles of:
  - 95°C, 15 seconds
  - 60°C, 30 seconds

Melting curve analysis (LUX™ Primers only): Refer to instrument documentation

2. Set up reactions as specified below. Volumes for a single 50-µl reaction are listed. For multiple reactions, prepare a master mix of common components, add the appropriate volume to each tube or plate well, and then add the unique probe or primer reactions (e.g., template).

**LUX™ Primers Reaction Mix**

- Platinum® Quantitative PCR SuperMix-UDG 25 µl
- LUX™ labeled primer, 10 µM 1 µl
- Unlabelled primer, 10 µM 1 µl
- ROX Reference Dye (optional) 1 µl/0.1 µl*
- Template (100 pg to 1 µg of genomic DNA, 10–10–1 copies of plasmid DNA, or cDNA generated from 10 pg to 1 µg of total RNA) ≤ 10 µl
- DEPC-treated water to 50 µl

**TagMan® Probes Reaction Mix**

- Platinum® Quantitative PCR SuperMix-UDG 25 µl
- Forward primer, 10 µM 1 µl
- Reverse primer, 10 µM 1 µl
- Fluorogenic probe, 10 µM 0.5 µl
- Template (100 pg to 1 µg of genomic DNA, 10–10–1 copies of plasmid DNA, or cDNA generated from 10 pg to 1 µg of total RNA) ≤ 10 µl
- DEPC-treated water to 50 µl

*See the table on page 2 for the amount/concentration of ROX to use for your specific instrument.

3. Cap or seal the reaction tube/PCR plate, and gently mix. Make sure that all components are at the bottom of the tube/plate; centrifuge briefly if needed.

4. Place reactions in a preheated real-time instrument programmed as described above. Collect data and analyze results.


Using melting curve analysis if possible, and/or run the PCR products on a 4% agarose gel after the reaction to identify contaminants. To reduce the risk of contamination, take standard precautions when preparing your PCR reactions.

No amplification curve appears on the qPCR graph

Run the reaction on a gel to determine whether PCR worked. Then proceed to the troubleshooting steps below.

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