PowerUp™ SYBR™ Green Master Mix

USER GUIDE

Universal 2X master mix for real-time PCR workflows

Catalog Numbers  A25741, A25742, A25743, A25776, A25777, A25778, A25779, A25780, A25918
Publication Number  MAN0013511
Revision  C.0
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Revision history
Table 1  Revision history of Pub. no. MAN0013511

<table>
<thead>
<tr>
<th>Revision</th>
<th>Date</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.0</td>
<td>February 2016</td>
<td>• Reorganized content according to current templates.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Clarified instructions for using genomic DNA in PCR.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Updated recommended action for primer-dimer formation and residual polymerase activity.</td>
</tr>
<tr>
<td>B.0</td>
<td>August 2015</td>
<td>Baseline for revision history</td>
</tr>
</tbody>
</table>

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Product information

IMPORTANT! Before using this product, read and understand the information in the “Safety” appendix in this document.

Product description

The Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix is formulated to provide superior specificity and sensitivity. It is supplied in a convenient 2X concentration pre-mix to perform real-time PCR using SYBR™ Green Dye. The master mix contains:

- SYBR™ Green Dye
- Dual-Lock™ DNA Polymerase, with a proprietary combination of two proprietary hot start modifications for exceptional specificity
- Heat-labile Uracil-DNA Glycosylase (UDG)
- ROX™ dye Passive Reference
- dNTP blend containing dUTP/dTTP
- Optimized buffer components

The user provides primers, template, and water.

See “Master mix components” on page 18 for more details about each component.

Contents and storage

Table 2  PowerUp™ SYBR™ Green Master Mix

<table>
<thead>
<tr>
<th>Cat. no.</th>
<th>Amount</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>A25741</td>
<td>1 mL</td>
<td></td>
</tr>
<tr>
<td>A25779 (2 × A25741)</td>
<td>2 × 1 mL</td>
<td></td>
</tr>
<tr>
<td>A25780 (5 × A25741)</td>
<td>5 × 1 mL</td>
<td></td>
</tr>
<tr>
<td>A25918 (10 × A25741)</td>
<td>10 × 1 mL</td>
<td>2–8°C</td>
</tr>
<tr>
<td>A25742</td>
<td>5 mL</td>
<td></td>
</tr>
<tr>
<td>A25776 (2 × A25742)</td>
<td>2 × 5 mL</td>
<td></td>
</tr>
<tr>
<td>A25777 (5 × A25742)</td>
<td>5 × 5 mL</td>
<td></td>
</tr>
</tbody>
</table>
### Cat. no. Amount Storage

<table>
<thead>
<tr>
<th>Cat. no.</th>
<th>Amount</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>A25778 (10 × A25742)</td>
<td>10 × 5 mL</td>
<td>2–8°C</td>
</tr>
<tr>
<td>A25743</td>
<td>50 mL</td>
<td></td>
</tr>
</tbody>
</table>

### Required materials

Unless otherwise indicated, all materials are available through [thermofisher.com](http://thermofisher.com). MLS: Fisher Scientific ([www.fisherscientific.com](http://www.fisherscientific.com)) or other major laboratory supplier.

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>One of the following Applied Biosystems™ instruments:</strong></td>
<td><strong>Source</strong></td>
</tr>
<tr>
<td>• QuantStudio™ Real-Time PCR System</td>
<td>Contact your local sales office.</td>
</tr>
<tr>
<td>• StepOnePlus™ Real-Time PCR System</td>
<td></td>
</tr>
<tr>
<td>• StepOne™ Real-Time PCR System</td>
<td></td>
</tr>
<tr>
<td>• 7500 Fast Real-Time PCR System</td>
<td></td>
</tr>
<tr>
<td>• 7500 Real-Time PCR Instrument</td>
<td></td>
</tr>
<tr>
<td>• 7900HT Fast Real-Time PCR Instrument</td>
<td></td>
</tr>
<tr>
<td>• 7900HT Real-Time PCR Instrument</td>
<td></td>
</tr>
<tr>
<td>• ViiA™ 7 Real-Time PCR System</td>
<td></td>
</tr>
<tr>
<td>Or use a compatible real-time PCR instrument from another supplier.</td>
<td></td>
</tr>
</tbody>
</table>

**Equipment**

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrifuge with adapter for 96- or 384-well plates</td>
<td>MLS</td>
</tr>
<tr>
<td>Laboratory mixer (Vortex or equivalent)</td>
<td>MLS</td>
</tr>
<tr>
<td>Microcentrifuge</td>
<td>MLS</td>
</tr>
<tr>
<td>Pipettors</td>
<td>MLS</td>
</tr>
</tbody>
</table>

**Plastics and other consumables**

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plates and seals for your instrument</td>
<td>Refer to <a href="http://thermofisher.com/plastics">thermofisher.com/plastics</a></td>
</tr>
<tr>
<td>Disposable gloves</td>
<td>MLS</td>
</tr>
<tr>
<td>Pipette tips with filters</td>
<td>MLS</td>
</tr>
<tr>
<td>Polypropylene tubes</td>
<td>MLS</td>
</tr>
</tbody>
</table>

**Reagents and kits**

One of the following reverse transcription kits, if performing gene expression analysis:
<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>• SuperScript™ VILO™ cDNA Synthesis Kit</td>
<td>• Cat. no. 11754-050 or 11754-250</td>
</tr>
<tr>
<td>• High-Capacity cDNA Reverse Transcription Kit</td>
<td>• Cat. no. 4368813 or 4368814</td>
</tr>
<tr>
<td>• High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor</td>
<td>• Cat. no. 4374966 or 4374967</td>
</tr>
<tr>
<td>• High-Capacity RNA-to-cDNA™ Kit</td>
<td>• Cat. no. 4387406</td>
</tr>
<tr>
<td>Nuclease-Free Water (not DEPC-Treated)</td>
<td>Cat. no. 4387936, AM9930, AM9932, AM9937, AM9938, or AM9939</td>
</tr>
<tr>
<td>TE, pH 8.0</td>
<td>Cat. no. AM9849 or AM9858</td>
</tr>
</tbody>
</table>

**Note:** Do not use plastics made of polyethylene terephthalate co-polyester, glycol modified (PTEG) for storage of PowerUp™ SYBR™ Green Master Mix or reaction mixes. SYBR™ dye has been shown to stick to this type of plastic material. Polypropylene, high density polyethylene (HDPE), and polystyrene are recommended for storage.
Workflow

Start with cDNA or gDNA

Create and set up a plate document

Prepare the PCR reaction plate

Run the PCR reaction plate

Analyze results

SDS Software

384-well plates, 96-well Standard or Fast plates, or 48-well plates

Real-Time PCR instrument

Amplification plot
Methods

Guidelines

Input DNA template requirements

Use 1–10 ng single-stranded cDNA or 10–100 ng gDNA per reaction.

See “RNA guidelines” on page 23 and “Template storage” on page 24 for additional information.

PCR reactions

• Four replicates of each reaction are recommended.
• Reaction mixes can be prepared depending upon experimental requirements. Scale the components according to the number of reactions and include 10% overage.
• If using smaller reaction volumes, scale all components proportionally. Reaction volumes <10 µL are not recommended.

Using NTC controls

No template control (NTC) reactions can be used to identify PCR contamination. NTC reactions contain all reaction components (PowerUp™ SYBR™ Green Master Mix, primers, water) except sample, and therefore should not return a C_T value.

Before you begin

Set up the plate document

Configure the plate document, following the guidelines for your instrument.

Prepare the reagents

1. Swirl the PowerUp™ SYBR™ Green Master Mix to mix thoroughly.

2. Thaw the DNA samples and primers on ice, vortex to mix, then centrifuge briefly.
Set up the PCR reactions

1. Prepare the appropriate number of reactions, plus 10% overage.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (10 µL/well)</th>
<th>Volume (20 µL/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PowerUp™ SYBR™ Green Master Mix (2X)</td>
<td>5 µL</td>
<td>10 µL</td>
</tr>
<tr>
<td>Forward and reverse primers[1]</td>
<td>Variable</td>
<td>Variable</td>
</tr>
<tr>
<td>DNA template + Nuclease-Free Water[2]</td>
<td>Variable</td>
<td>Variable</td>
</tr>
<tr>
<td>Total</td>
<td>10 µL</td>
<td>20 µL</td>
</tr>
</tbody>
</table>

[2] Use 1–10 ng cDNA or 10–100 ng gDNA for each reaction.

2. Mix the components thoroughly, then centrifuge briefly to spin down the contents and eliminate any air bubbles.

3. Transfer the appropriate volume of each reaction to each well of an optical plate.

4. Seal the plate with an optical adhesive cover, then centrifuge briefly to spin down the contents and eliminate any air bubbles.

PCR can be performed on the reaction plate up to 24 hours after completing the set-up, when stored at room temperature.

Set up and run the real-time PCR instrument

1. Place the reaction plate in the real-time PCR instrument.

2. Set the thermal cycling conditions using the default PCR thermal cycling conditions specified in the following tables according to the instrument cycling parameters and melting temperatures of the specific primers.

**Note:** Standard cycling conditions are recommended for genomic DNA templates. Use only standard cycling conditions for the 7900HT Real-Time PCR Instrument.

**Table 3** Fast cycling mode (primer $T_m \geq 60^\circ$C)

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Duration</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDG activation</td>
<td>50°C</td>
<td>2 minutes</td>
<td>Hold</td>
</tr>
<tr>
<td>Dual-Lock™ DNA polymerase</td>
<td>95°C</td>
<td>2 minutes</td>
<td>Hold</td>
</tr>
<tr>
<td>Denature</td>
<td>95°C</td>
<td>1 second[1] or 3 seconds[2]</td>
<td>40</td>
</tr>
<tr>
<td>Anneal/extend</td>
<td>60°C</td>
<td>30 seconds</td>
<td></td>
</tr>
</tbody>
</table>

### Table 4  Standard cycling mode \( \text{primer } T_m \geq 60°C \)

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Duration</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDG activation</td>
<td>50°C</td>
<td>2 minutes</td>
<td>Hold</td>
</tr>
<tr>
<td>Dual-Lock™ DNA polymerase</td>
<td>95°C</td>
<td>2 minutes</td>
<td>Hold</td>
</tr>
<tr>
<td>Denature</td>
<td>95°C</td>
<td>15 seconds</td>
<td></td>
</tr>
<tr>
<td>Anneal/extend</td>
<td>60°C</td>
<td>1 minute</td>
<td></td>
</tr>
</tbody>
</table>

### Table 5  Standard cycling mode \( \text{primer } T_m < 60°C \)

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Duration</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDG activation</td>
<td>50°C</td>
<td>2 minutes</td>
<td>Hold</td>
</tr>
<tr>
<td>Dual-Lock™ DNA polymerase</td>
<td>95°C</td>
<td>2 minutes</td>
<td>Hold</td>
</tr>
<tr>
<td>Denature</td>
<td>95°C</td>
<td>15 seconds</td>
<td></td>
</tr>
<tr>
<td>Anneal</td>
<td>55–60°C([1])</td>
<td>15 seconds</td>
<td></td>
</tr>
<tr>
<td>Extend</td>
<td>72°C</td>
<td>1 minute</td>
<td></td>
</tr>
</tbody>
</table>

\(\text{[1]}\) Anneal temperature should be set to the melting point for your primers.

3. Set the instrument to perform a default dissociation step.

A dissociation step can be performed up to 72 hours after the real-time PCR run if the plate is stored in the dark and up to 24 hours after the real-time PCR run if the plate is exposed to light.

### Table 6  Dissociation curve conditions (melt curve stage)

<table>
<thead>
<tr>
<th>Step</th>
<th>Ramp rate</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.6°C/second</td>
<td>95°C</td>
<td>15 seconds</td>
</tr>
<tr>
<td>2</td>
<td>1.6°C/second</td>
<td>60°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>3([1])</td>
<td>0.15°C/second</td>
<td>95°C</td>
<td>15 seconds</td>
</tr>
</tbody>
</table>

\(\text{[1]}\) Dissociation

Use the following settings for Applied Biosystems™ instruments:
- Experiment type: Standard curve
- Reagent: SYBR™ Green reagents
- Reporter: SYBR™
- Quencher: None
- Passive reference dye: ROX™
- Ramp speed: Standard or fast (choose the same setting as in step 2)
- Melt curve ramp increment: Continuous

4. Set the reaction volume appropriate for the type of plate being used for your PCR reaction.

5. Start the run.
Analyze results

1. View the amplification plots.
   See “A typical amplification plot“ on page 21 for more information.

2. Calculate the baseline and threshold cycles (C\textsubscript{T}) for the amplification curves using the instrument software.

3. Check for nonspecific amplification using dissociation curves.
   It is important to check for nonspecific amplification because SYBR™ Green dye detects any double-stranded DNA.
   See “Dissociation curves“ on page 22 for more information.

4. Perform relative or absolute quantitation.
   Visit thermofisher.com/qpcredution and click Absolute vs. Relative Quantitation for qPCR in the left menu for more information.
   • Relative quantitation — The target is compared to an internal standard, using either the standard curve or comparative C\textsubscript{T} method.
   • Absolute quantitation — The C\textsubscript{T} of the unknown samples is compared against a standard curve with known copy numbers.
## Troubleshooting

### General troubleshooting

<table>
<thead>
<tr>
<th>Observation</th>
<th>Possible cause</th>
<th>Recommended action</th>
</tr>
</thead>
<tbody>
<tr>
<td>The C&lt;sub&gt;T&lt;/sub&gt; values are high, there is poor precision, or the PCR reactions failed.</td>
<td>There is insufficient DNA template.</td>
<td>Use up to 100 ng of DNA template per 20-µL reaction. Typically, 1–10 ng cDNA or 10–100 ng genomic DNA per 20-µL reaction are sufficient.</td>
</tr>
<tr>
<td>The quality of the DNA template is poor.</td>
<td></td>
<td>Quantify the amount of DNA template (see &quot;Template quantitation using O.D. 260&quot; on page 24) and ensure the recommended amount is used. Test the DNA template for the presence of PCR inhibitors. Repeat the PCR reaction with a DNA template free of PCR inhibitors, if necessary.</td>
</tr>
<tr>
<td>The sample has degraded.</td>
<td></td>
<td>Prepare fresh cDNA or gDNA, then repeat the experiment.</td>
</tr>
<tr>
<td>Incorrect volumes of components were pipetted for the PCR reactions.</td>
<td></td>
<td>Prepare the PCR reactions as described in &quot;Set up the PCR reactions&quot; on page 10.</td>
</tr>
<tr>
<td>Too few PCR cycles were used.</td>
<td></td>
<td>Increase the number of PCR cycles to the default setting of 40 (see &quot;Set up and run the real-time PCR instrument&quot; on page 10) .</td>
</tr>
<tr>
<td>Low ΔR&lt;sub&gt;n&lt;/sub&gt; or R&lt;sub&gt;n&lt;/sub&gt; values are obtained.</td>
<td>The extension time was too short.</td>
<td>Use the recommended standard cycling thermal profile settings (see &quot;Set up and run the real-time PCR instrument&quot; on page 10).</td>
</tr>
<tr>
<td>There was primer-dimer formation and residual polymerase activity.</td>
<td></td>
<td>• Optimize the thermal cycling temperatures. • Reduce the primer concentration. • Redesign the primers.</td>
</tr>
<tr>
<td>ROX™ dye was not selected as the passive reference when the plate document was set up.</td>
<td></td>
<td>Select ROX™ dye as the passive reference when setting up the plate document.</td>
</tr>
</tbody>
</table>

*PowerUp™ SYBR™ Green Master Mix User Guide 13*
### Observation Possible cause Recommended action

<table>
<thead>
<tr>
<th>Observation</th>
<th>Possible cause</th>
<th>Recommended action</th>
</tr>
</thead>
<tbody>
<tr>
<td>The $\Delta R_n$ or $R_n$ values are extremely high.</td>
<td>ROX™ dye was not selected as the passive reference when the plate document was set up.</td>
<td>Select ROX™ dye as the passive reference when setting up the plate document.</td>
</tr>
<tr>
<td></td>
<td>There was evaporation from the reaction plate.</td>
<td>Ensure that the reaction plate is sealed completely, especially around the edges.</td>
</tr>
<tr>
<td>The $R_n$ values obtained in early cycles are low.</td>
<td>The $C_T$ value is less than 15.</td>
<td>Adjust the upper baseline range to a value less than 15.</td>
</tr>
<tr>
<td></td>
<td>ROX™ dye was not selected as the passive reference when the plate document was set up.</td>
<td>Select ROX™ dye as the passive reference when setting up the plate document.</td>
</tr>
<tr>
<td></td>
<td>There was evaporation from the reaction plate.</td>
<td>Ensure that the reaction plate is sealed completely, especially around the edges.</td>
</tr>
<tr>
<td>There is high variability across the reaction plate.</td>
<td>The reaction plate was not mixed well.</td>
<td>Mix the reaction mix gently by inversion, then centrifuge briefly before aliquoting to the reaction plate.</td>
</tr>
</tbody>
</table>

### Troubleshooting baseline settings

<table>
<thead>
<tr>
<th>Observation</th>
<th>Possible cause</th>
<th>Recommended action</th>
</tr>
</thead>
<tbody>
<tr>
<td>The baseline is set too low.</td>
<td>The baseline is set too low (cycles 3–5).</td>
<td>Manually adjust the baseline to a higher range of cycles (see Figure 3).</td>
</tr>
</tbody>
</table>

![Figure 1](image_url)
<table>
<thead>
<tr>
<th>Observation</th>
<th>Possible cause</th>
<th>Recommended action</th>
</tr>
</thead>
<tbody>
<tr>
<td>The baseline is set too high.</td>
<td>The baseline is set too high (cycles 5–20).</td>
<td>Manually adjust the baseline to a lower range of cycles (see Figure 3).</td>
</tr>
<tr>
<td><img src="image1.png" alt="Figure 2" /></td>
<td><img src="image2.png" alt="Figure 3" /></td>
<td></td>
</tr>
<tr>
<td>The baseline is set correctly.</td>
<td>The baseline is set correctly (cycles 3–15).</td>
<td>No action is required.</td>
</tr>
</tbody>
</table>
## Troubleshooting threshold settings

<table>
<thead>
<tr>
<th>Observation</th>
<th>Possible cause</th>
<th>Recommended action</th>
</tr>
</thead>
<tbody>
<tr>
<td>The threshold is set too low.</td>
<td>The threshold is set too low.</td>
<td>Manually adjust the threshold to a higher ( \Delta R_n ) (see Figure 6).</td>
</tr>
</tbody>
</table>

![Figure 4](image1.png)

The threshold is set too high.

![Figure 5](image2.png)

The threshold is set too high.

Manually adjust the threshold to a lower \( \Delta R_n \) (see Figure 6).
### Troubleshooting threshold settings

<table>
<thead>
<tr>
<th>Observation</th>
<th>Possible cause</th>
<th>Recommended action</th>
</tr>
</thead>
<tbody>
<tr>
<td>The threshold is set correctly.</td>
<td>The threshold is set correctly.</td>
<td>No action is required.</td>
</tr>
</tbody>
</table>

**Figure 6**

![Graph of ΔRn vs Cycle showing multiple curves](image)
# Master mix components

## Table 7  PowerUp™ SYBR™ Green Master Mix component function

<table>
<thead>
<tr>
<th>Component</th>
<th>Function</th>
</tr>
</thead>
</table>
| **Dual-Lock™ Taq DNA polymerase**  | • Utilizes a combination of two hot start mechanisms to control activity.  
• Provides tight control over Taq activation, preventing undesirable early activity of the polymerase at low temperatures that can lead to nonspecific amplification.  
• Allows flexibility in reaction set-up, including pre-mixing of PCR reagents and storage at room temperature for up to 72 hours prior to cycling.  
• Allows reactivation of polymerase after only 2 minutes at 95°C. |
| **Heat-labile uracil-DNA glycosylase (UDG)**  | • A 26 kDa recombinant enzyme derived from the thermolabile UDG gene isolated from marine bacteria, and expressed in E. coli.  
• Prevents reamplification of carryover PCR products by removing any uracil incorporated into single- or double-stranded amplicons (Longo, M.C, et al. 1990).  
• Acts on single- and double-stranded dU-containing DNA by hydrolyzing uracil-glycosidic bonds at dU-containing DNA site, creating an alkali-sensitive apyrimidic site in the DNA.  
• Prevents reamplification of carryover PCR products in an assay if all previous PCR for the assay was performed using a dUTP-containing master mix.  
• Allows stability of PCR products for 72 hours post-amplification.  
• Has no activity on RNA or dT-containing DNA. |
| **dUTP/dTTP**  | • Enables UDG activity and maintains optimal PCR results. |
| **SYBR™ Green**  | • Detects PCR products by fluorescing upon binding to double-stranded DNA formed during PCR (see “SYBR™ Green dye chemistry overview” on page 19). |
| **ROX™ passive reference**  | • Provides an internal reference to which the reporter-dye signal can be normalized during data analysis.  
• Normalization is necessary to correct for fluorescence fluctuations due to changes in concentration or volume. |
SYBR™ Green dye chemistry overview

The SYBR™ Green dye is used to detect PCR products by binding to double-stranded DNA formed during PCR.

1. When SYBR™ Green dye is added to a sample, it immediately binds to all double-stranded DNA (dsDNA) present in the sample. SYBR™ Green dye is only fluorescent when bound to dsDNA.

2. During PCR, DNA polymerase amplifies the target sequence which creates the PCR products.

3. SYBR™ Green dye then binds to each new copy of double-stranded DNA, generating a fluorescent signal.

4. As the PCR progresses, more PCR product is created. SYBR™ Green dye binds to all double-stranded DNA, so the result is an increase in fluorescence intensity proportional to the amount of PCR product produced.

Figure 7  Representation of how SYBR™ Green dye acts on double-stranded DNA during one extension phase of PCR
Two-step RT-PCR

Visit thermofisher.com/qpcrceducation for more information.

When performing a two-step RT-PCR reaction, total RNA or mRNA must first be reverse transcribed into cDNA.

1. In the reverse transcription (RT) step, cDNA is reverse transcribed from total RNA samples using random primers from the High-Capacity cDNA Reverse Transcription Kit or the SuperScript™ VILO™ cDNA Synthesis Kit.

2. In the PCR step, PCR products are synthesized from cDNA samples using the PowerUp™ SYBR™ Green Master Mix.
**A typical amplification plot**

Visit [thermofisher.com/qpcredducation](http://thermofisher.com/qpcredducation) and click *Essentials of Real-Time PCR* in the left menu for more information about amplification plots and dissociation curves.

A typical amplification plot is shown below.

Baseline and threshold values

- **Baseline** – the initial cycles of PCR in which there is little change in fluorescence signal.
- **C_T** – the intersection of the threshold with the amplification plot.
- **Threshold** – set above the background and within the exponential growth phase of the amplification curve.
Automatic calculation of the baseline and threshold can be conducted using your instrument’s software. Alternatively, baseline and threshold can be set manually. See “Troubleshooting baseline settings” on page 14 and “Troubleshooting threshold settings” on page 16 for examples of amplification plots where the baseline and threshold values are set too high or too low.

**Dissociation curves**

A dissociation curve is a graph that displays dissociation data from the amplicons of quantitative PCR runs. Change in fluorescence, due to a dye or probe interacting with double-stranded DNA, is plotted against temperature. A single peak indicates specific amplification, whereas multiple peaks or shoulders indicate nonspecific amplification or primer-dimer formation.

Because of the heat-labile UDG, you can generate a dissociation curve up to 72 hours after the real-time PCR run on any compatible Applied Biosystems™ Real-Time PCR system.

Primer-dimers are most prevalent in NTC wells and sample wells containing a low concentration of template.

This dissociation curve shows typical primer-dimer formation. The specific product is shown with a melting temperature \( T_m \) of 80.5°C, but the primer-dimer has a characteristically lower \( T_m \) of 75°C.

1. Dissociation curve of a specific product
2. Dissociation curve of a primer-dimer
Visit [thermofisher.com/qpcreducation](https://thermofisher.com/qpcreducation) for more information about DNA template quality, RNA guidelines, template quantitation, and template storage.

**DNA template quality**

Both agarose gel electrophoresis and spectrophotometry are used to examine DNA quality.

- **Agarose gel electrophoresis** – Purified DNA should run as a single band on an agarose gel. Agarose gels reveal contaminating DNAs and RNAs, but not proteins.
- **Spectrophotometry** – The $A_{260}/A_{280}$ ratio should be 1.8 to 2.0. Smaller ratios usually indicate contamination by protein or organic chemicals. Spectrophotometry can reveal protein contamination, but not DNA or RNA contamination.

**RNA guidelines**

RNA should be reverse transcribed into cDNA prior to use in a PowerUp™ SYBR™ Green Master Mix reaction. Use the SuperScript™ VILO™ cDNA Synthesis Kit or the High-Capacity cDNA Reverse Transcription Kit.

For optimal performance prior to reverse transcription, total RNA or mRNA should be:

- Between 0.002 µg/µL and 0.2 µg/µL
- Less than 0.005% of genomic DNA by weight
- Free of inhibitors of reverse transcription and PCR
- Dissolved in PCR-compatible buffer
- Free of RNase activity

**IMPORTANT!** If you suspect that the RNA contains RNase activity, add RNase inhibitor to the reverse transcription reaction at a final concentration of 1.0 U/µL.

- Nondenatured

**IMPORTANT!** It is not necessary to denature the RNA. Denaturation of the RNA may reduce the yield of cDNA for some gene targets.
Template quantitation using O.D. 260

Template quantitation is critical for successful PCR reactions. The most common way to determine DNA quantity is to measure the absorbance (optical density or O.D.) of a sample at 260 nm in a spectrophotometer.

One O.D. unit is the amount of substance dissolved in 1.0 mL that gives an absorbance reading of 1.00 in a spectrophotometer with a 1-cm path length. The wavelength is assumed to be 260 nm unless stated otherwise. $A_{260}$ values can be converted into $\mu g/\mu L$ using Beer’s Law:

$$Absorbance (260 \text{ nm}) = \text{sum of extinction coefficient contributions} \times \text{cuvette pathlength} \times \text{concentration}$$

The following formulas are derived from Beer’s Law (Ausubel, F.M. et al., eds. 1998):

- Concentration of single-stranded DNA = $A_{260} \times 33 \ \mu g/\mu L$
- Concentration of double-stranded DNA = $A_{260} \times 50 \ \mu g/\mu L$
- Concentration of single-stranded RNA = $A_{260} \times 40 \ \mu g/\mu L$

Note: Absorbance measurements of highly concentrated (O.D. >1.0) or very dilute (O.D. <0.05) DNA or RNA samples can be inaccurate. Dilute or concentrate the DNA/RNA to obtain a reading within the acceptable range.

Template storage

- Store purified RNA templates at −20°C or −70°C in Nuclease-Free Water.
- Store purified DNA templates at −20°C or −70°C in TE, pH 8.0.
Primer design guidelines

Primers should be designed using Primer Express™ Software or similar software. Refer to the Primer Express™ Software Version 3.0 Getting Started Guide (Pub. no. 4362460) and the software help.

- Keep the GC content in the 30–70% range.
- Do not overlap primer and probe sequences.
- The optimal primer length is 20 bases.
- Avoid runs of identical nucleotides. If repeats are present, there must be fewer than four consecutive G residues.
- Make sure the last five nucleotides at the 3’ end contain no more than two G and/or C bases.

<table>
<thead>
<tr>
<th>Template</th>
<th>Design guideline</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>Design the primers as described above.</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>Design the primers as described above.</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>Design the primers as described above and see “Selecting an amplicon site for cDNA” on page 26.</td>
</tr>
<tr>
<td>cDNA</td>
<td>Design the primers as described above.</td>
</tr>
<tr>
<td>RNA</td>
<td>Design the primers as described above.</td>
</tr>
</tbody>
</table>

Avoiding primer-dimers

Use primers that contain dA nucleotides near the 3’ ends so that any primer-dimer generated is efficiently degraded by UDG at least as well as any dU-containing PCR products. The farther a dA nucleotide from the 3’ end, the more likely partially degraded primer-dimer molecules can serve as a template for a subsequent PCR amplification.

Production of primer-dimers could lower the amplification yield of the desired target region. If primers cannot be selected with dA nucleotides near the ends, consider using primers with 3’ terminal dU-nucleotides. Single-stranded DNA with terminal dU nucleotides are not substrates for UDG (Delort, A.-M., et al. 1985), and therefore the primers are not degraded. Biotin-dUMP derivatives are not substrates for UDG.

For more information about designing primers, see “Primer design guidelines” on page 25.

Do not use UDG in subsequent amplifications of dU-containing PCR template, such as in nested PCR protocols. The UNG degrades the dU-containing PCR products, preventing further amplification.
Identifying target sequence and amplicon size

A target template is a DNA sequence, including cDNA, genomic DNA, or plasmid nucleotide sequence that you want to amplify.

Primers are designed to amplify amplicons (segments of DNA) within the target sequence using Primer Express™ Software. Shorter amplicons work best. Consistent results are obtained for amplicon size ranges from 50–150 bp.

Selecting an amplicon site for cDNA

Selecting a good amplicon site ensures amplification of the target cDNA without co-amplifying the genomic sequence, pseudogenes, and related genes.

- The amplicon should span one or more introns to avoid amplification of the target gene in genomic DNA.
- The primer pair must be specific to the target gene; the primer pair does not amplify pseudogenes or other related genes.
- Primers should be designed according to the guidelines in the Primer Express™ Software.
- Amplicons should be tested and those with the highest signal-to-noise ratio should be selected (low C_T with cDNA and no amplification with no template control or genomic DNA).
- The sequence may need to be examined and the amplicon redesigned if no good sequence is found. Alternatively, more sites may need to be screened.

If the gene of interest does not have introns, then an amplicon cannot be designed that amplifies the mRNA sequence without amplifying the genomic sequence. RT minus controls may need to be run.

Optimize primer concentrations for PCR

Overview

By independently varying the forward and reverse primer concentrations, you can identify the primer concentrations that provide optimal assay performance. The primer concentrations you select should provide a low C_T and a high ΔR_n when run against the target template, but should not produce nonspecific product formation with NTCs.

Quantitate primers

1. Measure the absorbance (at 260 nm of a 1:100 dilution) of each primer oligonucleotide in TE buffer.

2. Calculate the sum of extinction coefficient contributions for each primer:
   - Extinction coefficient contribution = Σ (extinction coefficient × number of bases in oligonucleotide sequence)
3. Calculate the oligonucleotide concentration in µM for each primer:
   • Absorbance at 260 nm = sum of extinction coefficient contribution × cuvette pathlength × concentration / 100
   • Rearrange to solve for concentration:
     \[ \text{Concentration} = 100 \left( \frac{\text{absorbance at 260 nm}}{\text{(sum of extinction coefficient contribution × cuvette pathlength)}} \right) \]

### Example calculation of primer concentration

In this example, the concentration of a primer (in TE buffer, diluted 1:100), with the sequence 5′-CGTACTCCTTTGCTGCTG-3′ is calculated using the following values:

<table>
<thead>
<tr>
<th>Chromophore</th>
<th>Extinction coefficient</th>
<th>Number of specific chromophores in example sequence</th>
<th>Extinction coefficient contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>15,200</td>
<td>1</td>
<td>15,200</td>
</tr>
<tr>
<td>C</td>
<td>7050</td>
<td>6</td>
<td>42,300</td>
</tr>
<tr>
<td>G</td>
<td>12,010</td>
<td>5</td>
<td>60,050</td>
</tr>
<tr>
<td>T</td>
<td>8400</td>
<td>6</td>
<td>50,400</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>—</strong></td>
<td><strong>—</strong></td>
<td><strong>167,950</strong></td>
</tr>
</tbody>
</table>

- Measured absorbance at 260 nm = 0.13
- Sum of extinction coefficient = 167,950 M⁻¹cm⁻¹ contributions for probe
- Cuvette pathlength = 0.3 cm
- Absorbance (260 nm) = sum of extinction coefficient contributions × cuvette pathlength × oligonucleotide concentration / 100
- \(0.31 = 167,950 \text{ M}^{-1}\text{cm}^{-1} \times 0.3 \text{ cm} \times C / 100\)
- \(C = 258 \text{ µM}\)

**Determine the optimal primer concentration for PCR**

1. Prepare a 96-well reaction plate.
   Use 10–100 ng of gDNA or 1–10 ng of cDNA template. The final concentration of PowerUp™ SYBR™ Green Master Mix is 1X.

   **Note:** The plate configuration accounts for four replicates of each of the following nine variations of primer concentration applied to both template and NTC wells:

<table>
<thead>
<tr>
<th>Reverse primer (nM)</th>
<th>Forward primer (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>800</td>
<td>800</td>
</tr>
</tbody>
</table>

2. Calibrate your instrument for SYBR™ Green Dye, if necessary.
   Refer to the instrument User Guide for calibration instructions. It is recommended to calibrate your instrument every six months.
3. Load the plate into the Applied Biosystems™ real-time PCR system.

4. Program the thermal-cycling conditions according to the information in “Set up and run the real-time PCR instrument” on page 10.

5. Run the plate.

6. Compile the results for $\Delta R_n$ and $C_T$, then select the minimum forward and reverse primer concentrations that yield the maximum $\Delta R_n$ values and low $C_T$ values.

Dissociation curves help you select the optimal primer concentrations for your SYBR™ quantification assays.

1. Review the linear view of the amplification plot in your NTC wells.

   Note: In Figure 8, the strong amplification of the NTC wells indicates that significant nonspecific amplification is occurring.

2. Generate a dissociation curve with your real-time PCR system.

   Note: In the example shown in Figure 9, the melting temperature of the product generated in the absence of template is lower than the melting temperature of the specific product generated with template. This variation is typical of primer-dimer formation, and it indicates that lower primer concentration may provide optimal results.

![Figure 8](image-url)  
**Figure 8** Amplification plot (linear view) demonstrating suspected nonspecific amplification in NTC wells
**Figure 9** Dissociation curve analysis confirming that product in NTC wells has a melting temperature different from the specific product.

1. Target amplification
2. NTC (nonspecific amplification)
WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the “Documentation and Support” section in this document.
Chemical safety

**WARNING! GENERAL CHEMICAL HANDLING.** To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the “Documentation and Support” section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

**IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.
Biological hazard safety

**WARNING! BIOHAZARD.** Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Safety equipment also may include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

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- Product documentation, including:
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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References


