

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

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Revision history: MAN0013511 F.0 (English)

Revision	Date	Description
F.0	11 December 2023	<ul style="list-style-type: none">The storage conditions were updated to include instructions to protect the master mix from light.The instructions to set up the PCR reactions were corrected to indicate that the reaction plate can be stored at room temperature for up to 72 hours. The plate should be protected from light.
E.0	19 January 2023	<ul style="list-style-type: none">The SuperScript™ VILO™ cDNA Synthesis Kit was removed from the list of reverse transcription kits.Information was added about the compatibility of the SuperScript™ IV VILO™ Master Mix with the PowerUp™ SYBR™ Green Master Mix (“Input DNA template requirements” on page 9).
D.0	30 September 2022	<ul style="list-style-type: none">The product description was updated to specify research applications.The list of compatible real-time PCR instruments was updated (“Required materials” on page 6).Information was added to indicate that cDNA generated with the SuperScript™ IV VILO™ Master Mix is not compatible with the PowerUp™ SYBR™ Green Master Mix (“Input DNA template requirements” on page 9).Reanalyzing the data with ROX™ dye set as the passive reference dye was added as an action in the troubleshooting section if the following items are observed:<ul style="list-style-type: none">The R_n vs. cycle plot is not displayed.The ΔR_n or R_n values are extremely high.
C.0	4 February 2016	<ul style="list-style-type: none">Reorganized content according to current templates.Clarified instructions for using genomic DNA in PCR.Updated recommended action for primer-dimer formation and residual polymerase activity.
B.0	1 August 2015	Baseline for revision history

The information in this guide is subject to change without notice.

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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 analytical specificity and sensitivity for your research applications. It is supplied in a convenient 2X concentration pre-mix to perform real-time PCR using SYBR™ Green Dye. The master mix contains the following components:

- 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.

For more information about each component, see “Master mix components” on page 19.

Contents and storage

Table 1 PowerUp™ SYBR™ Green Master Mix

Cat. No. ^[1]	Amount	Storage
A25741	1 mL	2–8°C Protect from light
A25779 (2 × A25741)	2 × 1 mL	
A25780 (5 × A25741)	5 × 1 mL	
A25918 (10 × A25741)	10 × 1 mL	
A25742	5 mL	
A25776 (2 × A25742)	2 × 5 mL	
A25777 (5 × A25742)	5 × 5 mL	

Table 1 PowerUp SYBR Green Master Mix (continued)

Cat. No. ^[1]	Amount	Storage
A25778 (10 × A25742)	10 × 5 mL	2–8°C
A25743	50 mL	Protect from light

^[1] Catalog numbers that appear as links open the web pages for those products.

Required materials

Unless otherwise indicated, all materials are available through thermofisher.com. "MLS" indicates that the material is available from fisherscientific.com or another major laboratory supplier.

Catalog numbers that appear as links open the web pages for those products.

Item	Source
One of the following Applied Biosystems™ instruments:	
<ul style="list-style-type: none"> QuantStudio™ 3 or 5 Real-Time PCR System QuantStudio™ 6 or 7 Flex Real-Time PCR System QuantStudio™ 6 Pro or 7 Pro Real-Time PCR System QuantStudio™ 12K Flex Real-Time PCR System StepOnePlus™ Real-Time PCR System StepOne™ Real-Time PCR System 7500 Fast Real-Time PCR System 7500 Real-Time PCR Instrument 7900HT Fast Real-Time PCR Instrument 7900HT Real-Time PCR Instrument ViiA™ 7 Real-Time PCR System <p>Or use a compatible real-time PCR instrument from another supplier.</p>	Contact your local sales office.
Equipment	
Centrifuge with adapter for 96- or 384-well plates	MLS
Laboratory mixer (Vortex or equivalent)	MLS
Microcentrifuge	MLS
Pipettors	MLS

Item	Source
Plastics and other consumables	
Plates and seals for your instrument	thermofisher.com/plastics
Disposable gloves	MLS
Pipette tips with filters	MLS
Polypropylene tubes	MLS
Reagents and kits	
One of the following reverse transcription kits, if performing gene expression analysis:	
<ul style="list-style-type: none"> High-Capacity cDNA Reverse Transcription Kit High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor High-Capacity RNA-to-cDNA™ Kit 	<ul style="list-style-type: none"> 4368813, 4368814 4374966, 4374967 4387406
Nuclease-Free Water (not DEPC-Treated)	4387936 , AM9930 , AM9932 , AM9937 , AM9938 , AM9939
TE, pH 8.0, RNase-free	AM9849 , AM9858

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



Set up the plate document (page 9)



Set up the PCR reactions (page 10)



Set up and run the real-time PCR instrument (page 11)



Analyze results (page 12)



Guidelines

Input DNA template requirements

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

See “RNA guidelines” on page 24 and “Template storage” on page 25 for additional information.

IMPORTANT! In some cases, cDNA generated with the SuperScript™ IV VILO™ Master Mix is not compatible with the PowerUp™ SYBR™ Green Master Mix. It can result in a high background signal. To ensure consistent results across different samples and assays, we recommend a different reverse transcription kit.

For kits recommended for reverse transcription of RNA to cDNA, see “Required materials” on page 6. If you use SuperScript™ IV VILO™ Master Mix and your results do not show a high background signal with your assay and sample, you can continue to use the SuperScript™ IV VILO™ Master Mix and the PowerUp™ SYBR™ Green Master Mix.

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.

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.

Component	Volume (10 µL/well)	Volume (20 µL/well)
PowerUp™ SYBR™ Green Master Mix (2X)	5 µL	10 µL
Forward and reverse primers ^[1]	Variable	Variable
DNA template + Nuclease-Free Water ^[2]	Variable	Variable
Total	10 µL	20 µL

^[1] For optimal performance in Fast and Standard modes, use 300–800 nM for each primer.

^[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 72 hours after completing the setup, when stored at room temperature. Protect the reaction plate from light if the PCR is not started immediately after the reactions are set up.

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 and the 7500 Real-Time PCR Instrument.

Table 2 Fast cycling mode (primer $T_m \geq 60^\circ\text{C}$)

Step	Temperature	Time	Cycles
UDG activation	50°C	2 minutes	1
Activation (Dual-Lock™ DNA polymerase)	95°C	2 minutes	1
Denature	95°C	1 second ^[1] or 3 seconds ^[2]	40
Anneal/extend	60°C	30 seconds	

^[1] When using a QuantStudio™ Real-Time PCR System or a ViiA™ 7 Real-Time PCR System.

^[2] When using a 7500 Fast Real-Time PCR System, 7900HT Fast Real-Time PCR Instrument, StepOnePlus™ Real-Time PCR System, or StepOne™ Real-Time PCR System.

Table 3 Standard cycling mode (primer $T_m \geq 60^\circ\text{C}$)

Step	Temperature	Time	Cycles
UDG activation	50°C	2 minutes	1
Activation (Dual-Lock™ DNA polymerase)	95°C	2 minutes	1
Denature	95°C	15 seconds	40
Anneal/extend	60°C	1 minute	

Table 4 Standard cycling mode (primer $T_m < 60^\circ\text{C}$)

Step	Temperature	Time	Cycles
UDG activation	50°C	2 minutes	1
Activation (Dual-Lock™ DNA polymerase)	95°C	2 minutes	1
Denature	95°C	15 seconds	40
Anneal	55–60°C ^[1]	15 seconds	
Extend	72°C	1 minute	

^[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 5 Dissociation curve conditions (melt curve stage)

Step	Ramp rate	Temperature	Time
1	1.6°C/second	95°C	15 seconds
2	1.6°C/second	60°C	1 minute
3 ^[1]	0.15°C/second	95°C	15 seconds

^[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 22 for more information.

2. Calculate the baseline and threshold cycles (C_T) for the amplification curves using the instrument software.

See “Baseline and threshold values” on page 22, “Troubleshooting baseline settings” on page 15, and “Troubleshooting threshold settings” on page 17 for more information.

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 23 for more information.

4. Perform relative or absolute quantitation.

Visit [thermofisher.com/qpcducation](https://www.thermofisher.com/qpcducation) 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_t method.
- Absolute quantitation—The C_t of the unknown samples is compared against a standard curve with known copy numbers.



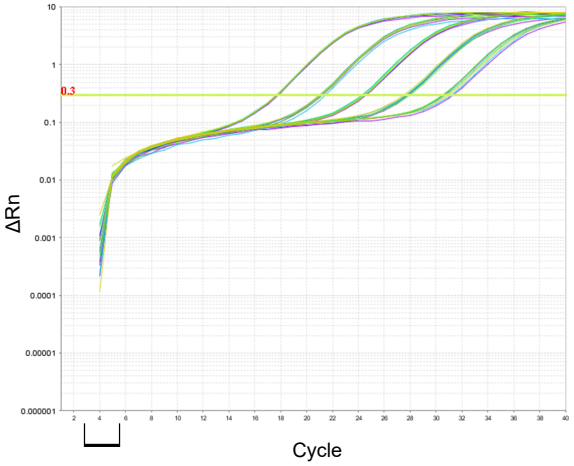
Troubleshooting

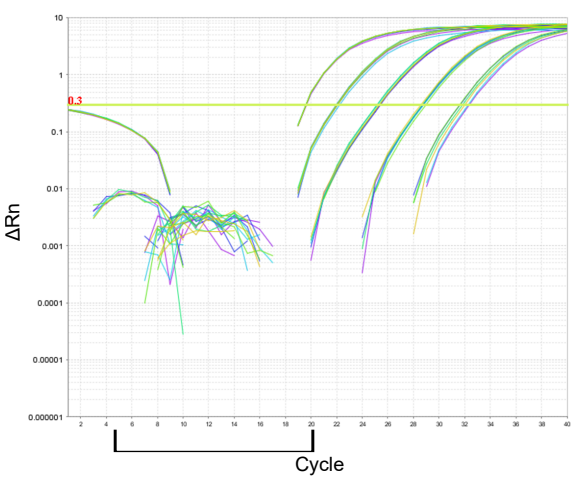
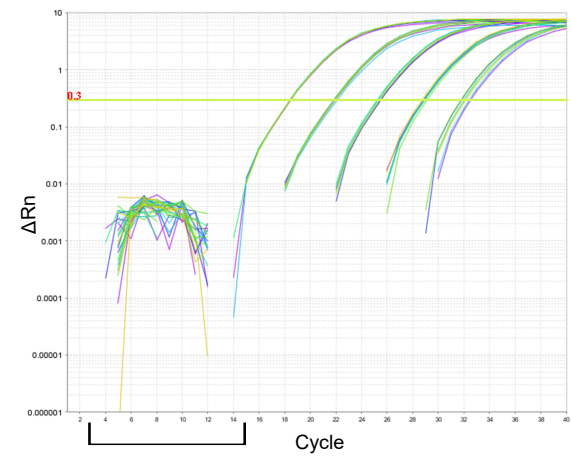
General troubleshooting

Observation	Possible cause	Recommended action
The C_t values are high, there is poor precision, or the PCR reactions failed	There is insufficient DNA template.	Use up to 100 ng of DNA template per 20- μ L reaction. Typically, 1–10 ng cDNA or 10–100 ng genomic DNA per 20- μ L reaction are sufficient.
	The quality of the DNA template is poor.	Quantify the amount of DNA template (see “Template quantitation using O.D. 260” on page 25) 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.
	The sample has degraded.	Prepare fresh cDNA or gDNA, then repeat the experiment.
	Incorrect volumes of components were pipetted for the PCR reactions.	Prepare the PCR reactions as described in “Set up the PCR reactions” on page 10.
	Too few PCR cycles were used.	Increase the number of PCR cycles to the default setting of 40 (see “Set up and run the real-time PCR instrument” on page 11) .
	There was primer-dimer formation and residual polymerase activity.	<ul style="list-style-type: none"> Optimize the thermal cycling temperatures. Reduce the primer concentration. Redesign the primers.
Low ΔR_n or R_n values are obtained	The extension time was too short.	Use the recommended standard cycling thermal profile settings (see “Set up and run the real-time PCR instrument” on page 11).
	There was primer-dimer formation and residual polymerase activity.	Optimize the thermal cycling temperatures.
		Reduce the primer concentration.
		Redesign the primers.

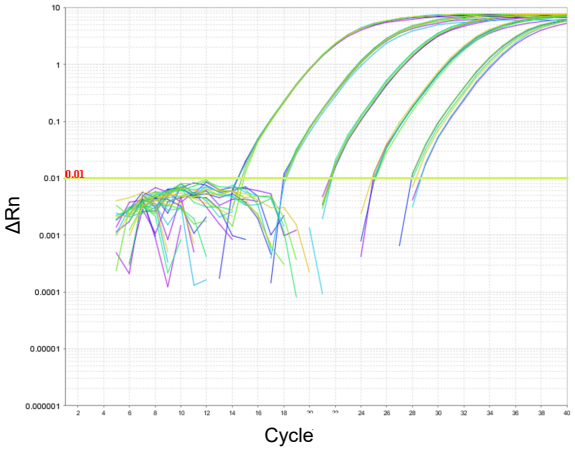
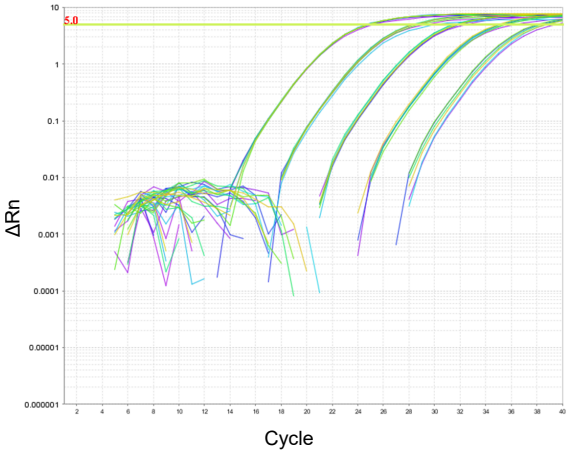
Observation	Possible cause	Recommended action
The R_n vs. cycle plot is not displayed	ROX™ dye was not selected as the passive reference when the plate document was set up.	Select ROX™ dye as the passive reference when setting up the plate document.
		Select ROX™ dye as the passive reference, then reanalyze the data. The run does not need to be repeated.
The ΔR_n or R_n values are extremely high	ROX™ dye was not selected as the passive reference when the plate document was set up.	Select ROX™ dye as the passive reference when setting up the plate document.
		Select ROX™ dye as the passive reference, then reanalyze the data. The run does not need to be repeated.
	There was evaporation from the reaction plate.	Ensure that the reaction plate is sealed completely, especially around the edges.
The R_n values obtained in early cycles are low	The C_t value is less than 15.	Adjust the upper baseline range to a value less than 15.
There is high variability across the reaction plate	ROX™ dye was not selected as the passive reference when the plate document was set up.	Select ROX™ dye as the passive reference when setting up the plate document.
	There was evaporation from the reaction plate.	Ensure that the reaction plate is sealed completely, especially around the edges.
There is high variability between replicates	The reaction plate was not mixed well.	Mix the reaction mix gently by inversion, then centrifuge briefly before aliquoting to the reaction plate.

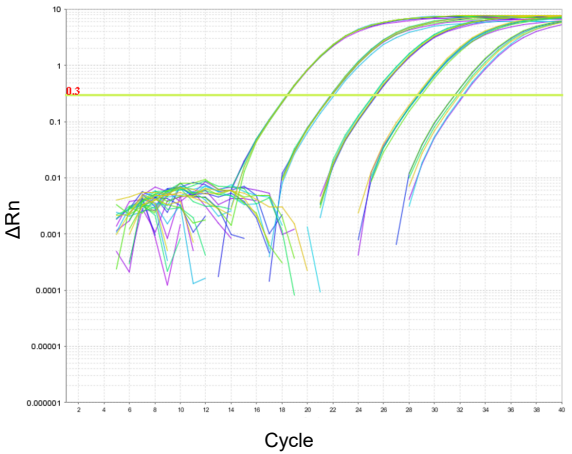
Troubleshooting baseline settings

Observation	Possible cause	Recommended action
<p>The baseline is set too low</p>  <p>Figure 1</p>	<p>The baseline is set too low (cycles 3–5).</p>	<p>Manually adjust the baseline to a higher range of cycles (see Figure 3 on page 16).</p>

Observation	Possible cause	Recommended action
<p>The baseline is set too high</p>  <p>Figure 2</p>	<p>The baseline is set too high (cycles 5–20).</p>	<p>Manually adjust the baseline to a lower range of cycles (see Figure 3 on page 16).</p>
<p>The baseline is set correctly</p>  <p>Figure 3</p>	<p>The baseline is set correctly (cycles 3–15).</p>	<p>No action is required.</p>

Troubleshooting threshold settings

Observation	Possible cause	Recommended action
<p>The threshold is set too low</p>  <p>Figure 4</p>	<p>The threshold is set too low.</p>	<p>Manually adjust the threshold to a higher ΔR_n (see Figure 6 on page 18).</p>
<p>The threshold is set too high</p>  <p>Figure 5</p>	<p>The threshold is set too high.</p>	<p>Manually adjust the threshold to a lower ΔR_n (see Figure 6 on page 18).</p>

Observation	Possible cause	Recommended action
<p>The threshold is set correctly</p>  <p>Figure 6</p>	<p>The threshold is set correctly.</p>	<p>No action is required.</p>



Background information

Master mix components

Table 6 PowerUp™ SYBR™ Green Master Mix component function

Component	Function
Dual-Lock™ <i>Taq</i> DNA polymerase	<ul style="list-style-type: none">• Utilizes a combination of two hot start mechanisms to control activity.• Provides tight control over <i>Taq</i> 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)	<ul style="list-style-type: none">• A 26 kDa recombinant enzyme derived from the thermolabile UDG gene isolated from marine bacteria, and expressed in <i>E. coli</i>.• Prevents reamplification of carryover PCR products by removing any uracil incorporated into single- or double-stranded amplicons.• 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	<ul style="list-style-type: none">• Enables UDG activity and maintains optimal PCR results.
SYBR™ Green	<ul style="list-style-type: none">• Detects PCR products by fluorescing upon binding to double-stranded DNA formed during PCR (see “SYBR™ Green dye chemistry overview” on page 20).
ROX™ passive reference	<ul style="list-style-type: none">• 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.

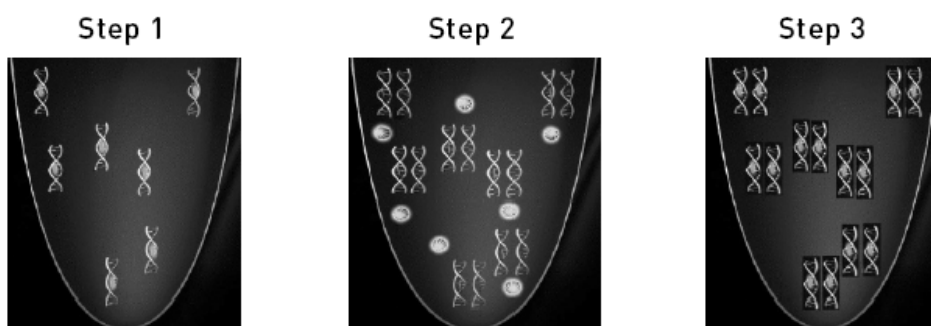


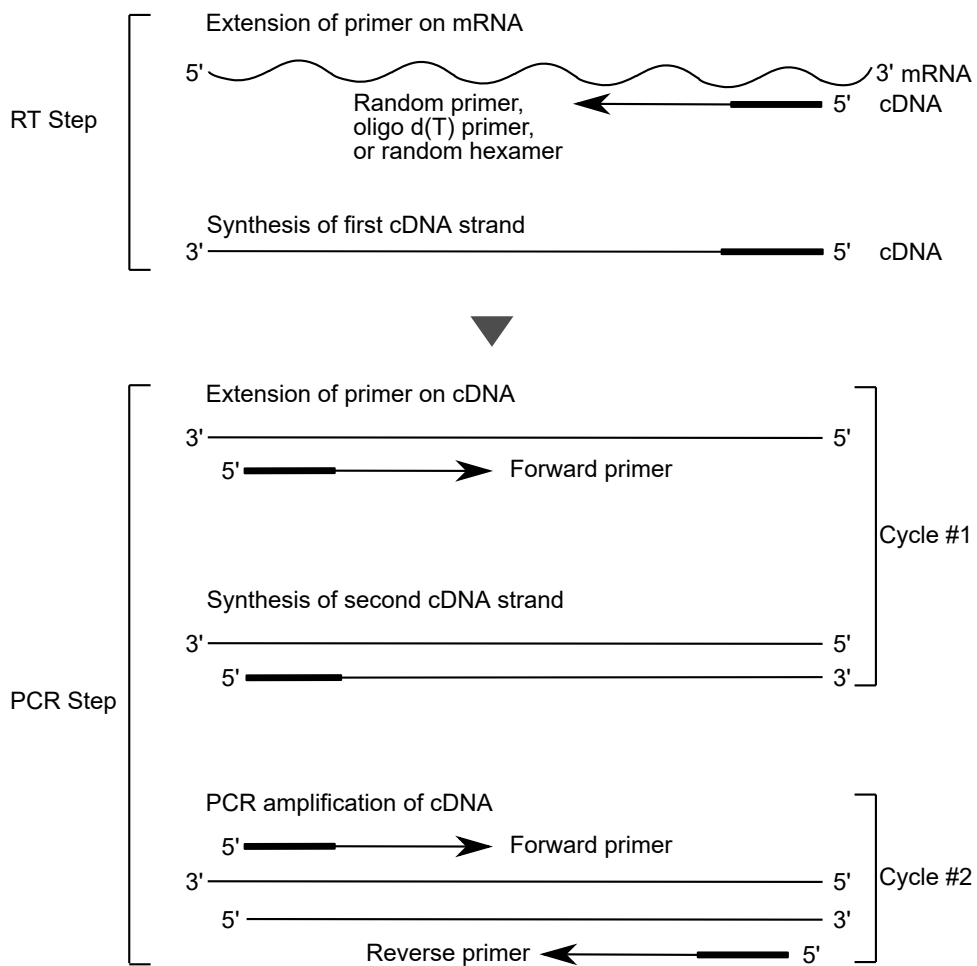
Figure 7 Representation of how SYBR™ Green dye acts on double-stranded DNA during one extension phase of PCR

Two-step RT-PCR

For more information, go to [thermofisher.com/qpcducation](https://www.thermofisher.com/qpcducation).

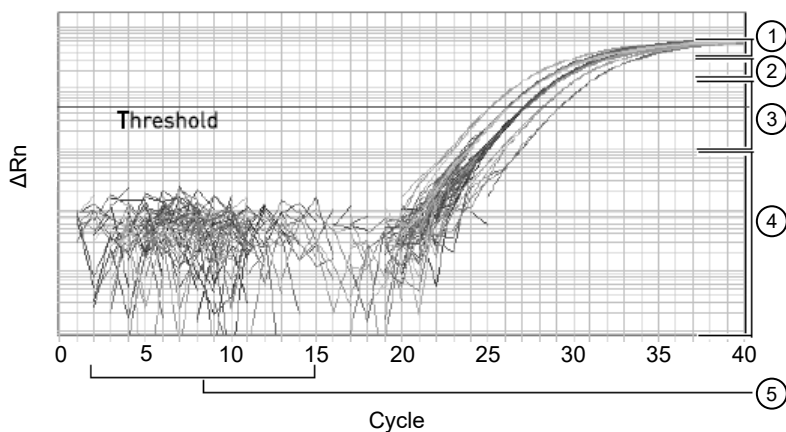
When performing a two-step RT-PCR reaction, total RNA or mRNA must first be reverse transcribed into cDNA.

1. In the reverse transcription (RT) step, cDNA is reverse transcribed from total RNA samples using random primers from the reverse transcription kit.
2. In the PCR step, PCR products are synthesized from cDNA samples using the master mix.



A typical amplification plot

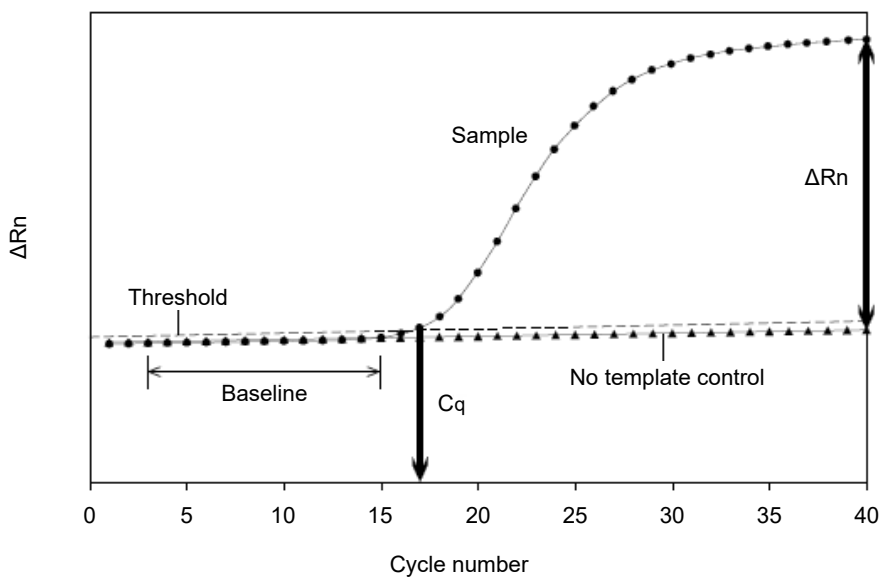
A typical amplification plot is shown below.



- ① Plateau phase
- ② Linear phase
- ③ Exponential phase (geometric phase)
- ④ Background
- ⑤ Baseline

Baseline and threshold values

- **Baseline**—The initial cycles of PCR in which there is little change in fluorescence signal.
- **C_q**—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 the software on your instrument. Alternatively, baseline and threshold can be set manually.

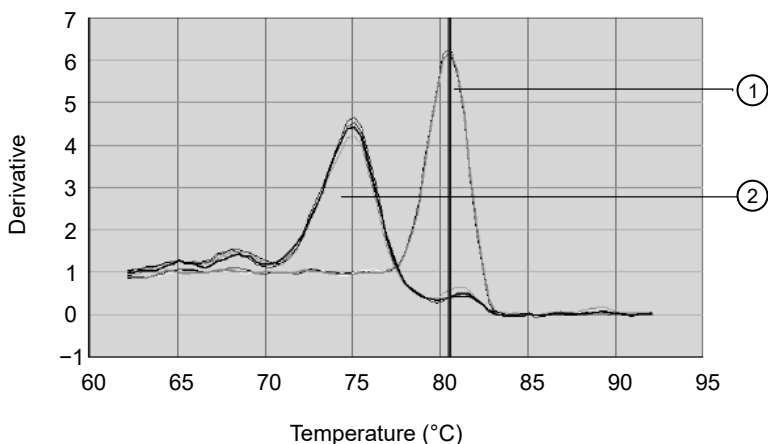
For examples of amplification plots where the baseline values and the threshold values are set too high or too low, see “Troubleshooting baseline settings” on page 15 and “Troubleshooting threshold settings” on page 17.

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



Template quality and quantity

Go to [thermofisher.com/qpcducation](https://www.thermofisher.com/qpcducation) 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 state otherwise. A_{260} values can be converted into $\mu\text{g}/\mu\text{L}$ using Beer's Law:

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

The following formulas are derived from Beer's Law:

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

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, target sequences, and optimizing primer concentration

Primer design guidelines

Primers should be designed using Primer Express™ Software or similar software. See *Primer Express™ Software Version 3.0 Getting Started Guide* (Pub. No. 4362460).

- Keep the GC content in the 30–70% range.
- 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.

Template	Design guideline
DNA	Design the primers as described above.
Plasmid DNA	
Genomic DNA	
cDNA	Design the primers as described above and see “Selecting an amplicon site for cDNA” on page 27.
RNA	Design the primers as described above.

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, 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 26.

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 \times 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 \times cuvette pathlength \times concentration / 100
- Rearrange to solve for concentration:
 - Concentration = 100 [absorbance at 260 nm / (sum of extinction coefficient contribution \times cuvette pathlength)]

Example calculation of primer concentration

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

Chromophore	Extinction coefficient	Number of specific chromophores in example sequence	Extinction coefficient contribution
A	15,200	1	15,200
C	7050	6	42,300
G	12,010	5	60,050
T	8400	6	50,400
Total	—	—	167,950

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

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:

Reverse primer (nM)	Forward primer (nM)		
	300	500	800
300	300 / 300	500 / 300	800 / 300
500	300 / 500	500 / 500	800 / 500
800	300 / 800	500 / 800	800 / 800

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 11.
5. Run the plate.
6. Compile the results for ΔR_n and C_t , then select the minimum forward and reverse primer concentrations that yield the maximum ΔR_n values and low C_t values.

Confirm the absence of nonspecific amplification

Melt curves help you select the optimal primer concentrations for your quantification assays with SYBR™ Green dye.

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

Note: In Figure 8 on page 29, the strong amplification of the NTC wells indicates that significant nonspecific amplification is occurring.

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

Note: In the example shown in Figure 9 on page 30, 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.

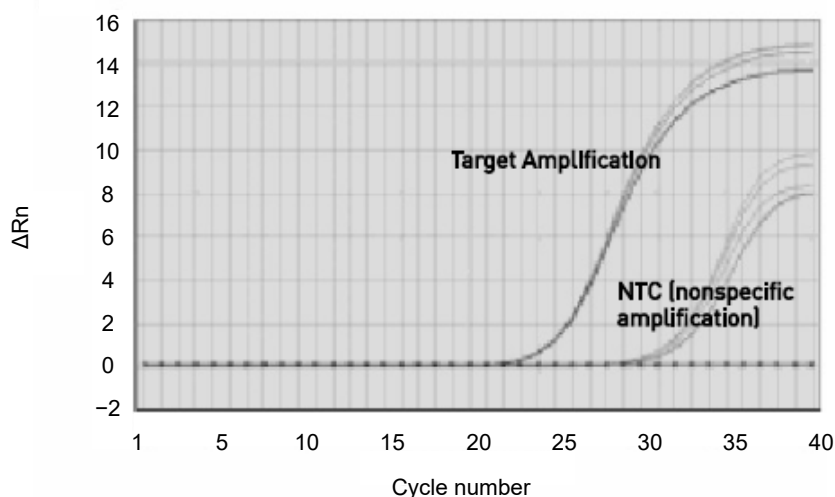


Figure 8 Amplification plot (linear view) demonstrating suspected nonspecific amplification in NTC wells

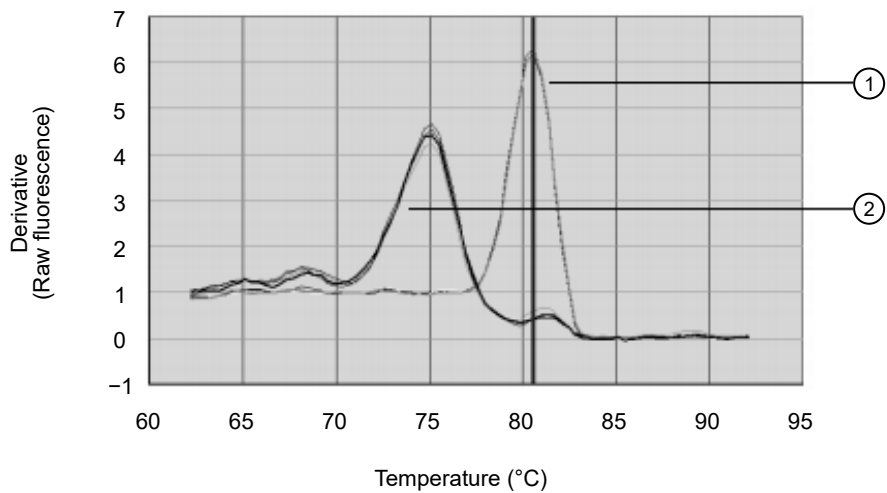


Figure 9 Melt curve analysis confirming that product in NTC wells has a melting temperature different from the specific product

- ① Target amplification
- ② NTC (nonspecific amplification)



Safety



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, and so on). To obtain SDSs, visit [thermofisher.com/support](https://www.thermofisher.com/support).

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. 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 sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer 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 needed) 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.



AVERTISSEMENT ! PRÉCAUTIONS GÉNÉRALES EN CAS DE MANIPULATION DE PRODUITS CHIMIQUES. Pour minimiser les risques, veiller à ce que le personnel du laboratoire lise attentivement et mette en œuvre les consignes de sécurité générales relatives à l'utilisation et au stockage des produits chimiques et à la gestion des déchets qui en découlent, décrites ci-dessous. Consulter également la FDS appropriée pour connaître les précautions et instructions particulières à respecter :

- Lire et comprendre les fiches de données de sécurité (FDS) fournies par le fabricant avant de stocker, de manipuler ou d'utiliser les matériaux dangereux ou les produits chimiques. Pour obtenir les FDS, se reporter à la section « Documentation et support » du présent document.
- Limiter les contacts avec les produits chimiques. Porter des équipements de protection appropriés lors de la manipulation des produits chimiques (par exemple : lunettes de sûreté, gants ou vêtements de protection).
- Limiter l'inhalation des produits chimiques. Ne pas laisser les récipients de produits chimiques ouverts. Ils ne doivent être utilisés qu'avec une ventilation adéquate (par exemple, sorbonne).
- Vérifier régulièrement l'absence de fuite ou d'écoulement des produits chimiques. En cas de fuite ou d'écoulement d'un produit, respecter les directives de nettoyage du fabricant recommandées dans la FDS.
- Manipuler les déchets chimiques dans une sorbonne.

- Veiller à utiliser des récipients à déchets primaire et secondaire. (Le récipient primaire contient les déchets immédiats, le récipient secondaire contient les fuites et les écoulements du récipient primaire. Les deux récipients doivent être compatibles avec les matériaux mis au rebut et conformes aux exigences locales, nationales et communautaires en matière de confinement des récipients.)
- Une fois le récipient à déchets vidé, il doit être refermé hermétiquement avec le couvercle fourni.
- Caractériser (par une analyse si nécessaire) les déchets générés par les applications, les réactifs et les substrats particuliers utilisés dans le laboratoire.
- Vérifier que les déchets sont convenablement stockés, transférés, transportés et éliminés en respectant toutes les réglementations locales, nationales et/ou communautaires en vigueur.
- **IMPORTANT !** Les matériaux représentant un danger biologique ou radioactif exigent parfois une manipulation spéciale, et des limitations peuvent s'appliquer à leur élimination.



WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.



WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety



WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.



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. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also 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.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
www.who.int/publications/i/item/9789240011311



Documentation and support

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 - Safety Data Sheets (SDSs; also known as MSDSs)

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

Limited product warranty

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