thermo scientific

Thermo Scientific™ SYBR™ Green qPCR Master Mix

USER GUIDE

Catalog Numbers A66732S, A66732

Publication Number MAN1000089

Revision A00

For Research Use Only. Not for use in diagnostic procedures.

ThermoFisher SCIENTIFIC

Thermo Fisher Scientific Baltics UAB | V.A. Graiciuno 8, LT-02241 | Vilnius, Lithuania
For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

Revision history: MAN1000089 A00 (English)

Revision	Date	Description
A00	11 April 2024	New document for the Thermo Scientific™ SYBR™ Green qPCR Master Mix.

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

Contents

1 Product information

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

Product description

The Thermo Scientific™ SYBR™ Green qPCR Master Mix is a 2X, ready-to-use master mix. It is designed for dye-based, quantitative amplification of DNA targets in cDNA and gDNA templates by real-time PCR. This master mix is formulated to provide robust performance with a variety of challenging templates and targets.

Direct detection of PCR product is monitored by measuring the increase in fluorescence of SYBR™ Green dye. The SYBR™ Green dye binds to double-stranded DNA formed during real-time PCR. For more information, see "Overview of the chemistry" on page 20.

The master mix provides flexibility for varying primer melting temperatures (Tm). The Tm can be between 55°C and 65°C. For recommended primer concentrations for different Tm, see "Guidelines" on page 9.

The master mix contains the following components:

- SYBR™ Green dye
- DNA polymerase, with an antibody-mediated hot start
- Heat-labile Uracil-DNA Glycosylase (UDG)
- ROX™ dye (passive reference dye)
- dNTP blend containing dUTP/dTTP
- Optimized buffer components

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

The user provides primers, template, and water.

Contents and storage

Table 1 Thermo Scientific™ SYBR™ Green qPCR Master Mix

Cat. No.	Amount	Number of 20–µL reactions	Storage
A66732S	1 mL	100	–30°C to –10°C
A66732	5 mL	500	

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.

IMPORTANT! Do not use plastics made of polyethylene terephthalate co-polyester, glycol modified (PTEG) for storage of the master mix or the reaction mixes. SYBR™ Green dye is not compatible with this type of plastic material. Polypropylene, high density polyethylene (HDPE), and polystyrene are recommended for storage.

Table 2 Required materials

Item	Source
Real-time qPCR instrument, such as	
• Applied Biosystems™ QuantStudio™ 7 Pro Real-Time PCR System • Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System • Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System • Applied Biosystems™ QuantStudio™ 12K Flex Real–Time PCR System • Applied Biosystems™ 7500 Fast Real-Time PCR System • Applied Biosystems™ 7500 Real-Time PCR System • Bio-Rad™ CFX Opus Real-Time PCR System • Roche™ LightCycler™ 480 System Or use a compatible real-time PCR instrument from another supplier.	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**
Plastics and other consumables	
PCR plates and seals for your instrument	**thermofisher.com/plastics**
Disposable gloves	**MLS**
Pipette tips with filters	**MLS**
Polypropylene tubes	**MLS**

Item	Source
Reagents and kits	
If performing a gene expression analysis, a reverse transcription kit, such as	
Maxima First Strand cDNA Synthesis Kit	K1642
SuperScript™ IV VILO™ Master Mix	11756050
SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme	11766050
High-Capacity cDNA Reverse Transcription Kit	4368814
High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor	4374967
High-Capacity RNA-to-cDNA™ Kit	4387406
iScript™ cDNA Synthesis Kit	Bio-Rad™, 1708890
LunaScript™ RT SuperMix Kit	New England BioLabs™, E3010S
QuantiTect™ Reverse Transcription Kit	QIAGEN™, 205311
PrimeScript™ RT Master Mix	Takara™, RR036A
Other reagents	
Nuclease-Free Water (not DEPC-Treated)	AM9930
TE, pH 8.0, RNase-free	AM9858

Workflow

Thermo Scientific™ SYBR™ Green qPCR Master Mix

Start with cDNA or gDNA

See “Requirements for input DNA” on page 9.

Set up the plate document or plate file, then prepare the reagents

See “Before you begin” on page 9.

Combine the components for PCR

See “Prepare the PCR reactions” on page 10.

Set up and run the real-time PCR instrument

See “Set up and run the real-time PCR instrument” on page 11.

Analyze the results

See “Analyze the results” on page 12.

2 Methods

Guidelines

Requirements for input DNA

Use 1–10 ng of cDNA or 10–100 ng of gDNA per reaction. See the recommendations in your reverse transcription kit for more information.

For more information, see “RNA guidelines” on page 24 and “Template storage” on page 25.

Guidelines for 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 of less than 10 µL are not recommended.
- The recommended final primer concentration for primers with a Tm of 55°C is 400 nM.

Guidelines for no-template control reactions

No-template control (NTC) reactions can be used to identify PCR contamination. NTC reactions contain all of the reaction components except for the sample.

Before you begin

Set up the plate document or plate file

Configure the plate document or plate file.

See the appropriate instrument user guide for detailed instructions.

Prepare the reagents

- Thaw the master mix.
- When the master mix is thawed, swirl it to mix thoroughly.
- Thaw the DNA samples and primers on ice, vortex to mix, then centrifuge briefly.

Prepare the PCR reactions

1. Combine the master mix, the primers, DNA template, and nuclease-free water, according to the one of the following tables.

Table 3 20–µL reaction

Component	Stock concentration	Final concentration	Volume for 1 reaction (20–µL reaction)	Volume for 4 reactions with 10% overage (20–µL reaction)[1]
Thermo Scientific™ SYBR™ Green qPCR Master Mix	2X	1X	10 µL	44.0 µL
Forward and reverse primers[2]	8,000 nM	400 nM	1 µL	4.4 µL
DNA[3]	5 ng/µL	0.5 ng/µL	2 µL[4]	8.8 µL[5]
Nuclease-free water	—	—	7 µL	30.8 µL
Total PCR volume	**—**	**—**	**20 µL**	**88 µL**

[1] 10% overage is recommended for pipetting variations.
[2] The final primer concentration can vary from 300–800 nM. A final concentration of 400 nM is recommended for primers with a Tm of 55°C.
[3] Use 1–10 ng of cDNA or 10–100 ng of gDNA. See the recommendations in your reverse transcription kit for more information.
[4] Does not exceed 9.0 µL.
[5] Does not exceed 39.6 µL.

Table 4 10–µL reaction

Component	Stock concentration	Final concentration	Volume for 1 reaction (10–µL reaction)	Volume for 4 reactions with 10% overage (10–µL reaction)[1]
Thermo Scientific™ SYBR™ Green qPCR Master Mix	2X	1X	5 µL	22.0 µL
Forward and reverse primers[2]	8,000 nM	400 nM	0.5 µL	2.2 µL
DNA[3]	5 ng/µL	0.5 ng/µL	1 µL[4]	4.4 µL[5]
Nuclease-free water	—	—	3.5 µL	15.4 µL
Total PCR volume	**—**	**—**	**10 µL**	**44 µL**

[1] 10% overage is recommended for pipetting variations.
[2] The final primer concentration can vary from 300–800 nM. A final concentration of 400 nM is recommended for primers with a Tm of 55°C.
[3] Use 1–10 ng of cDNA or 10–100 ng of gDNA. See the recommendations in your reverse transcription kit for more information.
[4] Does not exceed 4.5 µL.
[5] Does not exceed 19.8 µL.

2. Mix the components thoroughly, then centrifuge briefly to collect the contents at the bottom of the tube.

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

4. Seal the plate with an optical adhesive cover, then centrifuge briefly to collect the contents at the bottom of each well and eliminate any air bubbles.

PCR can be performed on the reaction plate up to 72 hours after completing the set-up, when stored at room temperature protected from light.

Note: Pre-PCR stability is influenced not only by the master mix, but also by the target being analyzed. For maximum confidence, confirm stability profiles of the specific targets.

Set up and run the real-time PCR instrument

1. Set up the thermal protocol, according to one of the following tables.

   Note: Standard cycling conditions are recommended for genomic DNA templates or long amplicons.

   Table 5 Fast cycling mode

Step	Temperature	Duration	Cycles
Enzyme activation	95°C	2 minutes	1
Denature	95°C	5 seconds	40
Anneal/extend	60°C	30 seconds	

   Table 6 Standard cycling mode

Step	Temperature	Duration	Cycles
Enzyme activation	95°C	2 minutes	1
Denature	95°C	15 seconds	40
Anneal/extend	60°C	60 seconds	

2. Set the instrument to perform a default dissociation step, according to one of the following tables.

   Note: Select the same mode used in step 1.

   Table 7 Fast cycling mode

Step	Ramp rate[1]	Temperature	Time
1	1.99°C/second	95°C	15 seconds
2	1.77°C/second	60°C	1 minute
3 (Dissociation)	0.075°C/second	95°C	15 seconds

[1] For the 7500 Real-Time PCR System, use the default ramp rate.

Table 8 Standard cycling mode

Step	Ramp rate[1]	Temperature	Time
1	1.6°C/second	95°C	15 seconds
2	1.6°C/second	60°C	1 minute
3 (Dissociation)	0.075°C/second	95°C	15 seconds

[1] For the 7500 Real-Time PCR System, use the default ramp rate.

Note: A dissociation step must be performed immediately after the real-time PCR run with Thermo Scientific™ SYBR™ Green qPCR Master Mix.

3. Set up the options.
   - Experiment type—Standard curve
   - Reagent—SYBR™ Green reagents
   - Reporter—SYBR™ Green
   - Quencher—None
   - Passive reference dye
     - For instruments compatible with ROX™ passive reference, select ROX™ dye.
     - For instruments not compatible with ROX™ passive reference, no selection is needed.
   - Ramp speed—Standard or fast
   - Melt curve ramp increment—Continuous
4. Set the reaction volume appropriate for the reaction plate.
5. Load the reaction plate into the real-time PCR instrument.
6. Start the run.

Analyze the results

1. View the amplification plots.

   For more information, see “A typical amplification plot” on page 22.
2. Determine the baseline and threshold cycles (C_q) for the amplification curves using the instrument software.

   For more information, see the following sections:
   - “Baseline and threshold values” on page 22
   - “Troubleshooting baseline settings” on page 16
   - “Troubleshooting threshold settings” on page 17
3. Check for nonspecific amplification using melt curves.

   It is important to check for nonspecific amplification because SYBR™ Green dye detects any double-stranded DNA.

For more information, see “Melt curves” on page 23.

4. Perform relative or absolute quantitation.

Option	Description
Relative quantitation	The target is compared to an internal standard using the comparative C_q method.
Absolute quantitation	The C_q of the unknown samples is compared against a standard curve with known copy numbers.

A

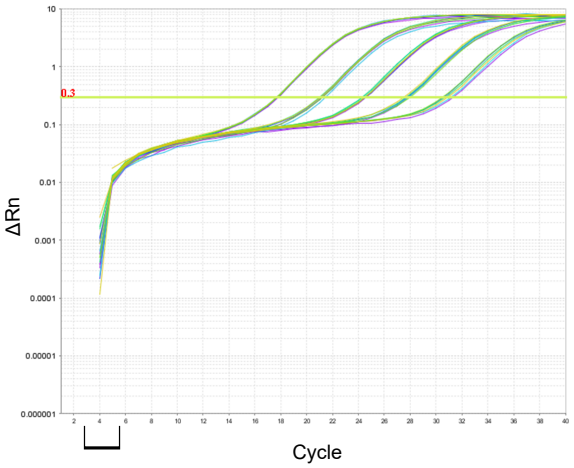
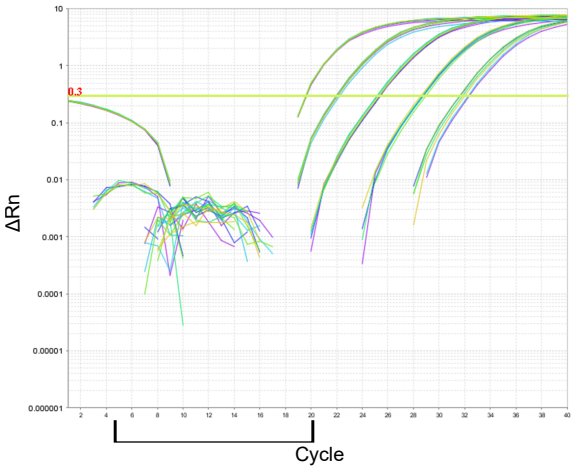
Troubleshooting

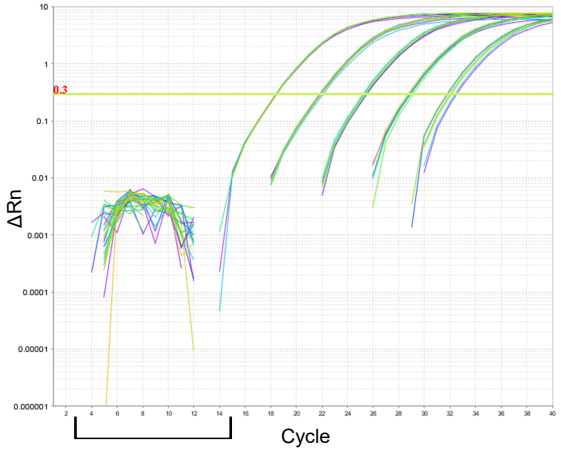
General troubleshooting

Observation	Possible cause	Recommended action
The C_q 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 reaction. Typically, 1–10 ng cDNA or 10–100 ng gDNA per reaction is sufficient.
	The quality of the DNA template is poor.	Quantify the amount of DNA template and ensure the recommended amount is used (see "Template quantitation using O.D. 260" on page 25).
		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 "Prepare 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.	• Optimize the thermal cycling temperatures. • Reduce the primer concentration. • Redesign the primer length, GC content, and/or Tm.
Low ΔR_n, R_n, or fluorescence signal 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 primer length, GC content, and/or Tm.

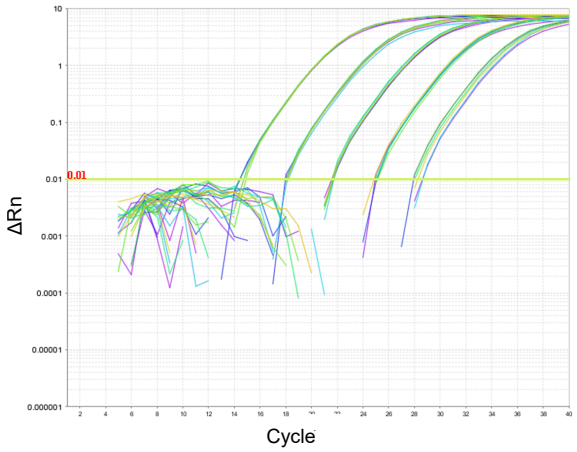
Observation	Possible cause	Recommended action
The R_n vs. cycle plot is not displayed (applies to only ROX™ dye-enabled instruments)	ROX™ dye was not selected as the passive reference when the plate document was set up.	Select ROX™ dye as the passive reference when setting up the plate document.
		Select 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_q 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. **Note:** This applies to real-time PCR instruments that are compatible with ROX™ passive reference dye.	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.
The **BADROX** flag is displayed (applies to only ROX™ dye-enabled instruments)	There were droplets on the sides of the wells of the reaction plate.	Set up and repeat the run. Centrifuge the plate to collect the contents at the bottom of the well.
	There was evaporation of the reagents.	Set up and repeat the run. Ensure that the plate is sealed properly.
	The incorrect concentration of passive reference dye was used.	Ensure that the correct concentration of the master mix is added to the reaction. See “Prepare the PCR reactions” on page 10.

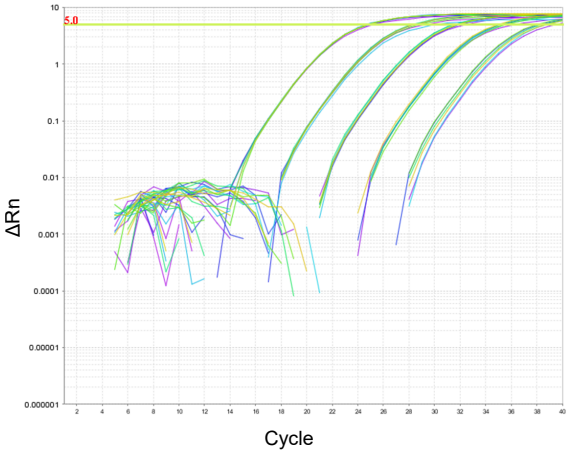
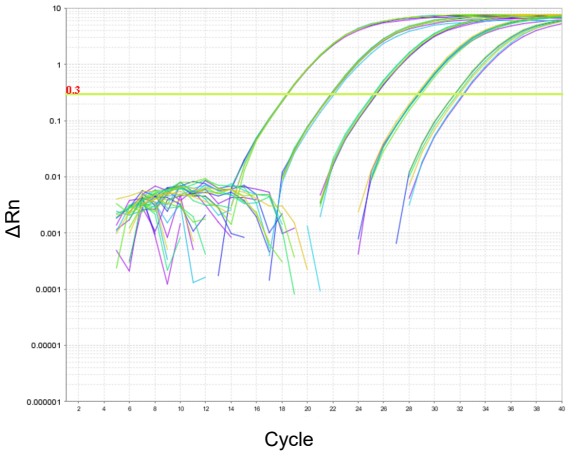
Troubleshooting baseline settings

Observation	Possible cause	Recommended action
The baseline is set too low  **Figure 1**	The baseline is set too low (cycles 3–5).	Manually adjust the baseline to a higher range of cycles (see Figure 3 on page 17).
The baseline is set too high  **Figure 2**	The baseline is set too high (cycles 5–20).	Manually adjust the baseline to a lower range of cycles (see Figure 3 on page 17).

Observation	Possible cause	Recommended action
The baseline is set correctly  **Figure 3**	The baseline is set correctly (cycles 3–15).	No action is required.

Troubleshooting threshold settings

Observation	Possible cause	Recommended action
The threshold is set too low  **Figure 4**	The threshold is set too low.	Manually adjust the threshold to a higher ΔR_n (see Figure 6 on page 18).

Observation	Possible cause	Recommended action
The threshold is set too high  **Figure 5**	The threshold is set too high.	Manually adjust the threshold to a lower ΔR_n (see Figure 6 on page 18).
The threshold is set correctly  **Figure 6**	The threshold is set correctly.	No action is required.

B

Background information

Master mix components

Table 9 Function of the components of the master mix

Component	Function
Antibody-mediated hot start polymerase	• Allows flexibility in reaction set-up. PCR reagents can be pre-mixed, then stored at room temperature and protected from light for up to 72 hours before cycling. • Allows activation 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. • 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. • Has no activity on RNA or dT-containing DNA.
dUTP/dTTP	• Enables UDG activity and maintains optimal PCR results.
SYBR™ Green dye	• Detects PCR products by fluorescing upon binding to double-stranded DNA formed during PCR (see “Overview of the chemistry” on page 20).
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 volume.

Overview of the chemistry

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

1. When the master mix is added to a sample, the SYBR™ Green dye immediately binds to all double-stranded DNA (dsDNA) present in the sample.
   The 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. The 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.
   The 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.

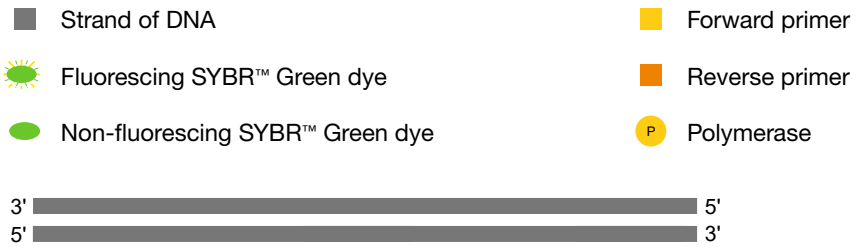


Figure 7 DNA template

Figure 8 SYBR™ Green dye binds to all double-stranded DNA

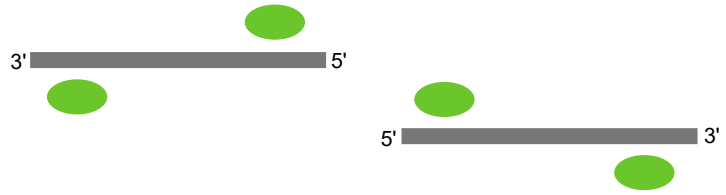


Figure 9 Denaturation

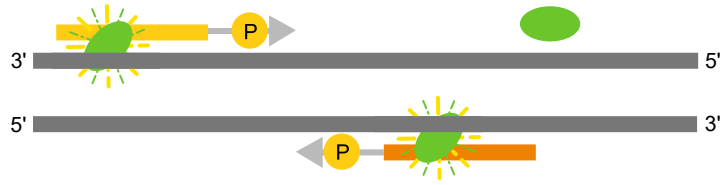


Figure 10 Polymerization

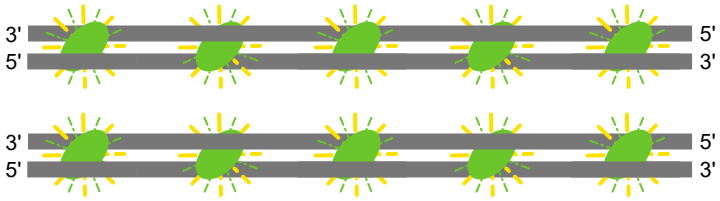
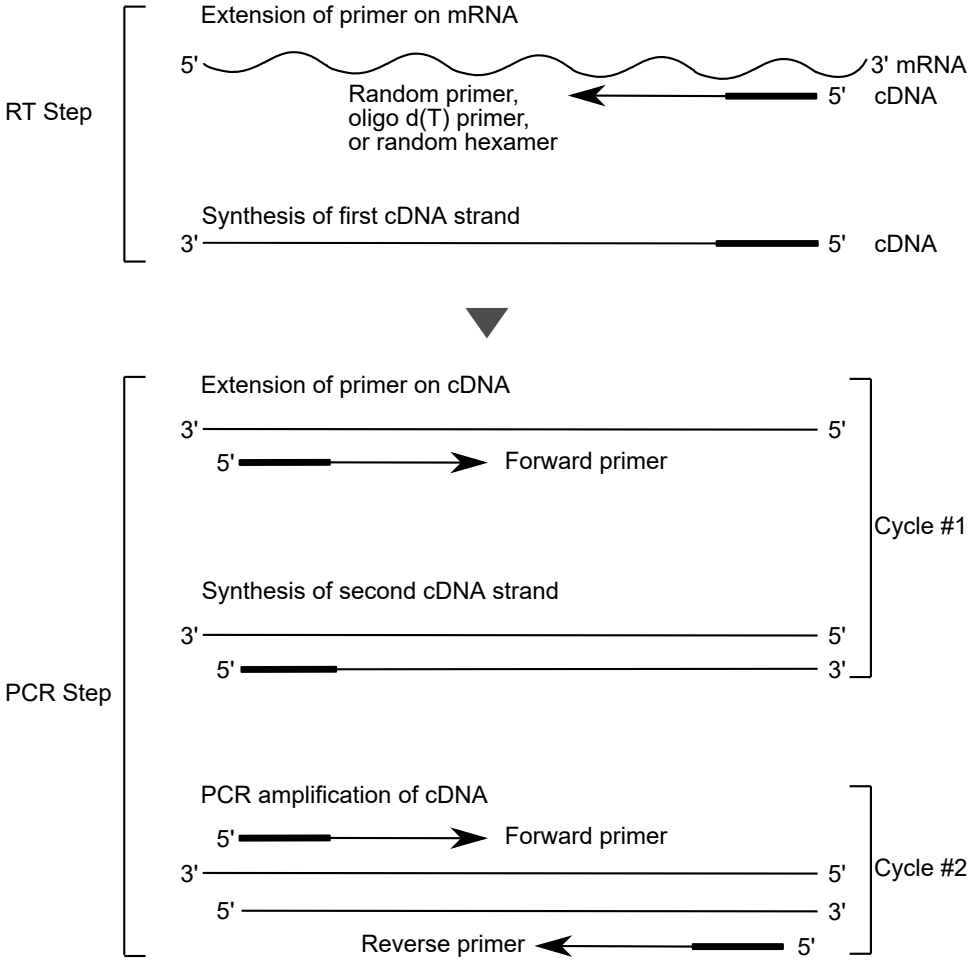


Figure 11 Completion

Two-step RT-PCR

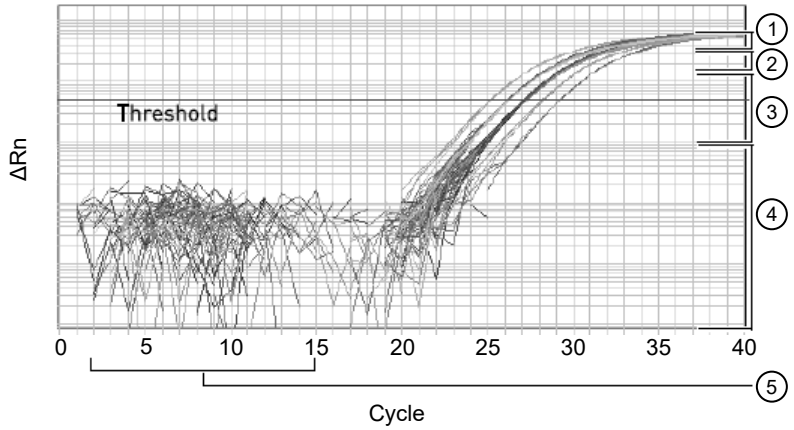
When performing a two-step RT-PCR reaction, total RNA or mRNA must first be reverse transcribed into cDNA.

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

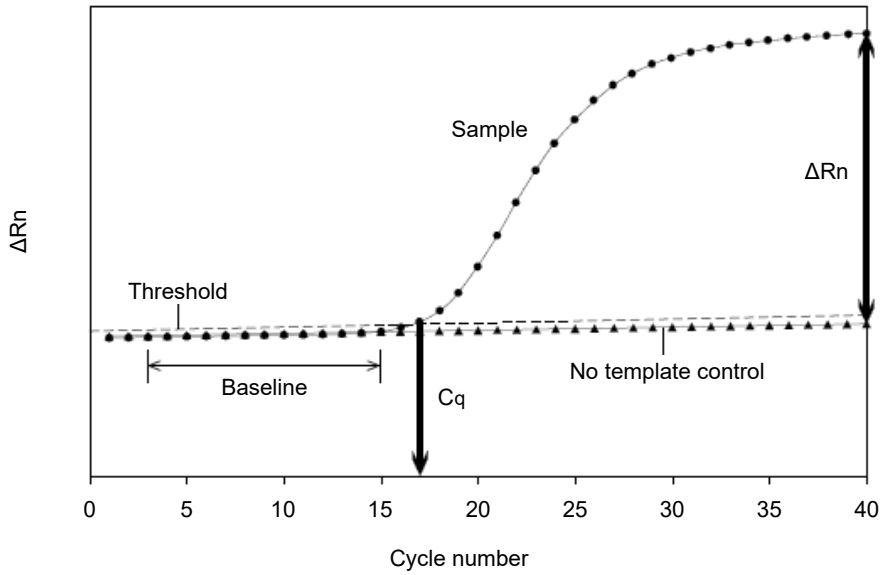
A typical amplification plot is shown below.



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.



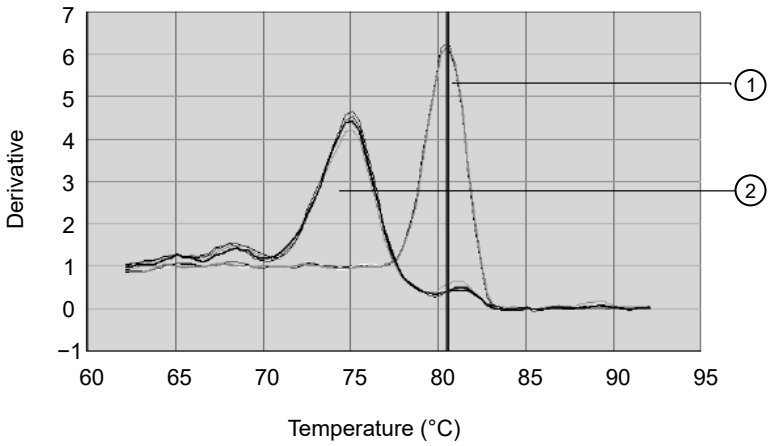
Automatic calculation of the baseline and threshold can be conducted using the software on your instrument. Alternatively, baseline and threshold can be set manually.

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 16 and “Troubleshooting threshold settings” on page 17.

Melt curves

A melt curve is a graph that displays dissociation data from the amplicons of quantitative PCR runs. Change in fluorescence, due to a dye 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.

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



This melt 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 Melt curve of a specific product
② Melt curve of a primer-dimer

C Template quality and quantity

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 Thermo Scientific™ SYBR™ Green qPCR Master Mix reaction. For recommended reverse transcription kits, see “Required materials” on page 6.

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 µg/µL using Beer's Law:

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

The following formulas are derived from Beer's Law:

- Concentration of single-stranded DNA = A_{260} × 33 µg/µL
- Concentration of double-stranded DNA = A_{260} × 50 µg/µL
- Concentration of single-stranded RNA = A_{260} × 40 µg/µL

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.

D 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.

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 helps to ensure 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_q 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_q 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:
     - Concentration = 100 [absorbance at 260 nm / (sum of extinction coefficient contribution × 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′-CGTACTCGTTCGTGCTGC-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	7,050	6	42,300
G	12,010	5	60,050
T	8,400	6	50,400
Total	—	—	167,950

- Measured absorbance at 260 nm = 0.13
- Sum of extinction coefficient = 167,950 $M^{-1}cm^{-1}$ 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 $M^{-1}cm^{-1}$ × 0.3 cm × C / 100
- C = 258 µM

Determine the optimal primer concentration for PCR

Calibrate your instrument for SYBR™ Green dye, if necessary. See the instrument user guide for calibration instructions. It is recommended to calibrate your instrument every six months.

1. Prepare a 96-well reaction plate.

   Use 10–100 ng of gDNA or 1–10 ng of cDNA template. The final concentration of the 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. Set up the thermal protocol (see "Set up and run the real-time PCR instrument" on page 11).
3. Load the plate into the real-time PCR instrument.
4. Start the run.
5. Compile the results for ΔR_n and C_q, then select the minimum forward and reverse primer concentrations that yield the maximum ΔR_n values and low C_q 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 12 on page 30, 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 13 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.

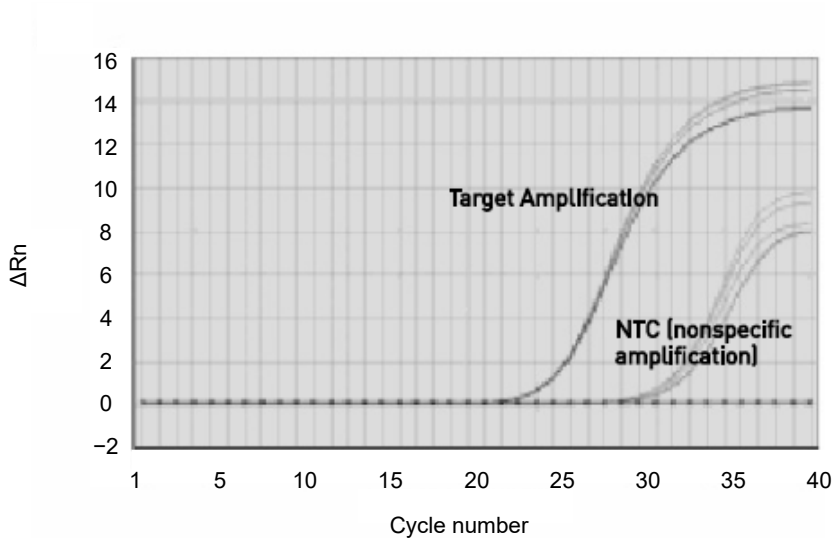


Figure 12 Amplification plot (linear view) demonstrating suspected nonspecific amplification in NTC wells

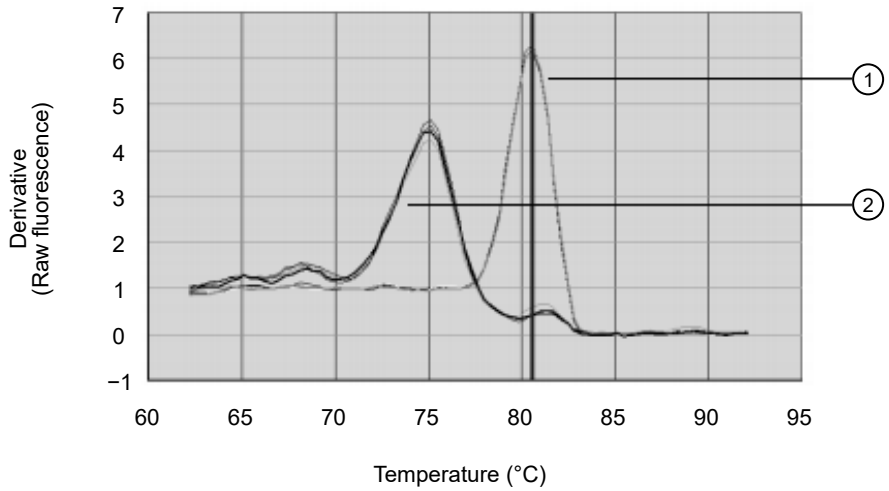


Figure 13 Melt curve analysis confirming that product in NTC wells has a melting temperature different from the specific product

① Target amplification
② NTC (nonspecific amplification)

E

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**.

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.

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. 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

F Documentation and support

Related documentation

Document	Pub. No.
Thermo Scientific™ SYBR™ Green qPCR Master Mix Quick Reference	MAN1000090

Customer and technical support

Visit **thermofisher.com/support** for the latest service and support information.

- Worldwide contact telephone numbers
- Product support information
  - Product FAQs
  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support
- Product documentation
  - 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.

Limited product warranty

Life Technologies Corporation and its affiliates warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have questions, contact Life Technologies at **www.thermofisher.com/support**.