applied biosystems

TaqPath™ 1-Step RT-qPCR Master Mix, CG

USER GUIDE

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For Laboratory Use.

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2.0	2 February 2023	• Streamlined the protocol. • Updated the legal information. • Rebranded the document.
1.0	10 September 2013	User Guide to accompany new product introduction.

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

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1 Product information

Product description

TaqPath™ 1-Step RT-qPCR Master Mix, CG is designed for one-step pathogen detection and gene expression workflows.

TaqPath™ 1-Step RT-qPCR Master Mix, CG contains the following components.

- Fast DNA Polymerase
- Thermostable MMLV enzyme
- Uracil-N glycosylase (UNG)
- dNTPs including dUTP
- RNase inhibitor
- ROX™ dye (passive reference)
- Buffer components optimized for maximum sensitivity and tolerance to several common RT-qPCR inhibitors

TaqPath™ 1-Step RT-qPCR Master Mix, CG is supplied at a 4X concentration.

Purpose of the product

Use TaqPath™ 1-Step RT-qPCR Master Mix, CG with any gene-specific primer/probe set to amplify RNA or DNA target sequences. This master mix in combination with a user-defined and supplied assay allows the user to perform one-step RT-qPCR for the following types of experiments:

- **Presence/absence—**An endpoint experiment that indicates the presence or absence of a specific nucleic acid sequence (target) in a sample. The actual quantity of target is not determined. Presence/absence experiments are commonly used to detect the presence or absence of a pathogen, such as a viral or bacterial pathogen. (Presence/absence experiments are also referred to as *plus/minus experiments*.)
- **Standard curve—**A type of quantification experiment that determines the absolute target quantity in samples. With the standard curve method, the real-time PCR system software measures amplification of the target in samples and in a standard dilution series. Data from the standard dilution series are used to generate the standard curve. Using the standard curve, the software interpolates the absolute quantity of target in the samples. Standard curve experiments are commonly used for quantifying viral load. (Standard curve experiments are also referred to as *absolute quantification* or *AQ experiments*.)

  You can also perform a standard curve experiment without running standards, if you only want to collect the quantification (C_t) values.

Note: A quantification experiment is a real-time experiment that measures the quantity of a target nucleic acid sequence (target) during each amplification cycle of the polymerase chain reaction (PCR).

Starting template

You can use this protocol for both RNA and DNA targets. During thermal cycling, the reverse transcription step will not affect performance with DNA targets.

About this protocol

TaqPath™ 1-Step RT-qPCR Master Mix, CG has been optimized for use with primers and hydrolysis probes. This protocol describes how to perform gene expression experiments with TaqPath™ 1-Step RT-qPCR Master Mix, CG.

Because analysis methods vary greatly between applications, this protocol provides general guidelines for analyzing data generated from experiments that use TaqPath™ 1-Step RT-qPCR Master Mix, CG in user-defined assays. For more detailed information about data analysis or the procedures outlined in this protocol, see the documentation for your instrument.

Contents and storage

Table 1 TaqPath™ 1-Step RT-qPCR Master Mix, CG

Cat. No.	Amount	Number of 20-µL reactions	Storage
A15299	5 × 1mL	1,000	–25°C to –15°C[1]
A15300	1 × 10 mL	2,000	

[1] The master mix does not freeze at –25°C to –15°C; gelling can occur.

Note:
- When stored at –25°C to –15°C, the performance of the master mix is guaranteed until the expiration date printed on the package and bottle labels.
- It is normal for this master mix to have a faint yellow hue.

Required materials not supplied

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:	
QuantStudio™ Dx Real-Time PCR Instrument[1]	Contact your local sales office
7500 Fast Dx Real-Time PCR Instrument[2]	

(continued)

Item	Source
Equipment	
Centrifuge with adapter for PCR plates	**MLS**
Laboratory mixer (vortex mixer or equivalent)	**MLS**
Microcentrifuge	**MLS**
Pipettors	**MLS**
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 assays	
Nuclease-Free Water (not DEPC-Treated)	4387936
TE, pH 8.0, RNase-free	AM9849

[1] The available run modes are standard and fast.
[2] Fast run mode only.

Workflow

Procedure for 1-Step™ RT-qPCR
Prepare samples (page 10)
Design the experiment and select the experiment type (page 10)
Determine the number of reactions (page 12)
Set up a plate document or experiment (page 13) Fast system or standard system.
Prepare the RT-qPCR reaction mix (page 14) Fast system or standard system.
Run the RT-qPCR plate (page 16)
Analyze the data (page 16)

Methods

General guidelines

Thermal-cycling conditions for your real-time PCR system

TaqPath™ 1-Step RT-qPCR Master Mix, CG can be run in either Fast or Standard cycling systems, provided the thermal-cycling profile and run mode are correctly set for the instrument being used. See "Set up a plate document or experiment" on page 13 for thermal cycling profiles for both fast and standard modes.

- **Run mode—**The run mode defines the ramp rate that is used to heat or cool the sample block between temperature changes.
- **Thermal-cycling profile—**The thermal-cycling profile defines the temperature and time for each step. Be sure to use the appropriate thermal-cycling profile for your system.

Procedural guidelines

This chapter provides a general protocol for performing one-step RT-qPCR using TaqPath™ 1-Step RT-qPCR Master Mix, CG. This protocol is suggested as a starting point. Optimal reaction conditions—incubation times and temperatures, primer/probe concentration, and the amount of template—can vary and should be optimized.

For more detailed information about the procedures outlined in this protocol, see the documentation for your instrument ("Related documentation" on page 34).

Before you begin

Assays and thermal-cycling conditions

Each assay should be independently optimized and validated to ensure appropriate performance. Validate your assays and re-optimize your thermal-cycling conditions as needed.

The reverse transcriptase enzyme contained in this kit is purified from *E.coli* expressing a proprietary version of the MMLV *pol* gene (GenBank™ accession no. J02255) expressed from pET-24(+) expression vector. It is possible that a minimal amount of the expression vector could be carried over into the final master mix formulation. If you are targeting MMLV, a related virus, or any of the plasmid sequence, we recommend designing primer sequences not contained in the expression vector.

Prevent contamination

Review Appendix C, "PCR good laboratory practices".

User-supplied materials

- Nuclease-free water and buffers
- User-defined expression assay
- Reaction plates and accessories for the real-time PCR instrument
- General laboratory equipment

Prepare samples

Isolate and purify the target nucleic acid samples according to your laboratory practices. We offer products appropriate for nucleic acid purification from a spectrum of sample types.

Storage conditions

Store the prepared samples at –85°C to –68°C in RT-PCR grade water. If you dilute your samples, use TE buffer or RT-PCR grade water as the diluent.

Design the experiment

User-defined assays

To design your own assay for use with TaqPath™ 1-Step RT-qPCR Master Mix, CG, see Appendix B, “Supplemental information” for more information.

Note: The term *assay* refers to the primer and probe set.

Select your experiment type

Select one of the following experiment types:

- Presence/absence
- Standard curve

Starting template

This protocol can be used for both RNA and DNA targets as the reverse transcription step will not affect thermal cycling performance with DNA targets. However, we recommend using TaqPath™ qPCR Master Mix, CG (Cat. No. A15297) when designing experiments to assay DNA targets.

RT-qPCR guidelines

Item	Guideline
Assays (primer and probe set)	Keep all assays in the freezer, protected from light, until you are ready to use them. Excessive exposure to light may affect the fluorescent probes.
	Just before use, allow the assays to thaw on ice.
	At initial use, aliquot the assays to avoid multiple freeze/thaw cycles.
TaqPath™ 1-Step RT-qPCR Master Mix, CG	Keep the master mix in the freezer, protected from light, until you are ready to use it.
	Just before use, allow the master mix to thaw on ice. The master mix does not freeze at –25°C to –15°C but gelling may occur. Thawing the master mix on ice allows the master mix to return to its liquid state.
Storing combined master mix and assay	You can combine TaqPath™ 1-Step RT-qPCR Master Mix, CG and the assay ahead of time and store at –25°C to –15°C for short periods. Stability varies depending on the assay, but storage for up to 4 weeks of the combined master mix and assay has been observed to have minimal effect on performance.
(For standard curve experiments) Standards	Standards are critical for accurate analysis of run data. Mistakes or inaccuracies in making the dilutions directly affect the quality of the results. The quality of pipettes and tips and the care used in measuring and mixing dilutions affect accuracy. Use TE buffer or RT-qPCR Grade Water to prepare the standard dilution series.
No-RT control	If you are concerned that your one-step real-time RT-qPCR is detecting genomic DNA rather than a particular RNA species, you can run a no-RT control reaction with TaqPath™ 1-Step RT-qPCR Master Mix, CG. To run a no-RT control: Heat-kill the RT enzyme by heating one aliquot of master mix at 95℃ for 5 minutes before mixing it with the assay and sample. The PCR hot-start mechanism will reactivate after the master mix has cooled to room temperature.
Thermal-cycling temperature ranges	The optimal temperatures for reverse transcription and annealing are recommended in this protocol (see "Set up a plate document or experiment" on page 13). However, in some instances you may wish to alter the temperatures; testing has shown that the: • RT enzyme will function best in the range of 48–55°C • Annealing temperature should be in the range of 56–62°C **Note:** Be sure the annealing temperature is consistent with the melting temperature (T_m) of your primer designs. For guidelines on designing primers, see "Probe and primer design" on page 21.
Multiplexing	TaqPath™ 1-Step RT-qPCR Master Mix, CG is designed to accommodate running multiple assays simultaneously.

Determine the number of reactions

Determine the total number of reactions in your experiment. For each experiment type, you need the following types of reactions:

Experiment type	Reaction type	Description
Presence/absence	Unknown	A well that contains: • Sample (DNA or RNA in which the presence of a target is unknown) • TaqPath™ 1-Step RT-qPCR Master Mix, CG • Assay of choice
	Exogenous Internal positive control (IPC)	A short synthetic DNA template that you can add to the reactions to distinguish between true negative results and reactions affected by PCR inhibitors, incorrect assay setup, or a reagent or instrument failure.
	No amplification control (NAC)	A well that contains all reaction components except the unknown sample and IPC. Alternatively, the well may contain the IPC plus a blocking agent for the IPC. No amplification should occur in NAC wells.
	No template control (NTC)	A well that contains all PCR components except the unknown sample. Only the IPC should amplify in NTC wells.
	Replicate	A well that is identical to another; it contains identical components and volumes. We recommend performing at least three replicates of each reaction.
Standard curve	Unknown	A well that contains: • Sample (DNA or RNA in which the quantity of the target is unknown) • TaqPath™ 1-Step RT-qPCR Master Mix, CG • Assay of choice
	Standard	A well that contains DNA of a known standard quantity; used in quantification experiments to generate standard curves. **Note:** You can perform a standard curve experiment without running standards, if you only want to collect the C_t values.
	Standard dilution series	A set of standards containing a range of known quantities. The standard dilution series is prepared by serially diluting standards.
	No template control (NTC)	A negative control well that contains water or buffer instead of sample. No amplification of the target should occur in negative control wells.
	Replicate	A well that is identical to another; it contains identical components and volumes. We recommend performing at least three replicates of each reaction.

Set up a plate document or experiment

For fast real-time PCR systems

In the real-time PCR system software, set up a plate document or experiment using the following parameters:

- Sample volume: 20 µL
- Auto Increment Settings: Accept the default value
- Data Collection: Accept the default value
- Ramp Rate Settings: Accept the default value
- Run mode: Use the default run mode for your system and sample block module (that is, Fast mode on Fast instruments and standard mode on standard instruments).
- Thermal-cycling conditions for sample volumes ≤30 µL:

Step	Temperature	Time	Cycles
UNG incubation	25°C	2 minutes	1
Reverse transcription[1]	50°C[1]	15 minutes	1
Polymerase activation[2]	95°C[2]	2 minutes	1
Amplification	95°C 60°C	3 seconds 30 seconds	40

[1] Reverse transcription works best between 48°C and 55°C.
[2] Required for RT inactivation, initial denaturation, and to activate the DNA polymerase.

For standard real-time PCR systems

In the real-time PCR system software, set up a plate document or experiment using the following parameters:

- Sample volume: 50 µL
- Auto Increment Settings: Accept the default value
- Data Collection: Accept the default value
- Ramp Rate Settings: Accept the default value
- Run mode: Standard
- Thermal-cycling conditions for sample volumes >30 µL:

Step	Temperature	Time	Cycles
UNG incubation	25°C	2 minutes	1
Reverse transcription[1]	50°C[1]	15 minutes	1
Polymerase activation[2]	95°C[2]	2 minutes	1
Amplification	95°C 60°C	15 seconds 60 seconds	40

[1] Reverse transcription works best between 48°C and 55°C.
[2] Required for RT inactivation, initial denaturation, and to activate the DNA polymerase.

Prepare RT-qPCR reaction mix

Fast real-time PCR systems

For Fast real-time PCR systems, you must use a volume of ≤30 µL per reaction.

1. Thaw all reagents on ice.
2. Vortex assays briefly to mix, then centrifuge to collect.
3. Mix thawed samples by gentle inversion or flicking 3–5 times, then briefly centrifuge to collect.
4. Ensure that there are no gel lumps remaining. Repeat steps 2 and 3, if necessary, to ensure a homogenous solution.
5. Calculate the total volume required for each reaction component:

   Note: Include 10% extra volume to compensate for the volume loss that occurs during pipetting.

Component	Volume[1]	Notes
4X TaqPath™ 1-Step RT-qPCR Master Mix, CG	5 µL	–
User-defined assay (primers and probe)	1 µL[2]	We recommend primer concentrations of 400–900 nM and a probe concentration of 100–250 nM.
Sample	Variable	Use as much sample as needed, up to the maximum allowed by the reaction volume.
RT-qPCR Grade Water	Variable	Fill to the total reaction volume.
Total volume per reaction	**20 µL**	–

[1] Volume for one 20 µL reaction.
[2] Volume for a pre-combined primer-probe mixture (ppmix) at a 20X stock concentration. Adjust the volume for different ppmix stock concentrations.

6. Working on ice, prepare a bulk RT-qPCR reaction mixture.
   a. Mix only the master mix, assay, and water that you calculated in the previous step.
      Exclude the sample.
   b. Invert the mixture at least 10 times to mix the components thoroughly.
   c. Centrifuge the mixture briefly to collect the contents.
7. Aliquot the appropriate volume of RT-qPCR reaction mix into each well of an optical reaction plate.
   The volume of reaction mix to add to each well is (20 µL – the volume of the sample).
8. Add sample to each well in the optical reaction plate that contains the RT-qPCR reaction mix .

9. Cover the reaction plate with an optical adhesive cover, invert the plate 3–5 times, making sure that the contents of the wells are moving back and forth between the seal and the bottom of the wells to ensure proper mixing, then centrifuge at 150 × *g* for 1 minute to spin down the contents and eliminate air bubbles.

IMPORTANT! TaqPath™ 1-Step RT-qPCR Master Mix, CG is a 4X formulation and is more viscous than most master mixes. Be sure that all of the components are thoroughly mixed in all the wells before proceeding. It has been observed that inverting the plate gives more uniform mixing across the reaction plate than vortexing.

Standard real-time PCR systems

1. Thaw all reagents on ice.
2. Vortex assays briefly to mix, then centrifuge to collect.
3. Mix thawed samples by gentle inversion or flicking 3–5 times, then briefly centrifuge to collect.
4. Ensure that there are no gel lumps remaining. Repeat steps 2 and 3, if necessary, to ensure a homogenous solution.
5. Calculate the total volume required for each reaction component:

Note: Include 10% extra volume to compensate for the volume loss that occurs during pipetting.

Component	Volume[1]	Notes
4X TaqPath™ 1-Step RT-qPCR Master Mix, CG	12.5 µL	–
User-defined assay (primers and probe)	2.5 µL[2]	We recommend primer concentrations of 400–900 nM and a probe concentration of 100–250 nM.
Sample	Variable	Use as much sample as needed, up to the maximum allowed by the reaction volume.
RT-qPCR Grade Water	Variable	Fill to the total reaction volume.
Total volume per reaction	**50 µL**	–

[1] Volume for one 50-µL reaction.
[2] Volume for a pre-combined primer-probe mixture (ppmix) at a 20X stock concentration. Adjust the volume for different ppmix stock concentrations.

6. Working on ice, prepare a bulk RT-qPCR reaction mixture.
   a. Mix only the master mix, assay, and water that you calculated in the previous step.
   Exclude the sample.
   b. Invert the mixture at least 10 times to mix the components thoroughly.
   c. Centrifuge the mixture briefly to collect the contents.
7. Aliquot the appropriate volume of RT-qPCR reaction mix into each well of an optical reaction plate.
   The volume of reaction mix to add to each well is (50 µL – the volume of the sample).

8. Add sample to each well in the optical reaction plate that contains the RT-qPCR reaction mix .

9. Cover the reaction plate with an optical adhesive cover, invert the plate 3–5 times, making sure that the contents of the wells are moving back and forth between the seal and the bottom of the wells to ensure proper mixing, then centrifuge at 150 × *g* for 1 minute to spin down the contents and eliminate air bubbles.

   IMPORTANT! TaqPath™ 1-Step RT-qPCR Master Mix, CG is a 4X formulation and is more viscous than most master mixes. Be sure that all of the components are thoroughly mixed in all the wells before proceeding. It has been observed that inverting the plate gives more uniform mixing across the reaction plate than vortexing.

Run the RT-qPCR plate

1. In the real-time PCR system software, open the plate document or experiment that corresponds to the reaction plate.

2. Load the reaction plate into the real-time PCR system.

3. Start the run.

Analyze the data

Data analysis varies depending on the real-time PCR system that you use. In general, to analyze the data in the real-time PCR system software, you:

1. View the amplification plot, and modify as needed:
   a. Set the baseline and threshold values. See "About baseline and threshold values" on page 17.

   b. Remove outliers from the analysis.

2. In the well table or results table, view the C_t values for each well and for each replicate group.

3. (For standard curve experiments) View the standard curve for:
   - Slope
   - Amplification efficiency
   - R^2 values
   - Y-intercept
   - C_t values
   - Outliers

About baseline and threshold values

You can use the real-time PCR system software to set the baseline and threshold values for the amplification plot, either automatically or manually.

- The *baseline* refers to the initial cycles of PCR in which there is slight change in fluorescence signal.
- The intersection of the *threshold* with the amplification plot defines the C_t in real-time PCR assays. The threshold is set above the background signal and within the exponential growth phase of the amplification curve.

For more information

For more information about data analysis, see "Related documentation" on page 34, or see the documentation for your instrument.

Troubleshooting

Observation	Possible cause	Recommended action
Amplification plot displays S-shaped curves	The RT-qPCR mix was not thoroughly combined.	When preparing the RT-qPCR mix ("Prepare RT-qPCR reaction mix" on page 14), be sure to invert the reaction plate firmly 3–5X to mix before centrifuging.
Amplification plot is truncated	The baseline was set too high.	When analyzing the data ("Analyze the data" on page 16), manually reset the baseline, or use the automatic baseline function.

Observation	Possible cause	Recommended action
C_t is low, but merges with the background signal (noise)	When automatic baseline is used, the software raises the threshold bar to avoid the elevated baseline.	When analyzing the data ("Analyze the data" on page 16), change analysis setting to allow manual adjustment of the threshold, then manually adjust the threshold bar.

Supplemental information

This appendix provides general guidelines to design primers and hydrolysis probes for quantification assays.

Assay design workflow

Assay design workflow
Select the amplicon site (page 21)
Design the probe and primer (page 21)
Determine the optimal primer concentrations (page 22)
Determine the optimal probe concentration (page 25)
Evaluate the results (page 26)

Amplicon site selection

General amplicon site selection guidelines

Using your preferred suite of software tools for sequence analysis and design, select an *amplicon site* (short segment of cDNA) within the target sequence. Selecting a good amplicon site ensures amplification of the target cDNA without coamplification of the genomic sequence, pseudogenes, or related genes.

- The amplicon should span one or more introns to avoid amplification of the target gene in genomic DNA.
- The shortest amplicons work the best. Consistent results are obtained for amplicon size ranges from 50–150 bp.
- Design the hydrolysis probe before determining primer pairs during assay design.
- Design hydrolysis probes and primer pairs according to the guidelines provided in “General probe design guidelines” on page 22.
- The primer pair must be specific to the target gene; the primer pair must not amplify pseudogenes or other related genes.
- Test the primer pairs, then select the primer pair that produces the highest signal-to-noise ratio (that is, earliest C_t with total RNA or mRNA and no amplification with genomic DNA or negative controls).

If the gene does not contain introns

If the gene you are studying does not contain introns, then you cannot ensure amplification of the target cDNA sequence without coamplification of the genomic sequence. In this case, you may need to run control reactions that do not contain reverse transcriptase (RT-controls) to determine whether your RNA sample contains DNA. Amplification in the RT-controls indicates that your RNA sample contains DNA. To remove the DNA from the RNA sample, treat the RNA sample with DNase I.

Probe and primer design

Using your preferred suite of software tools for sequence analysis and design, design a probe to detect amplification of the target sequence, then design primers to amplify the target sequence.

TaqPath™ 1-Step RT-qPCR Master Mix, CG has been optimized for use with primers and hydrolysis probes that have been designed according to our development guidelines. A concentration of 900 nM primers and a 250 nM fluorescent probe provides a highly reproducible and sensitive assay.

General probe design guidelines

- Keep the GC content in the 20–80% range.
- Avoid runs of identical nucleotides. If repeats cannot be avoided, there must be fewer than four consecutive G bases.
- The base at the 5′ end must not be a G.
- Select the strand in which the probe contains more C bases than G bases.
- For singleplex assays, keep the T_m between 68°C to 70°C.

General primer design guidelines

- Choose the primers after the probe.
- Do not overlap primer and probe sequences. The optimal primer length is 20 bases.
- Keep the GC content in the 20–80% range.
- Avoid runs of identical nucleotides. If repeats cannot be avoided, there must be fewer than four consecutive G bases.
- **IMPORTANT! Keep the T_m between 58–60°C**.
- Be sure the last 5 nucleotides at the 3′ end contain no more than two G and/or C bases.
- If you cannot find acceptable primer sequences, you may need to examine the sequence and select another amplicon site or screen for more sites.

Calculation of oligonucleotide concentrations

After you receive your primers and probe, use a spectrophotometric method to determine the concentrations of the oligonucleotides in your assay.

Calculate oligonucleotide concentrations

1. Calculate the sum of extinction coefficient contributions for each oligonucleotide sequence:

Chromophore	Extinction Coefficient
A	15,200
C	7,050
G	12,010
T	8,400
FAM™ dye	20,958
TAMRA™ dye	31,980
TET™ dye	16,255
JOE™ dye	12,000
VIC™ dye	30,100

2. Measure the absorbance at 260 nm (A_{260}) of each oligonucleotide diluted in TE buffer (for example, 1:100).

3. Calculate the oligonucleotide concentration using the formula:

   A_{260} = (sum of extinction coefficient contributions × cuvette pathlength × concentration) ÷ dilution factor

   Rearrange to solve for concentration:

   Concentration (C) = (dilution factor × A_{260}) ÷ (sum of extinction coefficient contributions × cuvette pathlength)

An example calculation of primer concentration

If the primer sequence is CGTACTCGTTCGTGCTGC:

- Sum of extinction coefficient contributions:
  = A × 1 + C × 6 + G × 5 + T × 6
  = 167,950 $M^{-1}cm^{-1}$
- Example A_{260} measurements:
  Dilution = 1:100
  Cuvette pathlength = 0.3 cm
  A_{260} = 0.13
- Primer concentration:
  = (100 × 0.13) ÷ (167,950 $M^{-1}cm^{-1}$ × 0.3 cm)
  = 2.58 × 10^{-4} M
  = 258 µM

An example calculation of probe concentration

If the probe sequence is CGTACTCGTTCGTGCTGC, FAM™ dye is attached to the 5′ end, and TAMRA™ dye is attached to the 3′ end:

- Sum of extinction coefficient contributions:
  = A × 1 + C × 6 + G × 5 + T × 6 + FAM™ × 1 + TAMRA™ × 1
  = 220,888 $M^{-1}cm^{-1}$
- Example A_{260} measurements:
  Dilution = 1:100
  Cuvette pathlength = 0.3 cm
  A_{260} = 0.13
- Probe concentration:
  = (100 × 0.13) ÷ (220,888 $M^{-1}cm^{-1}$ × 0.3 cm)
  = 1.96 × 10^{-4} M
  = 196 µM

Determine optimal primer concentrations

Purpose

To determine the minimum primer concentrations that yield the minimum threshold cycle (C_t) and the maximum baseline-corrected normalized reporter (ΔR_n) for the user-designed assay.

Primer concentrations to test

Use TaqPath™ 1-Step RT-qPCR Master Mix, CG to prepare at least three technical replicates of each of the conditions shown below:

Forward primer final concentration (nM)	Reverse primer final concentration (nM)		
	50	300	900
50	50/50	50/300	50/900
300	300/50	300/300	300/900
900	900/50	900/300	900/900

Prepare and run the RT-qPCRs

1. Prepare the RT-qPCRs as outlined in the following table:

   Note: Include 10% extra volume to compensate for the volume loss that occurs during pipetting.

Component	Volume for one reaction		Notes
	384-well or 96-well 0.1-mL plate	96-well 0.2 mL plate	
4X TaqPath™ 1-Step RT-qPCR Master Mix, CG	5 µL	12.5 µL	–
Forward primer (50–900 nM final)	1 µL	2.5 µL	–
Reverse primer (50–900 nM final)	1 µL	2.5 µL	
Hydrolysis probe (250 nM final)	2 µL	5 µL	
Sample	Variable	Variable	Use as much sample as needed, up to the maximum allowed by the reaction volume.
RT-qPCR Grade Water	Variable	Variable	Fill to the total reaction volume.
Total Volume	**20.0 µL**	**50.0 µL**	**–**

2. In the real-time PCR system software set up a plate document or experiment, see “Set up a plate document or experiment” on page 13.

3. Run the RT-qPCR plate, “Run the RT-qPCR plate” on page 16.

4. At the end of run, tabulate the results for the C_t and ΔR_n values. Choose the minimum forward- and reverse-primer concentrations that yield the maximum ΔR_n values and the lowest C_t.

Determine optimal probe concentration

Purpose

To determine the minimum probe concentration that yields the minimum threshold cycle (C_t) for the target sequence without decreasing the ΔR_n for the user-defined assay.

Probe concentrations to test

Use TaqPath™ 1-Step RT-qPCR Master Mix, CG to prepare at least three technical replicates at each 50 nM interval from 50–250 nM final probe concentration. Use the optimal primer concentrations you determined in the experiment you performed in “Determine optimal primer concentrations” on page 24.

Prepare and run the RT-qPCRs

1. Prepare the RT-qPCRs as outlined in the following table:

Note: Include 10% extra volume to compensate for the volume loss that occurs during pipetting.

Component	Volume for one reaction		Notes
	384-well or 96-well 0.1-mL plate	96-well 0.2-mL plate	
4X TaqPath™ 1-Step RT-qPCR Master Mix, CG	5 µL	12.5 µL	1X final concentration
Forward primer	1 µL	2.5 µL	Optimal[1]
Reverse primer	1 µL	2.5 µL	Optimal[1]
Hydrolysis probe (50–250 nM final)	2 µL	5 µL	–
Sample	Variable	Variable	Use as much sample as needed, up to the maximum allowed by the reaction volume.
RT-qPCR Grade Water	Variable	Variable	Fill to the total reaction volume.
Total Volume	**20.0 µL**	**50.0 µL**	–

[1] Use the forward- and reverse-primer concentrations determined in “Determine optimal primer concentrations” on page 24

2. In the real-time PCR system software set up a plate document or experiment, see "Set up a plate document or experiment" on page 13.

3. Run the RT-qPCR plate, "Run the RT-qPCR plate" on page 16.

4. Tabulate the results for the C_t and ΔR_n values. Choose the minimum probe concentration to yield the maximum ΔR_n and the minimum C_t values.

Evaluate the results

Review the results from the primer optimization and probe optimization experiments to identify the optimal assay (combination of hydrolysis probe and forward- and reverse-primers) based upon ΔR_n and C_t values.

An optimal assay should have:

- maximal PCR yield (ΔR_n).
- minimum C_t values.
- no nonspecific amplification in the negative controls.
- minimum forward- and reverse-primer concentrations that yield the maximum ΔR_n values and the lowest C_t.
- minimum probe concentrations that yield the minimum C_t and the highest ΔR_n values.

PCR good laboratory practices

Sample preparation

When preparing samples for PCR/RT-PCR amplification:

- Wear clean gloves and a clean lab coat (not previously worn while handling amplified PCR/RT-PCR products or used during sample preparation).
- Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation and PCR/RT-PCR setup
  - PCR/RT-PCR amplification and analysis of PCR/RT-PCR products
- Do not bring amplified PCR/RT-PCR products into the PCR/RT-PCR setup area.
- Open and close all sample tubes carefully. Avoid splashing or spraying PCR/RT-PCR samples.
- Keep reactions and components capped as much as possible.
- Use a positive-displacement pipette or aerosol-resistant barrier pipette tips.
- Clean lab benches and equipment periodically with 10% bleach solution or DNA*Zap*™ Solution (Cat. no. AM9890).

Preventing contamination

PCR/RT-PCR assays require special laboratory practices to avoid false positive amplifications. The high throughput and repetition of these assays can lead to amplification of a single DNA molecule.

False positives

Special laboratory practices are necessary in order to avoid false positive amplifications. This is because of the capability for single DNA molecule amplification provided by the PCR process. Because of the enormous amplification possible with PCR, amplicon carryover can result in sample contamination. Other sources of contamination could be from samples with high DNA levels or from positive control templates.

When dUTP replaces dTTP as a dNTP substrate in PCR and the method described below is used, UNG treatment can prevent the reamplification of carryover PCR products in subsequent experiments. This method uses enzymatic and chemical reactions analogous to the restriction-modification and excision-repair systems of cells to specifically degrade PCR products from previous PCR amplifications or to degrade mis-primed, non-specific products produced prior to specific amplifications, but not degrade native nucleic acid templates.

The method used to make PCR products susceptible to degradation involves substituting dUTP for dTTP in the PCR mix and treating subsequent PCR mixes with the enzyme uracil-N glycosylase (UNG, EC 3.2.2-) prior to amplification.

Although the protocol and reagents described here are capable of degrading or eliminating large numbers of carried over PCR products, we recommend that you continue to use the instruments and suggestions described in this user guide to minimize cross-contamination from non-dU-containing PCR products or other samples.

Uracil-N glycosylase (UNG)

The UNG provided in TaqPath™ 1-Step RT-qPCR Master Mix, CG is a pure, nuclease-free, 26-kDa recombinant enzyme encoded by the Escherichia coli uracil-N glycosylase gene which has been inserted into an E. coli host to direct the expression of the native form of the enzyme.

UNG acts on single- and double-stranded dU-containing DNA by hydrolyzing uracil-glycosidic bonds at dU-containing DNA sites. The enzyme causes the release of uracil, and creates an alkali-sensitive apyrimidic site in the DNA. Apyrimidic sites block replication by DNA polymerases. The enzyme has no activity on RNA or dT-containing DNA.

UNG incubation at 25°C is necessary to cleave any dU-containing PCR carryover products. Incubation at 95°C is necessary to substantially reduce UNG activity, and to denature the native DNA in the experimental sample. Because UNG is not completely deactivated during the 95°C incubation, it is important to keep the annealing temperatures greater than 55°C and to refrigerate PCR products at 2°C to 8°C to prevent amplicon degradation.

Prevention of PCR product carryover

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

Production of primer dimer could lower the amplification yield of the desired target region. If primers cannot be selected with dA nucleotides near the ends, the use of primers with 3′ terminal dU-nucleotides should be considered. Single-stranded DNA with terminal dU nucleotides are not substrates for UNG and thus the primers will not be degraded. Biotin-dUMP derivatives are not substrates for UNG.

The concentration of UNG and the time of the incubation step necessary to prevent amplification of contaminating dU-containing PCR product depends on the PCR conditions necessary to amplify your particular DNA sequence and the level of contamination expected. This mix has been developed to incorporate the optimal amount of UNG to prevent cross contamination while not affecting qPCR performance.

Do not attempt to use UNG in subsequent amplification of dU-containing PCR template, such as in nested-PCR protocols. The UNG will degrade the dU-containing PCR product, preventing further amplification.

Fluorescent contaminants

Since fluorescent contaminants may interfere with this assay and give false-positive results, it may be necessary to include a No-Amplification Control tube that contains sample, but no enzyme. If the absolute fluorescence of the No-Amplification Control is greater than that of the no template control (NTC) after PCR, fluorescent contaminants may be present in the sample or in the heat block of the real-time PCR instrument.

Glossary

ΔR_n

The difference between the R_n^+ value and the R_n^- value. It reliably indicates the magnitude of the signal generated by the given set of PCR conditions.

The following equation expresses the relationship of these terms:

$\Delta R_n = (R_n^+) - (R_n^-)$

where:

$$R_n^+ = \frac{\text{Emission Intensity of Reporter}}{\text{Emission Intensity of Passive Reference}} \quad \text{PCR with template}$$

$$R_n^- = \frac{\text{Emission Intensity of Reporter}}{\text{Emission Intensity of Passive Reference}} \quad \text{PCR without template or early cycles of a real-time reaction}$$

C_q

See quantification cycle (C_q).

C_t

See threshold cycle (C_t).

fold difference

The measured ratio of the quantity of template in Sample A over the quantity of template in Sample B, where quantity A > quantity B, so that the ratio is >1.

full replicate

Repeated wells of the same sample with the same assay, where the contents of each well go through all experimental steps (sample preparation, reverse transcription, and PCR) separately.

minimum fold difference

The smallest fold difference that is statistically significant.

multicomponenting

The term used to distinguish the contribution each individual dye makes to the fluorescent spectra. The overlapping spectra from the dye components generate the composite spectrum, which represents one reading from one well.

normalization

The Passive Reference 1, a dye included in the 4X TaqPath™ 1-Step RT-qPCR Master Mix, CG, does not participate in the 5′ nuclease PCR. The Passive Reference provides an internal reference to which the reporter-dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescent fluctuations due to changes in concentration or volume.

PCR/technical replicate

Identical reactions that contain identical components and volumes and evaluate the same sample.

quantification cycle (C_q)

The PCR cycle number at which the fluorescence meets the threshold in the amplification plot. Can also be referred to as threshold cycle (C_t).

R_n^+

The R_n value of a reaction containing all components including the template.

Normalization is accomplished by dividing the emission intensity of the reporter dye by the emission intensity of the Passive Reference to obtain a ratio defined as the R_n (normalized reporter) for a given reaction tube.

R_n^-

The R_n value of an unreacted sample. This value may be obtained from the early cycles of a real-time run (those cycles prior to a detectable increase in fluorescence). This value may also be obtained from a reaction not containing template.

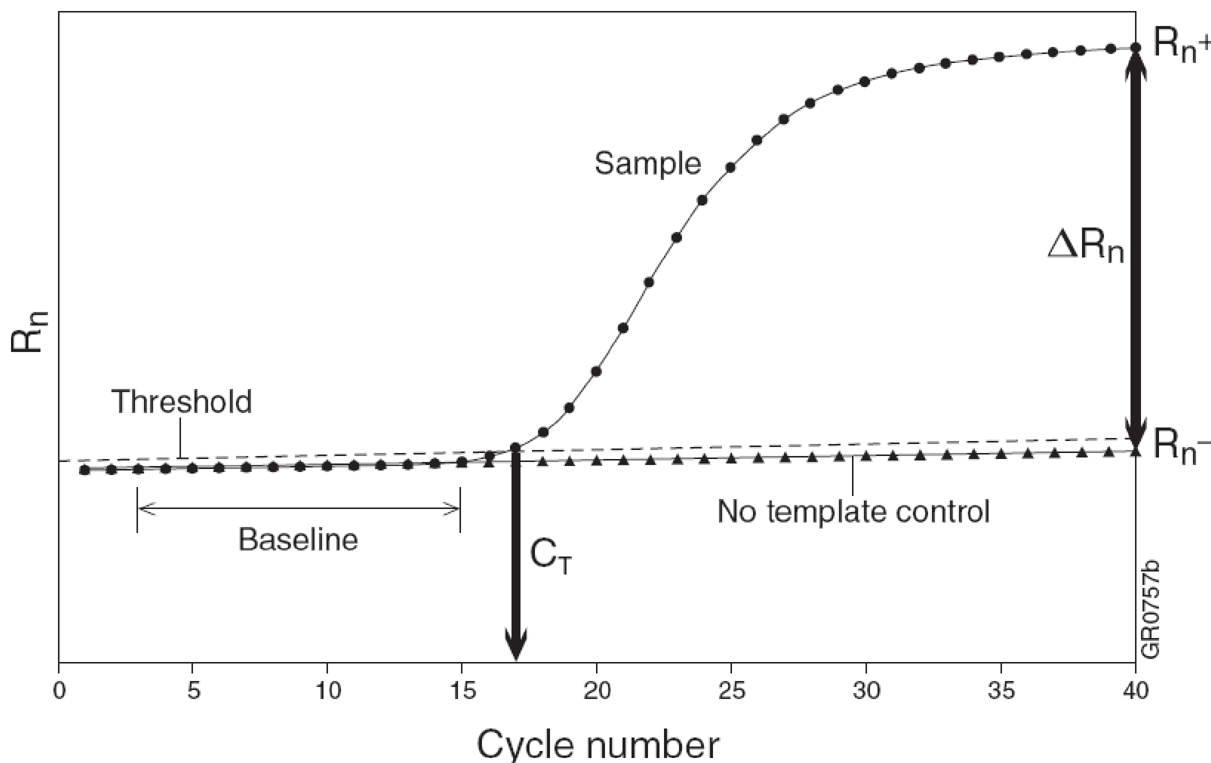
statistically significant (to a 99.7% confidence level)

A result with a low probability (0.3%) of resulting from chance.

threshold cycle (C_t)

The threshold cycle or C_t value is the cycle at which a statistically significant increase in ΔR_n is first detected. Threshold is defined as the average standard deviation of R_n for the early cycles, multiplied by an adjustable factor.

On the graph of R_n versus cycle number shown below, the threshold cycle occurs when the Sequence Detection System begins to detect the increase in signal associated with an exponential growth of PCR product.


Sample
R_n+
ΔR_n
R_n
Threshold
R_n-
Baseline
C_T
No template control
GR0757b
0
5
10
15
20
25
30
35
40
Cycle number

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

Documentation and support

Related documentation

Document	Pub. No.
TaqMan™ Gene Expression Assays User Guide—single-tube assays	4333458
Custom TaqMan™ Assays Design and Ordering Guide	4367671
Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument Reference Guide	4406991

Customer and technical support

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- 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/or its affiliate(s) 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 any questions, please contact Life Technologies at **www.thermofisher.com/support**.