applied biosystems

TaqPath™ BactoPure™ Microbial Detection Master Mix

USER GUIDE

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For Laboratory Use.

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Thermo Fisher Scientific Baltics UAB | V.A. Graiciuno 8, LT-02241 | Vilnius, Lithuania
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Revision history: Pub. No. MAN0025689

Revision	Date	Description
B.0	14 April 2022	• DTT (Cat. No. P2325) was added to the required materials. • The assay preparation procedure was updated. – A step to dilute the DTT was added. – The reagent volumes were updated. – A step to use a thermal block for dsDNase inactivation was added.
A.0	31 March 2022	New document for the TaqPath™ BactoPure™ Microbial Detection Master Mix.

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1 Product information

Product description

The Applied Biosystems™ TaqPath™ BactoPure™ Microbial Detection Master Mix is optimized for rapid, low-level microbial detection, even in the presence of inhibitors. The TaqPath™ BactoPure™ Microbial Detection Master Mix is validated for multiplexing and enables real-time PCR for presence/absence, standard curve, and relative quantification experiments.

The TaqPath™ BactoPure™ Microbial Detection Master Mix is formulated for the following purposes:

- Enable low-level detection (high sensitivity) of pan-bacterial, pan-fungal, and pan-mammalian targets, as well as viral DNA targets and antibiotic resistance markers
- Enable accurate detection and quantification of samples with low- or high-DNA target concentrations
- Enhance sensitivity and inhibitor tolerance during real-time PCR for the following samples:
  - Samples from crude lysates
  - Samples with inhibitors typically found in biopharmaceutical, molecular diagnostic, and research applications
  - Samples extracted from various human and animal sources, such as saliva, whole blood, and swabs

Versions of the master mix

The master mix is available in two versions:

Master mix formulation	Description
TaqPath™ BactoPure™ Microbial Detection Master Mix with ROX™	This master mix contains ROX™ dye as a passive reference dye.
TaqPath™ BactoPure™ Microbial Detection Master Mix (No ROX™)	This master mix does not contain a passive reference dye, enabling the measurement of the JUN™ dye, or a similar emission wavelength dye, in the channel previously used to measure ROX™ dye.

The master mix is supplied at a 2X concentration and contains the following components:

- Hot-Start AmpliTaq™ DNA Polymerase
- Heat-labile uracil-DNA glycosylase (UNG)
- dNTPs with dUTP
- Optimized buffer components
- ROX™ dye (not included in the version without ROX™ dye)

Note: For details about the master mix components, see “Master mix components” on page 17.

Recommended master mix formulation for single- and multiplex reactions

PCR option[1]	Compatible reporter dyes	Recommended master mix formulation
Singleplex (1 probe)	FAM™ dye, VIC™ dye, ABY™ dye	Either of the following: • TaqPath™ BactoPure™ Microbial Detection Master Mix with ROX™ • TaqPath™ BactoPure™ Microbial Detection Master Mix (No ROX™)
Multiplex (2-3 probes)	FAM™ dye, VIC™ dye, ABY™ dye	
Multiplex (≥3 probes)	JUN™ dye, FAM™ dye, VIC™ dye, ABY™ dye	TaqPath™ BactoPure™ Microbial Detection Master Mix (No ROX™)

[1] For detailed information about multiplex reactions, see the *TaqMan™ Assay Multiplex PCR Optimization Application Guide* (Pub. No. MAN0010189).

Contents and storage

The TaqPath™ BactoPure™ Microbial Detection Master Mix is supplied at a 2X concentration and is available in the following quantities:

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

Cat. No.	Amount	No. of 20-µL reactions	Storage
TaqPath™ BactoPure™ Microbial Detection Master Mix			
A52699	1 mL	100	2–8°C
A52700	5 mL	500	
A52701	5 × 1 mL[1]	500	
A52702	50 mL	5,000	
TaqPath™ BactoPure™ Microbial Detection Master Mix (No ROX™)			
A52703	1 mL	100	2–8°C
A52704	5 mL	500	
A52705	5 × 1 mL[1]	500	
A52706	50 mL	5,000	

[1] Five tubes, 1 mL each.

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™ 7 Flex Real-Time PCR System	Contact your local sales office
QuantStudio™ 5 Real-Time PCR System, 384-well	Contact your local sales office
QuantStudio™ 5 Real-Time PCR System, 96-well, 0.2 mL	Contact your local sales office
Equipment	
Centrifuge with plate adapter	**MLS**
Microcentrifuge	**MLS**
Vortex	**MLS**
Adjustable pipettors	**MLS**
Thermal block	**MLS**
Reagents	
dsDNase kit	EN0771
DNA Extract All Reagents Kit[1]	4402599
DNA*Zap*™ PCR DNA Degradation Solutions	AM9890
DNA buffer (TE Buffer)	12090015
DTT, 1 M	P2325
RT-PCR Grade Water	AM9935
Consumables	
Plastics consumables	**thermofisher.com/plastics**
Pipette tips	**thermofisher.com/pipettetips**
Disposable gloves	**MLS**
One of the following tubes: • Screw Cap Microcentrifuge Tubes, 1.5 mL • Screw Cap Micro Tubes, 2 mL	 • 3467TS • 3469-11

[1] Use the DNA Extract All Reagents Kit for crude lysate preparation.

2 Methods

General guidelines

Prevent contamination

Use stringent laboratory practices to avoid false positives that arise through the amplification of contaminants.

- Always use proper sterile technique in a laminar flow hood.
- Clean work surfaces, equipment, and consumables with DNA*Zap*™ PCR DNA Degradation Solutions (Cat. No. AM9890) to remove contaminating DNA.
- Wear clean gloves and a clean lab coat.
  - Do not wear the same gloves and lab coat that you have previously used when handling amplified products or preparing samples.
- Wipe gloves regularly with DNA*Zap*™ PCR DNA Degradation Solutions (Cat. No. AM9890).
- Maintain separate areas and dedicated equipment and supplies for the following tasks:
  - Sample preparation and reaction setup.
  - Amplification and analysis of products.
- Do not bring amplified products into the reaction setup area.
- Keep reactions and components capped as much as possible.
- Immediately dispose of pipette tips after one dispense cycle to reduce contamination.
- To avoid contamination, any sample collection (i.e., PBS) and/or DNA extraction (i.e., TE buffer) reagent should be aliquoted into screw cap microcentrifuge tubes and autoclaved (extended autoclave setting—approximately 15 PSI for 80 minutes at 121°C). Autoclaved DNA buffer should be used for sample dilutions and as a template for the non-template controls (NTC).
- Closed containers with autoclaved reagent tubes (extended autoclave setting—approximately 15 PSI for 80 minutes at 121°C) can be stored inside or outside a laminar flow hood, but once exposed to the outside they must be decontaminated with DNA*Zap*™ PCR DNA Degradation Solutions (Cat. No. AM9890) before bringing them into the laminar flow hood.

Run modes

The master mix can be run on either Fast or Standard cycling protocols, provided the thermal cycling profile and run mode are correctly set for the instrument being used.

Thermal cycling profile—The thermal-cycling profile defines the temperature and time for each step. Use the appropriate thermal cycling profile for your system.

Run mode—The run mode defines the ramp rate used to heat or cool the sample block between temperature changes, and the time at each step.

Real-Time PCR System[1]	Available run modes
QuantStudio™ 5 Real-Time PCR System	Standard/Fast
QuantStudio™ 7 Flex Real-Time PCR System	Standard/Fast

[1] For more information on available real-time PCR systems, accessories and consumables, go to thermofisher.com.

Prepare the assay

1. Dilute the DTT to a concentration of 20 mM according to the following table.

Reagent	Volume
DTT, 1 M	10 µL
RT-PCR Grade Water	490 µL
Total volume	**500 µL**

2. Prepare the assay in a 2-mL screw-cap tube according to the following table.

Reagent	Initial concentration	Final concentration	Final volume[1]
RT-PCR Grade Water	—	—	161.75 µL
Untreated assay	60X	20X[2]	100 µL
dsDNase Buffer	10X	1X	30 µL
dsDNase	2 U/µL	0.03 U/µL	4.5 µL
DTT[3]	20 mM	0.25 mM	3.75 µL
Total volume	—	—	**300 µL**

[1] Other volumes of assay can be treated using the same final concentrations of each reagent.
[2] Other final concentrations of the assay can be prepared.
[3] See step 1.

Note: The dsDNase is sensitive to physical denaturation. Pipette the dsDNase slowly. If using automated pipettes, set the aspiring and dispensing speeds at or below setting 2.

3. Gently invert the tube 10 times to mix.

IMPORTANT! Do not vortex. The dsDNase is sensitive to physical denaturation.

4. Centrifuge the tube for a few seconds.
5. In a thermal block, heat the tube for 60 minutes at 40°C for dsDNA digestion.
6. In a thermal block, heat the tube for 20 minutes at 60°C for dsDNase inactivation.

For short-term storage (up to one month), store the DTT dilution and the prepared assay at 2–8°C. For long-term storage (more than one month), store at –20°C.

Prepare the real-time PCR plate

Prepare all the samples with at least two replicates, a negative control, and a positive control (recommended).

1. Prepare the real-time PCR solution for 10–50 µL per reaction according to the following table. Resuspend the samples by inverting the tube.

Reagents	Final concentration	Volume per reaction	Volume per reaction (with 10% overage)
TaqPath™ BactoPure™ Microbial Detection Master Mix (2X)	1X	5–25 µL	5.5–27.5 µL
Prepared assay[1]	1X	Variable	Variable
Template	≥10 copies/reaction	Variable	Variable
RT-PCR Grade Water	—	Up to 10–50 µL	Up to 11–55 µL
Total volume	—	**10–50 µL**	**11–55 µL**

[1] See "Prepare the assay" on page 10.

2. Seal the plate with adhesive film using a film applicator, ensuring a tight seal around all edges.
3. Turn the plate upside down, then shake vigorously up and down three times to bring the liquid up to the film.
4. Turn the plate upright, then shake three more times up and down to bring the liquid back to the bottom of the plate.
5. Repeat step 3 and step 4 two more times to ensure the mixing of the real-time PCR solution.
6. Centrifuge the plate at ≥1,500 × *g* for at least one minute.

Set up and run the real-time PCR plate

1. Set up and run the plate in the instrument according to the following table.

Step	Temperature	Time	Cycles
Pre-read	60°C	30 seconds	1 (Hold)
Initial denaturation/Enzyme activation	95°C	2 minutes	
Denaturation	95°C	10 seconds	40
Annealing/Extension	60°C	30 seconds	
Post-read	60°C	30 seconds	1 (Hold)

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

3. Verify that the appropriate reaction volume is selected for your experiment.

4. Select the passive reference dye.

Option	Description
ROX	TaqPath™ BactoPure™ Microbial Detection Master Mix with ROX™
None	TaqPath™ BactoPure™ Microbial Detection Master Mix (No ROX™)

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

6. Start the run.

Analyze the results

Analysis methods vary between applications. This protocol provides general guidelines for analyzing data generated from experiments that use the TaqPath™ BactoPure™ Microbial Detection Master Mix within user-defined assays. For more detailed information about data analysis or the procedures outlined in this protocol, see the appropriate documentation for your instrument.

Guidelines for analysis

1. View the amplification plot, then modify as needed.
   - Remove outliers from the analysis.
   - Set the automatic baseline and threshold values.

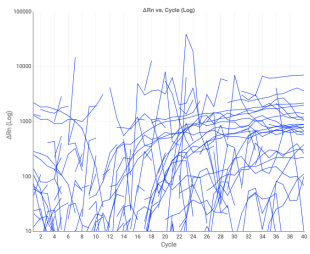
Master mix formulation	Recommended threshold value
TaqPath™ BactoPure™ Microbial Detection Master Mix with ROX™	0.2
TaqPath™ BactoPure™ Microbial Detection Master Mix (No ROX™)	Automatic

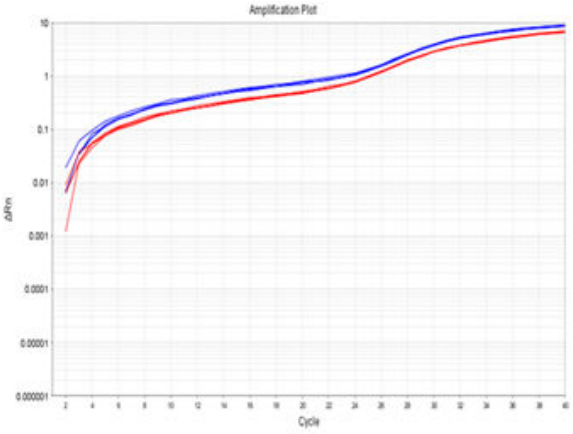
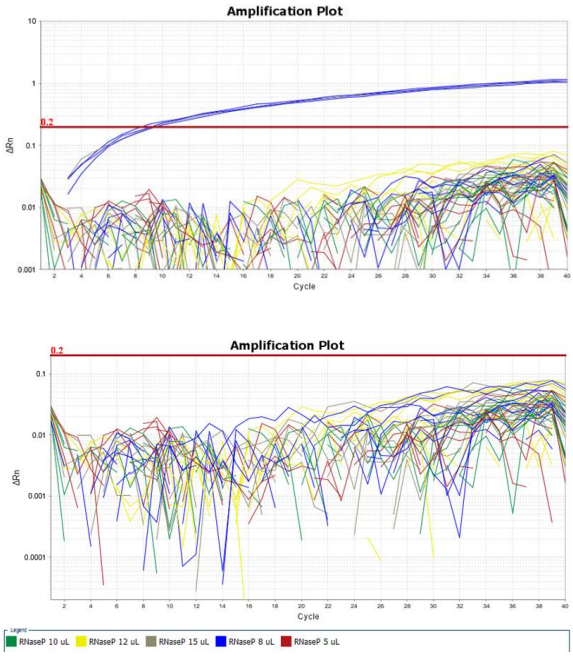
2. In the well table or results table, view the C_t values for each well and for each replicate group.

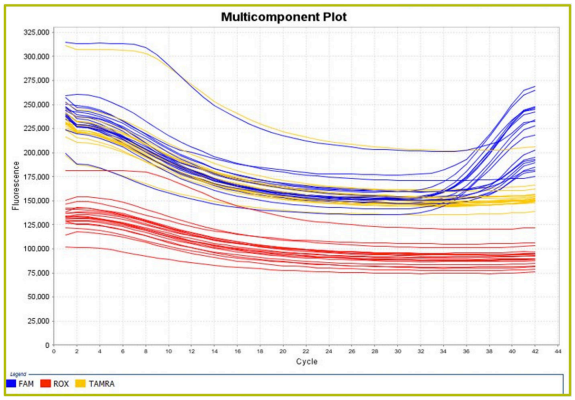
3. *(For standard curve experiments)* View the standard curve for the following items:
   - Slope
   - Amplification efficiency
   - R^2 values
   - Y-intercept
   - C_t values
   - Outliers

A

Troubleshooting

Observation	Possible cause	Recommended action
Small particles during reaction mix setup	The particles are components of the master mix.	No action is required. The particles do not interfere with the reaction and do not affect physical apparatus, including robotics.
Amplification plot shows a high level of noise 	The incorrect dye is set as the passive reference in the run file.	• If using TaqPath™ BactoPure™ Microbial Detection Master Mix with ROX™, change the passive reference to **ROX**, then reanalyze the data. • If using TaqPath™ BactoPure™ Microbial Detection Master Mix (No ROX™), change the passive reference to **None**, then reanalyze the data.
Unexpected results for microbial detection experiments using crude lysates	The assay that was used for the microbial detection experiment is not compatible with crude lysates.	Use a purified DNA sample input for use with the chosen microbial detection assay.
High NTC signal for microbial detection experiments	Some microbial detection assays have a significant NTC signal that is not due to amplification; rather, it is a property of the assay. In the absence of DNA template, some dye cleavage may occur.	No action is required. The signal does not contribute to or interfere with the cluster signal or analysis.

Observation	Possible cause	Recommended action
Amplification curves for samples appear as sigmoidal curves 	When automatic baseline is used, the software selects baseline cycle values that are too narrow.	Set the Amplification Plot's graph type to linear. If amplification appears to begin earlier than cycle 15, adjust the Baseline End Cycle to 2 cycles prior to the start of amplification. For example, if the amplification appears to begin at cycle 13, set the Start Cycle to 3 and End Cycle to 11.
The C_t is low **Note:** Related observations are false positives or high NTC signal. 	When automatic baseline is used, the software selects baseline cycle values that are too narrow.	Evaluate the multi-component plot to ensure that the C_t signal represents a true amplification and not part of the background signal noise.
The C_t value is less than 38 for the NTC	The assay was contaminated.	Follow the guidelines to prevent contamination (see "Prevent contamination" on page 9). Follow each step for assay preparation (see "Prepare the assay" on page 10).

Observation	Possible cause	Recommended action
Unexpected results and the multicomponent plot shows a distorted fluorescent pattern 	The reaction volume, in combination with the pate type, was not correct.	Modify the reaction volume. Add 0.2 µL of RNase *AWAY*™ Decontamination Reagent (Cat. No. 10328011) per reaction.

B

Supplemental information

Master mix components

Hot-Start AmpliTaq™ DNA Polymerase

By providing tight control over *Taq* activation, the hot-start approach helps to prevent undesirable early activity of the polymerase at low temperatures that can lead to nonspecific amplification.

The polymerase is provided in an inactive state to automate the hot-start PCR and allow flexibility in the reaction setup, including pre-mixing of PCR reagents at room temperature. The polymerase is activated after a brief hold step at 95°C.

Heat-labile uracil-N-glycosylase (UNG)

This master mix contains heat-labile uracil-N-glycosylase (UNG; also known as uracil-DNA glycosylase (UDG)). Heat-labile UNG prevents reamplification of carryover PCR products.

Treatment with UNG degrades dU-containing PCR carryover products and misprimed, nonspecific DNA molecules. 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 enzymatic activity occurs during the PCR reaction setup at room temperature; an activation step before thermal cycling is not necessary. Unlike standard UNG, heat-labile UNG is completely inactivated during the first ramp to the high-temperature step for template denaturation and polymerase activation.

PCR products from reactions that include heat-labile UNG are stable for up to 72 hours post-amplification.

dUTP

This Master Mix includes dUTP to enable uracil-N-glycosylase (UNG) activity and maintain optimal PCR results.

Optimized buffer components

This master mix is formulated with optimized buffer components to accommodate multiplex amplification of at least four DNA target sequences (for example, using FAM™ dye, VIC™ dye, ABY™ dye, and JUN™ dye) in a single reaction.

ROX™ Reference Dye

The ROX™ Reference Dye provides an internal reference to which the reporter-dye signal can be normalized during data analysis. Normalization is recommended to correct for fluorescent fluctuations due to noise factors such as changes in concentration or volume.

Best practices for PCR and RT-PCR experiments

Good laboratory practices for PCR and RT-PCR experiments

See “Prevent contamination” on page 9.

Use UNG to prevent false-positive amplification

Carryover amplicons can result in false-positive amplification during PCR. Use a master mix that contains heat-labile uracil-N-glycosylase (UNG; also known as uracil-DNA glycosylase (UDG)) to degrade many contaminating carryover amplicons.

To ensure the desired UNG activity:

- Use PCR components and thermal cycling conditions as specified.
  UNG-containing master mixes incorporate the optimal concentration of UNG to prevent cross-contamination while not affecting real-time PCR performance.
- Do not attempt to use UNG-containing master mixes in subsequent amplification of dU-containing PCR products, such as in nested-PCR protocols. The UNG will degrade the dU-containing PCR products, preventing further amplification.

Although treatment with UNG can degrade or eliminate large numbers of carryover PCR products, use good laboratory practices to minimize cross-contamination from non-dU-containing PCR products or other samples.

Detect fluorescent contaminants

Fluorescent contaminants can generate false positive results. To help detect these contaminants, we recommend including a no-amplification control reaction that contains sample, but no master mix.

After PCR, if the absolute fluorescence of the no-amplification control is greater than the fluorescence of the no template control (NTC), fluorescent contaminants may be present in the sample or in the heat block of the real-time PCR instrument.

C

Assay design guidelines

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

General amplicon site selection guidelines

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

- 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 on "Probe and primer design" on page 19.
- The primer pair must be specific to the target; 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).

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.

This master mix 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 generally 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 68–70°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.
- Ensure 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.

IMPORTANT! Keep the T_m between 58–62°C.

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 following formula:

$$A_{260} = \frac{\text{sum of extinction coefficient contributions} \times \text{cuvette pathlength} \times \text{concentration}}{\text{dilution factor}}$$

Rearrange to solve for concentration:

$$\text{Concentration (C)} = \frac{\text{dilution factor} \times A_{260}}{\text{sum of extinction coefficient contributions} \times \text{cuvette pathlength}}$$

An example calculation of primer concentration

If the primer sequence is `5′-CGTACTCGTTCGTGCTGC-3′`:

- 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 `5′-CGTACTCGTTCGTGCTGC-3′`, 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

Guidelines for multiplexing

Multiplex assays can be established for both quantitative and qualitative PCR assays. For relative quantification experiments, multiplexing can be used to determine the relative expression levels between different gene targets within a single sample. Normalization between different samples is achieved by using a reference gene (typically an abundant housekeeping gene). For qualitative presence/absence experiments, multiplexing can be used to determine the presence or absence of specific targets within a sample.

Up to four targets can be multiplexed in a single reaction depending upon the probes that are selected. A number of additional factors can also affect the results of a multiplex assay, including target abundance, and primer and probe concentrations. For both types of multiplex experiments, the goal is to minimize the difference in the C_t values between singleplex and multiplex reactions.

Target abundance

The amount of target (and endogenous control, if used) in a sample can affect the outcome of PCR results when performing multiplex assays. An example of target abundance arranged according to the C_t range for a typical 40-cycle PCR thermal protocol is shown in the following table. Values will differ for different experimental systems, so it is up to the user to determine the actual threshold to use for each expression level.

Target expression level	C_t range
High	$C_t \leq 20$
Medium	$20 < C_t \leq 27$
Low	$27 < C_t \leq 35$
No template control	$C_t > 38$

Different methods are recommended to offset the effects of target abundance when optimizing multiplex assays.

- **Some targets more abundant than others**
  When multiplex PCR is performed on a sample in which one (or more) target(s) is more abundant than the others, the assay(s) for the abundant species should be primer-limited. Typically, housekeeping genes/endogenous controls are high expressors. Using primer-limited reaction

conditions prevents consumption of reactants (dNTPs) before the less abundant species begins to amplify.

Applied Biosystems™ primer-limited assays have final primer concentrations of 150 nM each with 250 nM probe concentration. This is a suggested starting point for customer optimization.

Note: In addition to limiting primers, for very highly abundant transcripts, probe concentration may need to be adjusted.

- **Targets are of similar abundance**

  In situations where all targets are present in approximately equal abundance, no single assay need be primer-limited. However, assay optimization is recommended to minimize C_t difference between single and multiplex reactions. We recommend starting with 900 nM for each primer and 250 nM for the probe (in the final reaction mix).

- **Either target may be more abundant**

  If any of the targets could be more abundant than the others, depending on the samples being investigated, then all assays need to be primer-limited. Establishing reaction conditions for extreme cases (low/high abundance) is suggested for optimization.

Primer and probe concentration

Optimization of the concentrations of primers and probe for each target is an important first step in assembling a three- or four-color reaction.

In multiplexing, start with a standard condition (e.g., a concentration of 900 nM/900 nM/250 nM for the forward primer/reverse primer/probe in the final PCR reaction volume). Further optimization of the assay may be necessary depending upon your results.

If the required endogenous control target is available as a primer limited assay, you can begin validating your duplex PCR. However, if it is not, you must limit the primer concentration in the assay yourself. The goal of limiting the primer concentration in the assay is to find the primer concentration that gives the lowest (earliest) possible C_t value for the more abundant target without distorting the C_t value of the less abundant target. Limiting the primer concentration for the more abundant target has the effect of lowering its ΔRn; however, the C_t should remain unchanged under primer-limited conditions. A sample should be assayed using decreasing amounts of primer in order to determine the optimal primer concentration for each assay.

Dye selection

Make dye/target assignments to balance fluorescence levels in the multiplex reaction.

- FAM™ and ABY™ dyes can be used with low to medium expressors.
- VIC™ and JUN™ dyes can be used with medium to high expressors.

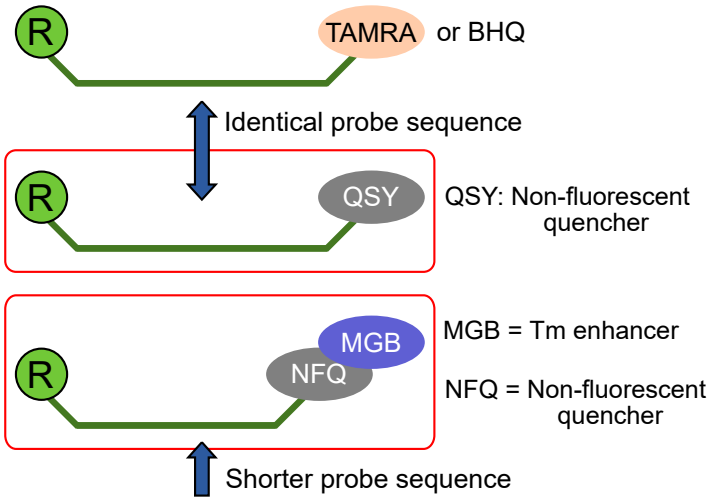
Probe selection

TaqMan™ Assays can use QSY™ probes or MGB probes.

Up to four targets can be multiplexed in a single reaction using QSY™ probes (FAM™ dye, VIC™ dye, ABY™ dye, and JUN™ dye).

Note: Use no more than two probes that contain the MGB group.

FAM™ dye and VIC™ dye are available with MGB/NFQ or QSY™ quenchers. For TaqMan™ Gene Expression Assays, order assays with a non-MGB probe at customorders@lifetech.com.



Verify singleplex reactions

The first step in a successful multiplex experiment is ensuring that your assays work in singleplex reactions with the dyes and quenchers that you have chosen to use in the multiplex reaction.

1. Prepare concentrated assay mix for each assay according to the expression level of the target. Suggested concentrations are given below. Slight changes may be required for optimal performance.

Target Expression Level	Concentration [1]			
	Assay Mix (Final)	Primer 1	Primer 2	Probe 1
High	20X	3 µM	3 µM	5 µM
Medium	20X	6 µM	6 µM	5 µM
Low	20X	18 µM	18 µM	5 µM

[1] Using a 20X assay mix, the respective concentrations of primers and probes in the reactions will be 150 nM/150 nM/250 nM (High), 300 nM/300 nM/250 nM (Medium), and 900 nM/900 nM/250 nM (Low).

2. Prepare the reaction mixtures according to one of the tables below.

96-well (0.2-mL) plate

Component	Singleplex reaction 1	Singleplex reaction 2	Singleplex reaction 3	Singleplex reaction 4
TaqPath™ BactoPure™ Microbial Detection Master Mix	10 µL	10 µL	10 µL	10 µL
FAM™ Assay Mix (20X)	1.0 µL	—	—	—
VIC™ Assay Mix (20X)	—	1.0 µL	—	—
ABY™ Assay Mix (20X)	—	—	1.0 µL	—
JUN™ Assay Mix (20X)	—	—	—	1.0 µL
Template	Up to 9 µL (1 pg to 100 ng)			
Water	To total volume			
Total volume	**20 µL**	**20 µL**	**20 µL**	**20 µL**

384-well plate or 96-well (0.1-mL) plate

Component	Singleplex reaction 1	Singleplex reaction 2	Singleplex reaction 3	Singleplex reaction 4
TaqPath™ BactoPure™ Microbial Detection Master Mix	5 µL	5 µL	5 µL	5 µL
FAM™ Assay Mix (20X)	0.5 µL	—	—	—
VIC™ Assay Mix (20X)	—	0.5 µL	—	—
ABY™ Assay Mix (20X)	—	—	0.5 µL	—
JUN™ Assay Mix (20X)	—	—	—	0.5 µL
Template	Up to 4.5 µL (1 pg to 100 ng)			
Water	To total volume			
Total volume	**10 µL**	**10 µL**	**10 µL**	**10 µL**

3. Mix the components thoroughly, then centrifuge briefly to collect the contents at the bottom of the tube and eliminate any air bubbles.

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

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

6. Perform PCR (see “Prepare the real-time PCR plate” on page 11).

7. Analyze the results.

Verify the multiplex reaction (see “Verify multiplex reaction” on page 26).

Verify multiplex reaction

After performing verification of singleplex reactions, proceed to evaluation and optimization of the multiplex reaction.

For the appropriate concentrations, see “Verify singleplex reactions” on page 24.

1. Combine verified singleplex concentrations in a multiplex reaction and confirm that they work together.

Component	Multiplex (four-plex) reaction	
	384-well plate 96-well plate (10 µL/well)	96-well plate (20 µL/well)
TaqPath™ BactoPure™ Microbial Detection Master Mix	5 µL	10 µL
FAM™ Assay Mix (20X)	0.5 µL	1 µL
VIC™ Assay Mix (20X)	0.5 µL	1 µL
ABY™ Assay Mix (20X)	0.5 µL	1 µL
JUN™ Assay Mix (20X)	0.5 µL	1 µL
Template	Up to 3 µL (1 pg to 100 ng)	Up to 6 µL (1 pg to 100 ng)
Water	To total volume	
Total volume	**10 µL**	**20 µL**

2. Mix the components thoroughly, then centrifuge briefly to collect the contents at the bottom of the tube 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 the plate briefly collect the contents at the bottom of the wells and eliminate any air bubbles.
5. Perform PCR (see “Prepare the real-time PCR plate” on page 11).

Evaluate PCR results

Evaluate multiplex the real-time PCR results to verify that the reaction efficiency, ΔC_t between singleplex and multiplex reactions, and standard deviation of replicates are not compromised using the selected multiplex conditions. Ideally there should be no difference between the results from single and multiplex reactions under the selected conditions.

- Reaction efficiency
  - Make a dilution series of the sample containing seven 10-fold dilutions in triplicate. Run each assay individually and in multiplex using each dilution in the series.
  - The standard curve method is recommended to evaluate (optimize) multiplex assays. Run as many 10-fold dilution points, in triplicate, as possible for the sample(s) and assays being investigated for each assay singly and in multiplex. A minimum of 3 logs should be used, but up to 6 logarithmic units is ideal. Ensure that the dynamic range of the standard curve is broad enough to encompass most of the experimental samples, bearing in mind that the expression levels of the target(s) of interest may vary widely between samples
  - Take a careful look at the standard curve to verify that there is a good fit of the line to all the dilution points, and that the correlation coefficient (R^2) of the line is 0.98 or higher. A lower R^2 value indicates that some of the dilutions (usually the lowest, highest, or both) do not fall within the range of the standard curve. For more information, go to **thermofisher.com/qpcreducation**.
  - Results are analyzed in a plot of *log*[template amount]$_{(x\text{-axis})}$ against C_t value$_{(y\text{-axis})}$.
  - The slope of the line is used to calculate the PCR efficiency using the formula:
    Efficiency = $10^{(-1/\text{slope})}$ - 1
  - The target efficiency for 5 to 6 logarithmic units should be 100% +/- 10% in both singleplex and multiplex reactions. If there are significant differences, re-optimize the primer and probe concentrations.
- ΔC_t between singleplex and multiplex reactions
  - Using the dilution series, calculate ΔC_t value between the target in singleplex and multiplex reactions. The ΔC_t values between multiplex and singleplex reactions should be as close as possible (e.g., $\Delta C_t \leq 1$).
  - Differences in C_t between single and multiplex reactions can often be mitigated by adjusting primer concentrations. Adjustments following the general guidelines provided under may be required (see “Verify singleplex reactions” on page 24).
- Standard deviation
  - A high standard deviation of the C_t indicates that other factors, such as competition or inhibition in the multiplex reaction, are contributing to the lack of reproducibility. In general, a C_t standard deviation variation of less than 3% indicates good reproducibility.
  - Determine the standard deviations of samples assayed as single and multiplex reactions. High standard deviations of C_t values in multiplex reactions can often be minimized by adjustments to the cycling conditions. Increasing the anneal/extend times (to 30 to 45 seconds) is suggested if the standard deviations in multiplex reactions increase relative to singleplex.

D Experimental design guidelines

User-defined assays

To design your own assay for use with the master mix, see *TaqMan™ Multiplex PCR Optimization User Guide* (Pub. No. MAN0010189).

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

Select your experiment type

Select one of the following experiment types:

- Presence/absence
- Relative quantification
- Standard curve

Guidelines for real-time PCR

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 might 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™ BactoPure™ Microbial Detection Master Mix	Keep the master mix at 4°C, protected from light, until you are ready to use it.

(continued)

Item	Guideline
Storing combined master mix and assay	You can combine the master mix and the assay ahead of time and store at room temperature for up to 24 hours, or 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.
Thermal-cycling temperature ranges	The optimal temperatures for annealing are recommended in this protocol (see "Prepare the real-time PCR plate" on page 11). However, in some instances you may wish to alter the temperatures. The 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 19.
Multiplexing	TaqPath™ BactoPure™ Microbial Detection Master Mix is designed to accommodate running multiple assays simultaneously. For guidelines on designing multiplex reactions, see *TaqMan™ Multiplex PCR Optimization User Guide* (Pub. No. MAN0010189).

Recommended types of reactions

For each experiment type, the following types of reactions are needed.

Experiment type	Reaction type	Description
Presence/absence	Unknown	A well that contains: • Sample (DNA in which the presence of a target is unknown) • TaqPath™ BactoPure™ Microbial Detection Master Mix • 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.

(continued)

Experiment type	Reaction type	Description
Presence/absence	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. The wells contain identical components and volumes. Performing at least three replicates of each reaction is recommended.
Relative quantification	Unknown	A well that contains: • Sample (DNA for which the quantity of a target is unknown) • TaqPath™ BactoPure™ Microbial Detection Master Mix • Assay of choice • Assay for reference gene
	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. The wells contain identical components and volumes. Performing at least three replicates of each reaction is recommended.
Standard curve	Unknown	A well that contains: • Sample (DNA in which the quantity of the target is unknown) • TaqPath™ BactoPure™ Microbial Detection Master Mix • 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. The wells contain identical components and volumes. Performing at least three replicates of each reaction is recommended.

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, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. 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.

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
  https://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.
QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide	MAN0010407
QuantStudio™ 6 and 7 Flex Real-Time PCR Systems Maintenance and Administration Guide	4489821
QuantStudio™ Design and Analysis Desktop Software User Guide	MAN0010408
QuantStudio™ Design and Analysis v2 User Guide	MAN0018202
QuantStudio™ Real-Time PCR Software Getting Started Guide (for use with QuantStudio™ 6 and 7 Flex Real-Time PCR Systems)	4489822
TaqMan™ Assay Multiplex PCR Optimization Application Guide	MAN0010189

Customer and technical support

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  - 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/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**.