TaqPath™ 1-Step Multiplex Master Mix

USER GUIDE

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For Laboratory Use.

ThermoFisher SCIENTIFIC

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For descriptions of symbols on product labels or product documents, go to **thermofisher.com/symbols-definition**.

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

Revision history: Pub. No. MAN0014269

Revision	Date	Description
B.0	20 April 2020	• Correct volume of master mix to be added to verify singleplex and multiplex reactions. • Added QuantStudio™ 6 Pro and 7 Pro Real-Time PCR Systems. • Updated information about the components of the master mix. • Updated information about good laboratory practices. • Added related documentation.
A.0	16 March 2016	New document.

Contents

1 Product information

Product description

The Applied Biosystems™ TaqPath™ 1-Step Multiplex Master Mix is designed to perform 1-step reverse transcription real-time PCR applications with any gene-specific primer and probe sets. This master mix is formulated with optimized buffer components to accommodate multiplex amplification of up to four RNA or DNA target sequences (for example, using FAM™ dye, VIC™ dye, ABY™ dye, and JUN™ dye) in a single reaction.

The master mix is designed for use with a user-defined and user-supplied assay to perform 1-step multiplex real-time PCR 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*.
- **Relative quantification**—A quantification experiment performed to determine the relative expression levels between different gene targets within a single sample. Results are then compared across different samples to determine differences in target levels. Normalization between different samples is achieved by using a reference gene (typically an abundant housekeeping gene such as β-actin). Relative quantification experiments are also referred to as *gene expression analysis 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 cycle threshold (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 PCR.

Types of the master mix

The master mix is available in two versions.

- TaqPath™ 1-Step Multiplex Master Mix containing MUSTANG PURPLE™ dye as a passive reference dye.
- TaqPath™ 1-Step Multiplex Master Mix (No ROX™), which does not contain a passive reference dye.

The master mix contains the following components:

- Fast DNA Polymerase
- Thermostable MMLV enzyme
- Uracil-N-glycosylase (UNG)
- dNTPs including dUTP
- RNase inhibitor
- MUSTANG PURPLE™ dye (not included in the no ROX™ dye version)
- Buffer components optimized for maximum sensitivity and tolerance to several common RT and real-time PCR inhibitors

About the kit

Contents and storage

The master mix is supplied at a 4X concentration.

Product	Cat. No.	Amount	No. of 20–µL reactions	Storage
TaqPath™ 1-Step Multiplex Master Mix (No ROX™)	A28521	1 × 0.5 mL	100	–30°C to –10°C
	A28522	5 × 1 mL	1000	
	A28523	1 × 10 mL	2000	
TaqPath™ 1-Step Multiplex Master Mix	A28525	1 × 0.5 mL	100	
	A28526	5 × 1 mL	1000	
	A28527	1 × 10 mL	2000	

Stability

Performance of the master mix is guaranteed until the expiration date printed on the package and bottle labels.

Note: The TaqPath™ 1-Step Multiplex Master Mix might have a faint green hue. The TaqPath™ 1-Step Multiplex Master Mix (No ROX™) might have a faint yellow hue.

Required materials not supplied

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

Item	Source
One of the following Applied Biosystems™ instruments:	
• QuantStudio™ 6 Pro Real-Time PCR System or QuantStudio™ 7 Pro Real-Time PCR System • QuantStudio™ 3 or 5 Real-Time PCR System [1] • QuantStudio™ 6 / QuantStudio™ 7 Flex Real-Time PCR System • QuantStudio™ 12K Flex Real–Time PCR System • QuantStudio™ Dx Real-Time PCR Instrument • ViiA™ 7 Real-Time PCR System • 7500 Real-Time PCR System • 7500 Fast Real-Time PCR System • 7500 Fast Dx Real-Time PCR instrument Or use a compatible real-time PCR instrument from another supplier.	Contact your local sales office.
Equipment	
Centrifuge with adapter for 96- or 384-well plates	MLS
Laboratory mixer (vortex 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 kits	
Nuclease-free Water (not DEPC-Treated)	4387936
TE, pH 8.0	AM9849

[1] The QuantStudio™ 3 System can be used for limited multiplexing. It is not compatible with MUSTANG PURPLE™ dye or Cy5™ dye.

Workflow

Perform assay screening and multiplex assay optimization

See the *TaqMan™ Assay Multiplex PCR Optimization User Guide* (Pub. No. MAN0010189)

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2 Methods

General guidelines

Prevent contamination

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

For guidelines on recommended laboratory procedures, see Appendix E, “Best practices for PCR and RT-PCR experiments”.

Assays and thermal cycling conditions

The master mix is optimized for use with primers and hydrolysis probes.

Optimize and validate each assay independently to ensure appropriate performance. Validate multiplexed assays and re-optimize your thermal cycling conditions as needed (see “Guidelines for multiplexing” on page 25).

Run modes

The master mix can be run on either Fast or Standard cycling systems, 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. For thermal cycling profiles for fast and standard modes, see “Set up a plate document or experiment” on page 12.

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
7500 Real-Time PCR System	Standard
7500 Fast Real-Time PCR System or 7500 Fast Dx Real-Time PCR instrument	Standard/Fast
QuantStudio™ Dx Real-Time PCR Instrument	Standard/Fast
ViiA™ 7 Real-Time PCR System	Standard/Fast

Real-Time PCR System[1]	Available run modes
QuantStudio™ 6 Pro Real-Time PCR System or QuantStudio™ 7 Pro Real-Time PCR System	Standard/Fast
QuantStudio™ 3 or 5 Real-Time PCR System	Standard/Fast
QuantStudio™ 6 / QuantStudio™ 7 Flex Real-Time PCR System	Standard/Fast
QuantStudio™ 12K 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 samples

Thermo Fisher Scientific offers products appropriate for nucleic acid purification from different sample types.

Isolate and purify the target nucleic acid samples according to your laboratory practices.

Starting template

The master mix can be used with both RNA and DNA targets.

The reverse transcription step does not affect thermal cycling performance with DNA targets. We recommend using TaqPath™ qPCR Master Mix, CG (Cat. No. A15297) when performing experiments designed to assay only DNA targets.

Sample storage conditions

Store the prepared RNA samples at –85°C in RT-PCR Grade Water. Store DNA samples at –20°C in TE buffer.

If you dilute your samples, use TE buffer or RT-PCR Grade Water as the diluent.

Set up a plate document or experiment

Fast real-time PCR systems

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

- Recommended 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 (e.g. Fast mode on fast instruments and Standard mode on standard instruments)

- Thermal-cycling conditions for sample volumes ≤30 µL:

Step	Stage	Cycles	Temperature	Time
UNG incubation	1	1	25°C	2 minutes
Reverse transcription[1]	2	1	53°C	10 minutes
Polymerase activation[2]	3	1	95°C	2 minutes
Amplification	4	40	95°C	3 seconds
			60°C	30 seconds

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

Standard real-time PCR systems

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

- Recommended 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	Stage	Cycles	Temperature	Time
UNG incubation	1	1	25°C	2 minutes
Reverse transcription[1]	2	1	53°C	10 minutes
Polymerase activation[2]	3	1	95°C	2 minutes
Amplification	4	40	95°C	15 seconds
			60°C	1 minute

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

Prepare RT–real-time PCR reaction mix

Prepare the RT–real-time PCR reaction mix for 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 the contents at the bottom of the tube.
4. Ensure that there are no gel lumps remaining.

   Repeat step 2 and step 3, if necessary, to ensure a homogeneous solution.
5. Calculate the total volume required for each reaction component according to the table below:

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

Component	Volume (one 20–µL reaction)	Notes
TaqPath™ 1-Step Multiplex Master Mix (4X)	5 µL	—
Up to four user-defined assays (primers and probe)[1]	1 µL	Use primer concentrations of 150–900 nM and a probe concentration of 100–250 nM.[2]
RNA Sample	Variable	Use as much sample as needed, up to the maximum allowed by the reaction volume.
RT-PCR Grade Water	Variable	Fill to the total reaction volume.
Total volume per reaction	**20 µL**	**—**

[1] Potential assays include the TaqMan™ Assay Mix, FAM™ dye (20X); TaqMan™ Assay Mix, VIC™ dye (20X); TaqMan™ Assay Mix, ABY™ dye (20X); and TaqMan™ Assay Mix, JUN™ dye (20X).

[2] Determine optimal primer and probe concentrations (see “Guidelines for multiplexing” on page 25).

6. Working on ice, add the components directly to each well of an optical reaction plate.

7. Cover the reaction plate with an optical adhesive cover and 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.

   IMPORTANT! The TaqPath™ 1-Step Multiplex Master Mix is a 4X formulation and is more viscous than most master mixes. Ensure that all of the components are thoroughly mixed in all the wells before proceeding. Inverting the plate gives more uniform mixing across the reaction plate than vortexing.

8. Centrifuge at 150 × *g* (1000 rpm) for 1 minute to spin down the contents and eliminate air bubbles.

Prepare the RT–real-time PCR reaction mix for 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 the contents at the bottom of the tube.
4. Ensure that there are no gel lumps remaining.

   Repeat step 2 and step 3, if necessary, to ensure a homogeneous solution.
5. Calculate the total volume required for each reaction component according to the table below:

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

Component	Volume (one 50–µL reaction)	Notes
TaqPath™ 1-Step Multiplex Master Mix (4X)	12.5 µL	—
Up to four user-defined assays (primers and probe)[1]	2.5 µL	Use primer concentrations of 150–900 nM and a probe concentration of 100–250 nM.[2]
RNA Sample	Variable	Use as much sample as needed, up to the maximum allowed by the reaction volume.
RT-PCR Grade Water	Variable	Fill to the total reaction volume.
Total volume per reaction	**50 µL**	**—**

[1] Potential assays include the TaqMan™ Assay Mix, FAM™ dye (20X); TaqMan™ Assay Mix, VIC™ dye (20X); TaqMan™ Assay Mix, ABY™ dye (20X); and TaqMan™ Assay Mix, JUN™ dye(20X).

[2] Determine optimal primer and probe concentrations (see "Guidelines for multiplexing" on page 25).

6. Working on ice, add the components directly to each well of an optical reaction plate.

7. Cover the reaction plate with an optical adhesive cover and 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.

   IMPORTANT! The TaqPath™ 1-Step Multiplex Master Mix is a 4X formulation and is more viscous than most master mixes. Ensure that all of the components are thoroughly mixed in all the wells before proceeding. Inverting the plate gives more uniform mixing across the reaction plate than vortexing.

8. Centrifuge at 150 × *g* (1000 rpm) for 1 minute to spin down the contents and eliminate air bubbles.

Run the RT–real-time PCR plate

1. In the real-time PCR system software, open the plate document or experiment that corresponds to the reaction plate.
2. Verify that the appropriate reaction volume is selected for your experiment.
3. Select the passive reference dye.

Option	Description
MUSTANG PURPLE™	TaqPath™ 1-Step Multiplex Master Mix
None	TaqPath™ 1-Step Multiplex Master Mix (No ROX™)

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

Analyze the data

Because analysis methods vary between applications, this protocol provides general guidelines for analyzing data generated from experiments that use TaqPath™ 1-Step Multiplex Master Mix in 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, and modify as needed.
   - Set the baseline and threshold values.
   - 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 the following items:
   - Slope
   - Amplification efficiency
   - R^2 values
   - Y-intercept
   - C_t values
   - Outliers

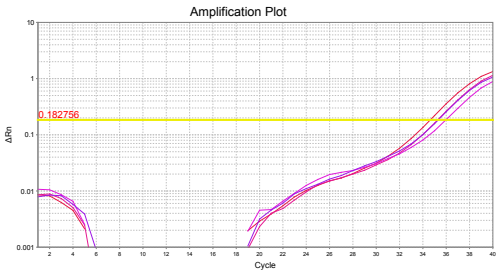
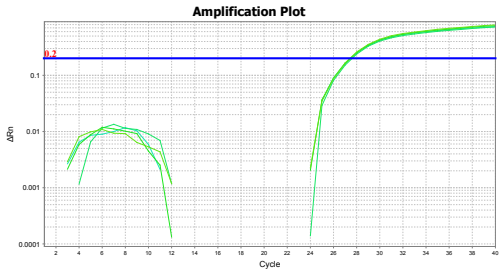
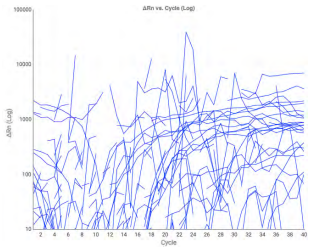
Overview of baseline and threshold values

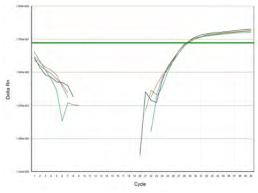
You can use the real-time PCR system software to set the baseline and threshold values for the amplification plot, either automatically or manually.

- 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.

Troubleshooting

This section provides troubleshooting information for the TaqPath™ 1-Step Multiplex Master Mix. For general real-time PCR troubleshooting information, see the appropriate guide for your instrument.

Observation	Possible cause	Recommended action
The amplification plot displays S-shaped curves. 	The RT-qPCR mix was not thoroughly combined.	When preparing the RT-qPCR mix, be sure to invert the reaction plate firmly 3–5 times to mix before centrifuging (see "Prepare RT–real-time PCR reaction mix" on page 14).
The amplification plot is truncated. 	The baseline was set too high.	When analyzing the data, manually reset the baseline, or use the automatic baseline function (see "Analyze the data" on page 16).
Amplification plot shows a high level of noise when the No-ROX mix is used. 	ROX is set as the passive reference in the run file.	Change the passive reference to "None" and reanalyze the data.

Observation	Possible cause	Recommended action
The 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, change analysis setting to allow manual adjustment of the threshold, then manually adjust the threshold bar (see "Analyze the data" on page 16).
		Evaluate the multi-component plot to ensure that the C_t signal represents a true amplification and not part of the background signal noise.

B

Master mix components

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/dTTP

This master mix includes a blend of dUTP/dTTP to enable uracil-N-glycosylase (UNG) activity and maintain optimal PCR results.

AmpliTaq™ DNA Polymerase

The AmpliTaq™ DNA Polymerase, UP, is provided in an inactive state to automate the hot start PCR technique and allow flexibility in the reaction setup, including pre-mixing of PCR reagents at room temperature.

The polymerase is equipped with a proprietary hot start mechanism that improves specificity. The polymerase is re-activated after a brief hold at 95°C.

MUSTANG PURPLE™ dye

MUSTANG PURPLE™ dye can act as a passive reference (absorption 647 nm; emission 654 nm) to provide an internal reference for normalizing the reporter-dye signal during data analysis for multiplex reactions. Normalization corrects for fluorescence fluctuations due to changes in concentration or volume.

For multiplex reactions, MUSTANG PURPLE™ dye can be used instead of the typical ROX™ reference dye. This replacement allows the use of JUN™ dye which is detected by the same channel as ROX™ dye.

For more information about multiplex reactions, see the *TaqMan™ Assay Multiplex PCR Optimization User Guide* (Pub. No. MAN0010189).

Assay design guidelines

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

Amplicon site selection

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.

- For gene expression assays, 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 on “Probe and Primer Design” on page 23.
- 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).

If the gene does not contain introns

For gene expression assays, if the gene you are studying does not contain introns, then you cannot ensure amplification of the target cDNA sequence without co-amplification 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.

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 provides a highly reproducible and sensitive assay.

Note: The reverse transcriptase enzyme 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. A minimal amount of the expression vector may potentially 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.

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.
- 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 58–60°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	8400
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 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	$C_t > 35$

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.

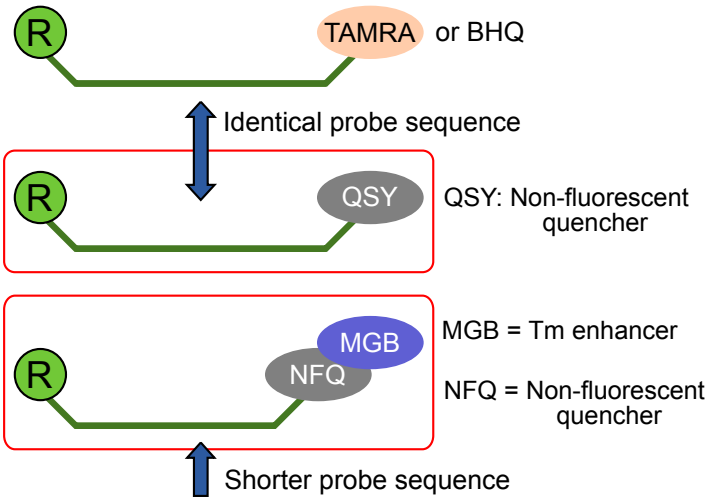
Probe selection

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.

Table 1 96-well Standard (0.2-mL) Plate

Component	Singleplex reaction 1	Singleplex reaction 2	Singleplex reaction 3	Singleplex reaction 4
TaqPath™ 1-Step Multiplex Master Mix	5 µL	5 µL	5 µL	5 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 11 µL (1 pg to 100 ng)			
Water	To total volume			
Total	**20 µL**	**20 µL**	**20 µL**	**20 µL**

Table 2 384-well plate or 96-well Fast (0.1-mL) Plate

Component	Singleplex reaction 1	Singleplex reaction 2	Singleplex reaction 3	Singleplex reaction 4
TaqPath™ 1-Step Multiplex Master Mix	2.5 µL	2.5 µL	2.5 µL	2.5 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 5.5 µL (1 pg to 100 ng)			
Water	To total volume			
Total	**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 “Run the RT–real-time PCR plate” on page 16).

7. Analyze the results.

Verify the multiplex reaction (see “Verify multiplex reaction” on page 29).

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 27.

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 Fast plate (10 µL/well)	96-well Standard plate (20 µL/well)
TaqPath™ 1-Step Multiplex Master Mix	2.5 µL	5 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 5.5 µL (1 pg to 100 ng)	Up to 11 µL (1 pg to 100 ng)
Water	To total volume	
Total	**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 "Run the RT–real-time PCR plate" on page 16).

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 27).

- 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™ 1-Step Multiplex Master Mix	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. **Note:** 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.

Item	Guideline
Storing combined master mix and assay	You can combine the master mix 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 1-step RT real-time PCR is detecting genomic DNA rather than a particular RNA species, you can run a no-RT control reaction with the master mix. To run a no-RT control, heat-kill the RT enzyme by heating one aliquot of master mix at 95°C 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 12). However, in some instances you may wish to alter the temperatures. • The RT enzyme will function best in the range of 48–55°C. • 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 23.
Multiplexing	TaqPath™ 1-Step Multiplex 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 or RNA in which the presence of a target is unknown) • TaqPath™ 1-Step Multiplex Master Mix • Assay of choice

Experiment type	Reaction type	Description
Presence/absence	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. 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 or RNA for which the quantity of a target is unknown) • TaqPath™ 1-Step Multiplex 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 or RNA in which the quantity of the target is unknown) • TaqPath™ 1-Step Multiplex 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.

Experiment type	Reaction type	Description
Standard curve	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.

E Best practices for PCR and RT-PCR experiments

Good laboratory practices for PCR and RT-PCR

- 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.
- Change gloves if you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation and reaction setup.
  - Amplification and analysis of products.
- Do not bring amplified products into the reaction setup area.
- Open and close all sample tubes carefully. Avoid splashing or spraying samples.
- Keep reactions and components capped as much as possible.
- Use a positive-displacement pipettor or aerosol-resistant barrier pipette tips.
- Clean lab benches and equipment periodically with 10% bleach solution or DNA decontamination solution.

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.

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.

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.

F

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.

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

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

Biological hazard safety

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

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

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2009-P.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

Documentation and support

Related documentation

Document	Pub. No.
TaqPath™ 1-Step Multiplex Master Mix Quick Reference	MAN0014389
TaqPath™ 1-Step Multiplex Master Mix Product Information Sheet	MAN0014069
TaqMan™ Multiplex PCR Optimization User Guide	MAN0010189

Customer and technical support

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

- Worldwide contact telephone numbers
- Product support information
  - Product FAQs
  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support
- Product documentation
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

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

Limited product warranty

Life Technologies Corporation and/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**.