

# TaqMan™ Assay Multiplex PCR Optimization

## APPLICATION GUIDE

for use with:

TaqMan™ Gene Expression Assays

TaqMan™ SNP Genotyping Assays

TaqMan™ MicroRNA Assays

TaqMan™ Advanced miRNA Assays

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Revision F



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For descriptions of symbols on product labels or product documents, go to [thermofisher.com/symbols-definition](https://thermofisher.com/symbols-definition).

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Revision	Date	Description
F	26 August 2025	<ul style="list-style-type: none"><li>The branding was updated to TrueMark™ OpenArray™ Plate.</li><li>The list of products to support multiplex PCR was updated (“Thermo Fisher Scientific products for multiplex PCR” on page 7).</li><li>The information in the following sections was updated for the available products:<ul style="list-style-type: none"><li>“Overview of TaqMan™ Assays” on page 8</li><li>“Probes” on page 9</li><li>“Overview of reporter dyes and quenchers” on page 10</li><li>“TaqMan™ QSY™ probes and TaqMan™ QSY2™ probes” on page 10</li><li>“Probe selection” on page 29</li></ul></li><li>The Genotyping Analysis Module in Diomni™ Design and Analysis (RUO) Software 3 was added as an option to analyze SNP genotyping assays, with the limitation of automatic calling only if more than one SNP genotyping assay is assigned to one well.</li><li>The primer concentration for a target with medium expression level was updated to 350 nM (“Prepare singleplex PCR reactions” on page 35).</li><li>The primer concentration was updated to from 300 nM to 350 nM in the list of conditions when determining the optimal primer concentration (“Determine the optimal primer concentration” on page 55).</li></ul>
E00	14 March 2024	Updates made for additional dye and probe information.
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## About this guide

This guide is designed to give guidance to researchers for performing multiplex PCR with TaqMan™ Assays. It covers the following items:

- Guidance on optimizing multiplex PCR reactions
- Information to facilitate the performance of multiplex PCR and decrease the amount of time required to perform optimization
- Guidance on verification of multiplex PCR reaction performance
- Performing multiplex PCR with up to six probes

## Overview of multiplex PCR

Multiplex PCR is the simultaneous amplification of multiple targets in a single reaction tube. Different reporters with distinct fluorescence spectra are used in multiplex PCR to track each individual amplification reaction. The real-time PCR instrument detects a signal from each reporter dye and determines the amount of each target.

Multiplex PCR can reduce the amount of sample that is required for real-time PCR because it measures more than one target in a single reaction. It also can reduce reaction costs compared to performing 6 singleplex reactions independently.

## Applications for multiplex PCR

The simplest application for multiplexing is to amplify one target gene and one endogenous control in a shared reaction well, using probes labeled with two distinct fluorescent dyes and the same pool of enzymes, nucleotides, and other reagents.

Multiplex PCR with multiple reporter dyes in one well is best suited for non-quantitative applications, such as genotyping and pathogen detection. For quantitative reactions, higher throughput formats such as 384-well plates and TrueMark™ OpenArray™ Plates provide an alternative methodology (spatial multiplexing). The disadvantages of spatial multiplexing, where the sample is divided into smaller reactions, is that the sensitivity of the assay is typically reduced.

## Optimization and verification of multiplex PCR

Multiplex PCR is as sensitive and as accurate as amplifying a single gene, but it is more technically complex. Multiplex PCR conditions must be optimized, and it is necessary to verify the multiplex PCR.

The following factors can affect the reliability of multiplex PCR assays:

- Competition or inhibition between assays through interactions among the various primer pairs, probes, targets, amplicons, or any combination of these factors
- The relative expression levels of targets, including endogenous controls, and the range of their expression
- The efficiency of the multiplex assays
- Primer concentration affected by polymerase saturation

## Thermo Fisher Scientific products for multiplex PCR

Thermo Fisher Scientific offers a complete multiplex PCR solution using TaqMan™ predesigned or custom assays that enables up to a 6-plex reaction for gene expression and up to a 6-plex reaction for genotyping analysis. The solution includes the following products, all of which are described in this guide.

- Up to six dyes optimized to work together with minimal spectral overlap
- Probes with an MGB-NFQ quencher
- Custom probes with an MGB-NFQ quencher
- Custom probes with a QSY™ quencher
- Custom probes with a QSY2™ quencher
- A set of master mixes optimized for multiplex PCR
- Spectral calibration plates designed for multiplex PCR

These, combined with our custom services and real-time PCR instruments and software, decrease the amount of time required to optimize your multiplex PCR. Optimization of reaction components such as magnesium is typically not necessary.

## Overview of *TaqMan*™ Assays

Predesigned primer and probe sets are available for gene expression, genotyping, and microRNA analysis.

**Table 1** Reporter and quencher dye options for predesigned *TaqMan*™ Gene Expression Assays

Reporter dye options	Standard	Primer-limited	Quencher dye	Ordering
FAM™ dye	Yes	No	MGB-NFQ	<a href="http://thermofisher.com">thermofisher.com</a>
FAM™ dye	No	Yes	MGB-NFQ	<a href="mailto:specialty_oligos@thermofisher.com">specialty_oligos@thermofisher.com</a>
VIC™ dye	Yes	Yes	MGB-NFQ	<a href="http://thermofisher.com">thermofisher.com</a>
ABY™ dye	Yes	Yes		<a href="mailto:specialty_oligos@thermofisher.com">specialty_oligos@thermofisher.com</a>
JUN™ dye			MGB-NFQ	
Cyanine 5 dye				
FAM™ dye	Yes	Yes		<a href="mailto:specialty_oligos@thermofisher.com">specialty_oligos@thermofisher.com</a>
VIC™ dye			QSY™ quencher	
ABY™ dye				
JUN™ dye				
Cyanine 5 dye	Yes	Yes	QSY2™ quencher	<a href="mailto:specialty_oligos@thermofisher.com">specialty_oligos@thermofisher.com</a>
Cyanine 5.5 dye				

**Table 2** Reporter and quencher dye options for predesigned *TaqMan*™ SNP Genotyping Assays

Reporter dye options	Quencher dye	Ordering
FAM™ dye VIC™ dye	MGB-NFQ	<a href="http://thermofisher.com">thermofisher.com</a>
ABY™ dye JUN™ dye	MGB-NFQ	<a href="mailto:specialty_oligos@thermofisher.com">specialty_oligos@thermofisher.com</a>
FAM™ dye VIC™ dye ABY™ dye JUN™ dye	QSY™ quencher	<a href="mailto:specialty_oligos@thermofisher.com">specialty_oligos@thermofisher.com</a>

We recommend redesigning *TaqMan*™ SNP Genotyping Assays when using a QSY™ quencher. The probes must be longer with a QSY™ quencher. This can affect specificity.

**Table 3** Reporter and quencher dye options for predesigned TaqMan™ MicroRNA Assays and TaqMan™ Advanced miRNA Assays

Reporter dye options	Available as primer-limited	Quencher dye	Ordering
FAM™ dye	No	MGB-NFQ	<a href="http://thermofisher.com">thermofisher.com</a>
VIC™ dye	No	MGB-NFQ	<a href="mailto:specialty_oligos@thermofisher.com">specialty_oligos@thermofisher.com</a>

Custom versions of the assays can be ordered ([specialty\\_oligos@thermofisher.com](mailto:specialty_oligos@thermofisher.com)).

Custom assays with different dyes and quenchers are required for more targets.

## Probes

Product	Reporter dye	Quencher dye	Cat. No.	Amount
TaqMan™ MGB Probe	FAM™ dye or VIC™ dye	MGB-NFQ	4316034	6,000 pmol
			4316033	20,000 pmol
			4316032	50,000 pmol
TaqMan™ ABY™ MGB Probe	ABY™ dye	MGB-NFQ	A40005000	6,000 pmol
			A40005001	20,000 pmol
			A40005002	50,000 pmol
TaqMan™ JUN™ MGB Probe	JUN™ dye	MGB-NFQ	A40005003	6,000 pmol
			A40005004	20,000 pmol
			A40005005	50,000 pmol
TaqMan™ Cy®5 MGB Probe	Cyanine 5 dye	MGB-NFQ	A40005006	6,000 pmol
			A40005007	20,000 pmol
			A40005008	50,000 pmol
TaqMan™ QSY™ Probe	FAM™ dye, VIC™ dye, ABY™ dye, or JUN™ dye	QSY™ quencher	4482777	6,000 pmol
			4482778	20,000 pmol
			4482779	50,000 pmol
TaqMan™ QSY2™ Probe	Cyanine 5 dye or Cyanine 5.5 dye	QSY2™ quencher	A56899	6,000 pmol
			A56900	20,000 pmol
			A56901	50,000 pmol

## Overview of reporter dyes and quenchers

The sequence, the reporter dye, and the quencher must be appropriate for the probe used in the assay. The quencher should be able to quench the reporter dye. The absorbance range of the quencher must sufficiently overlap with the emission range of the reporter dye.

The choice of quencher can affect the Tm of the probe and therefore its compatibility with the real-time PCR experiment. For example, a probe sequence paired with MGB-NFQ has a higher Tm compared to the same sequence paired with a QSY™ quencher or QSY2™ quencher. It is important to consider quencher choice when designing probes and calculating Tm. For questions about reporter dye and quencher compatibility, contact Technical Support.

For the available combinations of reporter dyes and quencher dyes for each assay, see “Overview of TaqMan™ Assays” on page 8.

Alternatively, you can design assays with Cyanine 5 and/or Cyanine 5.5 dyes and the QSY2™ quencher. If used with MGB-NFQ, the probe sequence needs to be shorter than if used with a QSY™ non-fluorescent quencher to obtain a similar Tm.

MicroRNA assays are predesigned with a FAM™ reporter dye and MGB-NFG quencher. Due to the short length of the microRNA, longer additional probes are not recommended.

## TaqMan™ QSY™ probes and TaqMan™ QSY2™ probes

QSY™ and QSY2™ probe non-fluorescent quenchers can be used for designing custom TaqMan™ QSY™ and QSY2™ probes. QSY™ and QSY2™ custom probes can be substituted for the 3' TAMRA™ probe, the 3' BHQ™-1 probe, and some BHQ™-2 probes without redesigning the probe sequence. These probes maintain the same Tm.

Changing from a QSY™ or QSY2™ probe to an MGB-NFQ probe requires a redesign to maintain the same Tm.

Custom TaqMan™ QSY™ Probes are available with FAM™ dye, VIC™ dye, ABY™ dye and JUN™ dye. Custom TaqMan™ QSY2™ probes are available with Cyanine 5 and Cyanine 5.5 dyes.

**Table 4** Absorbance and emission wavelengths for recommended dyes

Dye <sup>[1]</sup>	Absorbance	Emission
FAM™ dye	496 nm	520 nm
VIC™ dye	532 nm	552 nm
ABY™ dye	568 nm	583 nm
JUN™ dye	606 nm	618 nm
Cyanine 5	649 nm	667 nm
Cyanine 5.5	678 nm	695 nm

<sup>[1]</sup> Other dyes can function in multiplex PCR reactions but the dyes listed in this table are recommended.

They can be used to detect up to the following number of targets:

- Six targets with TaqMan™ Gene Expression Assays
- Two SNPs in TaqMan™ SNP Genotyping Assays

# Master mixes for multiplex PCR

When assays are amplified together, they compete for the same reagents, including dNTPs, Mg<sup>2+</sup>, and polymerase. The more targets that are assayed in a multiplex reaction, the more likely it is that the assays will compete for reagents and be inhibited. A master mix that is developed for specific multiplex PCR assays is recommended.

**Note:** The master mixes listed in the table below all contain heat-labile UNG.

Master Mix	Assays	Template	Passive reference dye	Inhibitor tolerance	Stability of fully assembled reaction	Cycling mode
TaqPath™ BactoPure™ Microbial Detection Master Mix	Microbial detection	DNA	Available without a passive reference dye or with ROX™ passive reference dye.	Tolerant of PCR inhibitors typically found in biopharmaceutical, molecular diagnostic, and research applications from both purified samples and crude lysates	72 hours	Standard or fast
TaqPath™ ProAmp™ Master Mix	Genotyping	gDNA	Available with ROX™ or MUSTANG PURPLE™ passive reference dye <sup>[1]</sup>	Compatible with samples prepared from human or animal sources (buccal swabs, blood, and card punches)	72 hours	
TaqPath™ DuraPlex™ 1-Step RT-qPCR Master Mix	Gene expression Pathogen detection	RNA	Available without a passive reference dye or with ROX™ passive reference dye	Tolerant of inhibitors commonly found in clinical samples (VTM, blood, stool, and urine)	8 hours	

<sup>[1]</sup> MUSTANG PURPLE™ dye is not compatible with StepOne™ and StepOnePlus™ instruments, 7300 Real-Time PCR System, or QuantStudio™ 3 Real-Time PCR System.

## TaqPath™ BactoPure™ Microbial Detection Master Mix

TaqPath™ BactoPure™ Microbial Detection Master Mix is optimized for rapid low-level microbial detection even in the presence of PCR inhibitors.

The master mix is available in two formulations:

- TaqPath™ BactoPure™ Microbial Detection Master Mix (No ROX™)—Does not contain a passive reference dye
- TaqPath™ BactoPure™ Microbial Detection Master Mix—Contains ROX™ passive reference dye

Both versions of the master mix have the following benefits:

- Consistent low-level detection of bacterial, fungal, mammalian, and viral DNA, as well as antibiotic resistance markers
- Tolerant of PCR inhibitors typically found in biopharmaceutical, molecular diagnostic, and research applications from both purified samples and crude lysates
- Wide linear dynamic range<sup>[1]</sup> (up to 8 logs) enabling accurate detection with both low- and high-DNA-concentration samples
- Optimized for multiplexing—Available in two formulations that enable one- to four-target detection per reaction
- 72 pre-PCR benchtop stability of pre-assembled<sup>[2]</sup> reactions, enabled by our proprietary hot-start mechanism
- Compliance package containing the most frequently requested documents, available via the product page

The master mix contains the following components:

- Hot-start AmpliTaq<sup>™</sup> Fast DNA Polymerase
- Heat-labile Uracil-DNA Glycosylase (UNG)
- dNTPs (with dUTP)
- Optimized buffer

For more information, see *TaqPath<sup>™</sup> BactoPure<sup>™</sup> Microbial Detection Master Mix User Guide* (Pub. No. MAN0025689).

**Table 5 TaqPath<sup>™</sup> BactoPure<sup>™</sup> Microbial Detection Master Mix (No ROX<sup>™</sup>)**

Cat. No.	Unit size	Number of 20- $\mu$ L reactions	Storage
A52703	1 x 1 mL	100	2–8°C
A52704	1 x 5 mL	500	
A52705	5 x 1 mL	500	
A52706	1 x 50 mL	5,000	

<sup>[1]</sup> Dynamic range is a function of the assay and template concentration in the sample, as well as the formulation of the master mix; thus, individual results may vary.

<sup>[2]</sup> Pre-PCR stability is influenced not only by the master mix, but also by the target being analyzed. Stability profiles of specific targets should be confirmed.



**Table 6** **TaqPath™ BactoPure™ Microbial Detection Master Mix**

Cat. No.	Unit size	Number of 20- $\mu$ L reactions	Storage
A52699	1 x 1 mL	100	2–8°C in the dark
A52700	1 x 5 mL	500	
A52701	5 x 1 mL	500	
A52702	1 x 50 mL	5,000	

## TaqPath™ ProAmp™ Master Mix

TaqPath™ ProAmp™ Master Mix is used for high-throughput genotyping experiments that require accurate results from samples containing PCR inhibitors. It is designed to deliver sensitive and reproducible results from genomic DNA targets. It is compatible with multiplex PCR for up to four targets.

The master mix is available in two formulations:

- TaqPath™ ProAmp™ Master Mix—Contains ROX™ passive reference dye
- TaqPath™ ProAmp™ Multiplex Master Mix—Contains MUSTANG PURPLE™ passive reference dye

Both formulations of the master mix contains the following components:

- Dual-Lock™ Taq DNA Polymerase
- Heat-labile uracil-DNA glycosylase (UDG or UNG)
- dNTPs with dUTP
- Optimized buffer components

For more information, see *TaqPath™ ProAmp™ Master Mixes User Guide* (Pub. No. MAN0015758).

**Table 7** TaqPath<sup>™</sup> ProAmp<sup>™</sup> Multiplex Master Mix

Cat. No.	Amount	Number of 20- $\mu$ L reactions	Storage
A30868	1 mL	100	2–8°C in the dark
A30869	1 x 10 mL	1,000	
A30870	1 x 50 mL	5,000	
A30873	2 x 10 mL	2,000	
A30874	2 x 50 mL	10,000	

**Table 8** TaqPath<sup>™</sup> ProAmp<sup>™</sup> Master Mix

Cat. No.	Amount	Number of 20- $\mu$ L reactions	Storage
A30865	1 mL	100	2–8°C in the dark
A30866	1 x 10 mL	1,000	
A30867	1 x 50 mL	5,000	
A30871	2 x 10 mL	2,000	
A30872	2 x 50 mL	10,000	

## TaqPath<sup>™</sup> DuraPlex<sup>™</sup> 1-Step RT-qPCR Master Mix

TaqPath<sup>™</sup> DuraPlex<sup>™</sup> 1-Step RT-qPCR Master Mix allows cDNA synthesis and PCR amplification in the same well. This minimizes pipetting and hands-on time.

The master mix is available in two formulations.

- TaqPath<sup>™</sup> DuraPlex<sup>™</sup> 1-Step RT-qPCR Master Mix (No ROX<sup>™</sup>)—Does not contain a passive reference dye
- TaqPath<sup>™</sup> DuraPlex<sup>™</sup> 1-Step RT-qPCR Master Mix—Contains ROX<sup>™</sup> passive reference dye

Both formulations of the master mix are supplied at a 4X concentration and contain the following components:

- AmpliTaq™ DNA Polymerase with Hot-start
- Thermostable MMLV reverse transcriptase
- dNTPs including dATP, dGTP, dCTP, dTTP, dUTP
- Recombinant ribonuclease inhibitor
- ROX™ passive reference dye (not included in TaqPath™ DuraPlex™ 1-Step RT-qPCR Master Mix (No ROX™))
- Buffer components optimized for sensitivity and tolerance to several common RT-PCR inhibitors

Both versions of the master mix have the following benefits:

- Assembled reaction can be stored at room temperature for up to 8 hours
- Workflows requiring benchtop stable reagents
- High sensitivity to detect low-copy targets with reproducible  $C_t$  results
- Six logs of dynamic range with both RNA and DNA targets
- Multiplex PCR of up to five targets simultaneously with a passive reference dye or six targets without a passive reference dye
- Tolerance of inhibitors commonly found in clinical samples

For more information, see *TaqPath™ DuraPlex™ 1-Step RT-qPCR Master Mix User Guide* (Pub. No. [MAN0028387](#)).

**Table 9 TaqPath™ DuraPlex™ 1-Step RT-qPCR Master Mix (No ROX™)**

Cat. No.	Amount	Number of 20- $\mu$ L reactions	Storage
A58669	0.5 mL	100	-25°C to -15°C
A58670	5 x 1 mL	1,000	
A58671	10 mL	2,000	

**Table 10 TaqPath™ DuraPlex™ 1-Step RT-qPCR Master Mix**

Cat. No.	Amount	Number of 20- $\mu$ L reactions	Storage
A58666	0.5 mL	100	-25°C to -15°C
A58667	5 x 1 mL	1,000	
A58668	10 mL	2,000	

## MUSTANG PURPLE™ passive reference dye

The MUSTANG PURPLE™ passive reference dye has an absorption wavelength of 647 nm and an emission wavelength of 654 nm. It provides an internal reference for normalizing the reporter-dye signal during data analysis. Normalization corrects for fluctuations in fluorescence due to changes in concentration or volume.

MUSTANG PURPLE™ dye is used in place of the typical ROX™ passive reference dye to allow the use of JUN™ dye. ROX™ dye and JUN™ dye are detected with the same filter.

## Required materials not supplied

### Real-time PCR instruments

The following real-time PCR instruments can be used for multiplex PCR:

- QuantStudio™ 3 and 5 Real-Time PCR Systems
- QuantStudio™ 6 / QuantStudio™ 7 Flex System
- QuantStudio™ 6 Pro and 7 Pro Real-Time PCR Systems
- QuantStudio™ 12K Flex Real-Time PCR System
- ViiA™ 7 Real-Time PCR System
- 7500 Real-Time PCR System
- 7500 Fast Real-Time PCR System

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**Note:** The software for the 7500 Fast Real-Time PCR System does not support assigning two SNP assays to one well, but analysis can be done with the Genotyping application on Thermo Fisher™ Connect.

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- StepOne™ and StepOnePlus™ Real-Time PCR Systems

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**Note:** The StepOne™ Real-Time PCR System has three filters, which permits it to run a duplex with FAM™ dye and VIC™ dye, and use a master mix that contains ROX™ as the passive reference dye. The StepOnePlus™ Real-Time PCR System has four filters and can be used for three dyes with ROX™ as the passive reference dye. Neither system supports assigning two SNP assays to one well.

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**IMPORTANT!** Do not use the StepOne™ Real-Time PCR System and StepOnePlus™ Real-Time PCR System for more than two concurrent assays.

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## Filter selection

Table 11 Filters for QuantStudio<sup>™</sup> 3 and 5 Real-Time PCR Systems

Dye	System and filter	
	QuantStudio <sup>™</sup> 3 System	QuantStudio <sup>™</sup> 5 System
FAM <sup>™</sup> dye	x1m1	x1m1
VIC <sup>™</sup> dye	x2m2	x2m2
ABY <sup>™</sup> dye	x3m3	x3m3
ROX <sup>™</sup> <sup>[1]</sup> or JUN <sup>™</sup> dye	x4m4	x4m4
MUSTANG PURPLE <sup>™</sup> <sup>[1]</sup> or Cyanine 5 dye	N/A	x5m5
Cyanine 5.5 dye <sup>[2]</sup>	N/A	x6m6

<sup>[1]</sup> Used as a passive reference dye.

<sup>[2]</sup> The QuantStudio<sup>™</sup> 5 384-well instrument has only five filters.





Table 12 Filters for ViiA™ 7 Real-Time PCR System, QuantStudio™ 6 and 7 Flex Real-Time PCR Systems, QuantStudio™ 6 Pro and 7 Pro Real-Time PCR Systems, and QuantStudio™ 12K Flex Real-Time PCR System

Dye	System and filter			
	ViiA™ 7 Real-Time PCR System	QuantStudio™ 6 Flex System and QuantStudio™ 6 Pro System	QuantStudio™ 7 Flex System and QuantStudio™ 7 Pro System	QuantStudio™ 12K Flex Real-Time PCR System
FAM™ dye	x1m1	x1m1	x1m1	x1m1
VIC™ dye	x2m2	x2m2	x2m2	x2m2
ABY™ dye	x3m3	x3m3	x3m3	x3m3
ROX™ <sup>[1]</sup> or JUN™ dye	x4m4	x4m4	x4m4	x4m4
MUSTANG PURPLE™ <sup>[1]</sup> or Cyanine 5 dye	x5m5	x5m5	x5m5	x5m5
Cyanine 5.5 dye	x6m6	N/A	x6m6	x6m6

<sup>[1]</sup> Used as a passive reference dye.

Table 13 Filters for 7500 Real-Time PCR System and 7500 Fast Real-Time PCR System

Dye	System and filter	
	7500 Real-Time PCR System	7500 Fast Real-Time PCR System
FAM™ dye	Filter A	Filter A
VIC™ dye	Filter B	Filter B
ABY™ dye	Filter C	Filter C
ROX™ <sup>[1]</sup> or JUN™ dye	Filter D	Filter D
MUSTANG PURPLE™ <sup>[1]</sup> or Cyanine 5 dye	Filter E	Filter E

<sup>[1]</sup> Used as a passive reference dye.

**Table 14** Filters for StepOne<sup>™</sup> and StepOnePlus<sup>™</sup> Real-Time PCR Systems

Dye	System and filter	
	StepOne <sup>™</sup> Real-Time PCR System	StepOnePlus <sup>™</sup> Real-Time PCR System
FAM <sup>™</sup> dye	Filter 1	Filter 1
VIC <sup>™</sup> dye	Filter 2	Filter 2
ABY <sup>™</sup> dye	N/A	Filter 3
ROX <sup>™</sup> <sup>[1]</sup> dye	Filter 3	Filter 4

[1] Used as a passive reference dye.

## Spectral calibration plates

It is recommended that calibration and verification of your real-time instrument be performed as outlined in the instrument user guide.

For spectral calibration on the 7500 Real-Time PCR System or the 7500 Fast Real-Time PCR System, set up custom calibration files for FAM<sup>™</sup> dye and VIC<sup>™</sup> dye plates using new file names.

**Note:** Cyanine 5.5 dye calibration material at 1X concentration can be ordered through [specialty\\_oligos@thermofisher.com](mailto:specialty_oligos@thermofisher.com).

For use with ViiA<sup>™</sup> 7 Real-Time PCR Systems, QuantStudio<sup>™</sup> 6 and 7 Flex Real-Time PCR Systems running software older than v1.5, order the Cyanine 5 dye calibration plate or 1x material through [specialty\\_oligos@thermofisher.com](mailto:specialty_oligos@thermofisher.com).

**IMPORTANT!** Do not overwrite the original system FAM<sup>™</sup> dye and VIC<sup>™</sup> dye calibration files. See your instrument user guide.

**Note:** The plates listed below are multi-dye checkerboard plates.

Calibration Plate	Source	Real-Time PCR System	
		QuantStudio™ 3 and 5 Real-Time PCR Systems <sup>[1]</sup>	QuantStudio™ 6 Pro and 7 Pro Real-Time PCR Systems and ViiA™ 7 Real-Time PCR Systems <sup>[2]</sup>
<b>Standard 96-well plates (0.2 mL)</b>			
QuantStudio™ 3/5 Spectral Calibration Plate 1, 96-Well 0.2-mL (FAM™, VIC™, ROX™, SYBR™ dyes)	<a href="#">A26331</a>	✓	✓
QuantStudio™ 3/5 Spectral Calibration Plate 2, 96-Well 0.2-mL (ABY™, JUN™, MUSTANG PURPLE™ dyes)	<a href="#">A26332</a>	✓	✓
QuantStudio™ 3/5 Spectral Calibration Plate 3, 96-Well 0.2-mL (TAMRA™, NED™, Cyanine 5 dyes)	<a href="#">A26333</a>	✓	✓
<b>Fast 96-well plates (0.1 mL)</b>			
QuantStudio™ 3/5 Spectral Calibration Plate 1, (FAM™, VIC™, ROX™, and SYBR™ dyes), 96-well, 0.1 mL	<a href="#">A26336</a>	✓	✓
QuantStudio™ 3/5 Spectral Calibration Plate 2 (ABY™, JUN™, MUSTANG PURPLE™ dyes), 96-well (0.1-mL) plate	<a href="#">A26337</a>	✓	✓
QuantStudio™ 3/5 Spectral Calibration Plate 3 (TAMRA™, NED™ and Cyanine 5 dyes) 96-well (0.1-mL) plate	<a href="#">A26340</a>	✓	✓
<b>384-well plates</b>			
QuantStudio™ 5 Spectral Calibration Plate 1, (FAM™, VIC™, ROX™, TAMRA™, and SYBR™ dyes), 384-well	<a href="#">A26334</a>	✓	✓
QuantStudio™ 5 Spectral Calibration Plate 2 (ABY™, JUN™, MUSTANG PURPLE™, NED™, and Cyanine 5 dyes), 384-well	<a href="#">A26335</a>	✓	✓

[1] The instrument must be configured with the appropriate block.

[2] QuantStudio™ 6 Pro and 7 Pro Real-Time PCR Systems and ViiA™ 7 Real-Time PCR Systems require software v1.6 or later to use these multi-dye calibration plates.

Calibration Plate	Source	Real-Time PCR System		
		7500 Real-Time PCR System	7500 Fast Real-Time PCR System	QuantStudio™ 6 and 7 Flex Real-Time PCR Systems, QuantStudio™ 12K Flex Real-Time PCR System, and ViiA™ 7 Real-Time PCR Systems <sup>[1, 2]</sup>
<b>Standard 96-well plates (0.2 mL)</b>				
ABY™ Dye Spectral Calibration Plate for Multiplex qPCR, 96-well	<a href="#">A24738</a>	✓	—	✓
JUN™ Dye Spectral Calibration Plate for Multiplex qPCR, 96-well	<a href="#">A24737</a>	✓	—	✓
FAM™ Dye Spectral Calibration Plate, 96-well	<a href="#">4432327</a>	✓	—	✓
VIC™ Dye Spectral Calibration Plate, 96-well	<a href="#">4432334</a>	✓	—	✓
MUSTANG PURPLE™ Dye Spectral Calibration Plate for Multiplex qPCR, 96-well	<a href="#">4461599</a>	✓	—	✓
<b>Fast 96-well plates (0.1 mL)</b>				
ABY™ Dye Spectral Calibration Plate for Multiplex qPCR, Fast 96-well	<a href="#">A24734</a>	—	✓	✓
JUN™ Dye Spectral Calibration Plate for Multiplex qPCR, Fast 96-well	<a href="#">A24735</a>	—	✓	✓
FAM™ Dye Spectral Calibration Plate, Fast 96-well	<a href="#">4432389</a>	—	✓	✓
VIC™ Dye Spectral Calibration Plate, Fast 96-well	<a href="#">4432396</a>	—	✓	✓
MUSTANG PURPLE™ Dye Spectral Calibration Plate for Multiplex qPCR, Fast 96-well	<a href="#">4457328</a>	—	✓	✓
<b>384-well plates</b>				
ABY™ Dye Spectral Calibration Plate for Multiplex qPCR, 384-well	<a href="#">A24736</a>	—	—	✓

(continued)



Calibration Plate	Source	Real-Time PCR System		
		7500 Real-Time PCR System	7500 Fast Real-Time PCR System	QuantStudio™ 6 and 7 Flex Real-Time PCR Systems, QuantStudio™ 12K Flex Real-Time PCR System, and ViiA™ 7 Real-Time PCR Systems <sup>[1, 2]</sup>
JUN™ Dye Spectral Calibration Plate for Multiplex qPCR, 384-well	<a href="#">A24733</a>	—	—	✓
FAM™ Dye Spectral Calibration Plate, 384-well	<a href="#">4432271</a>	—	—	✓
VIC™ Dye Spectral Calibration Plate, 384-well	<a href="#">4432278</a>	—	—	✓
MUSTANG PURPLE™ Dye Spectral Calibration Plate for Multiplex qPCR, 384-well	<a href="#">4457334</a>	—	—	✓

<sup>[1]</sup> The instrument must be configured with the appropriate block.

<sup>[2]</sup> ViiA™ 7 Real-Time PCR Systems require software v1.5 or earlier to use these spectral dye calibration plates.

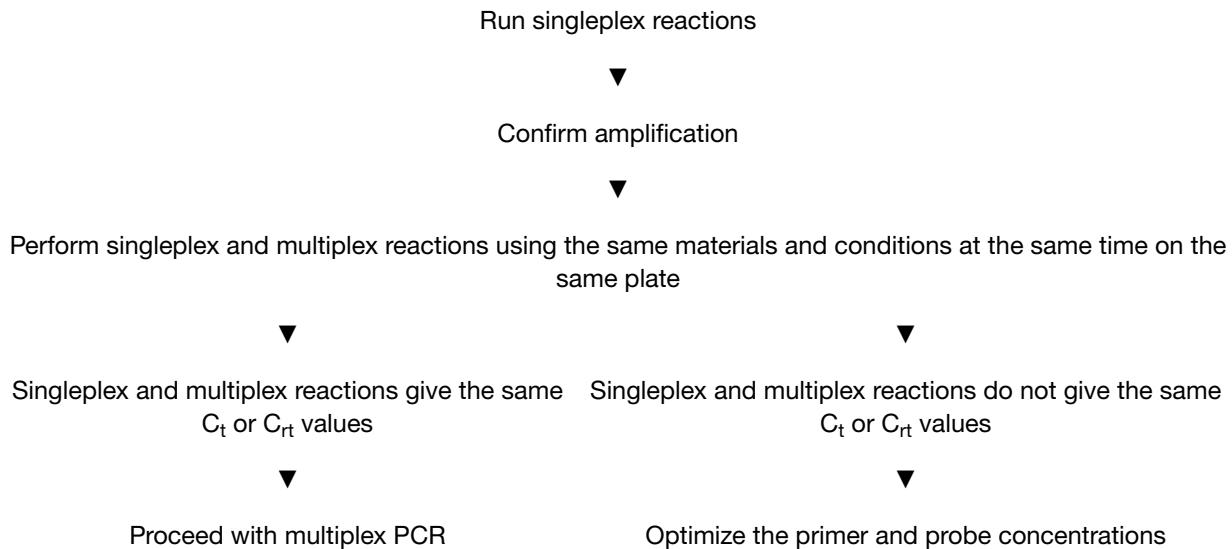
## Other materials and equipment not supplied

For compatible instruments, see “Real-time PCR instruments” on page 18.

**Table 15 Other materials and equipment required for the workflow**

Item	Source
<b>Software</b>	
Primer Express™ Software	<a href="#">4363991</a>
TaqMan™ Genotyper Software	<a href="#">thermofisher.com/qpcrsoftware</a>
Diomni™ Design and Analysis (RUO) Software 3 with Genotyping Analysis Module	<a href="#">thermofisher.com/qpcrsoftware</a>
<b>Equipment</b>	
Centrifuge, with adapter for 96-well plates or adapter for 384-well plates	MLS
Microcentrifuge	MLS
Vortex mixer	MLS
Pipettes	MLS
<b>Master Mixes</b>	
TaqPath™ BactoPure™ Microbial Detection Master Mix	<a href="#">A52699, A52700, A52701, A52702, A52703 A52704, A52705, A52706</a>
TaqPath™ ProAmp™ Multiplex Master Mix	<a href="#">A30870</a>
TaqPath™ DuraPlex™ 1-Step RT-qPCR Master Mix	<a href="#">A58667</a>
<b>Tubes, plates, and other consumables</b>	
Tubes, plates, and film	<a href="#">thermofisher.com/plastics</a>
Aerosol-resistant barrier pipette tips	MLS
Disposable gloves	MLS
<b>Reagents</b>	
Nuclease-free water	<a href="#">AM9930</a>
TE, pH 8.0, RNase-free	<a href="#">AM9849</a>

## Workflow



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**Note:** For gene expression assays, it is acceptable for the  $C_t$  or  $C_{rt}$  values to be within one cycle of each other.

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# Overview of component selection for multiplex PCR

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## Primer design

We recommend starting with predesigned TaqMan™ Assay primer and probe sets, which are optimized for reaction components such as magnesium. A bioinformatics check should be done on TaqMan™ Assays that are intended for multiplexing.

Refer to the multiplexing support link (<https://www.thermofisher.com/us/en/home/global/forms/life-science/assay-support.html>) available on the Thermo Fisher Assay Design Hub website.

If a custom-designed assay is needed, use the following guidelines.

- Primer design should include bioinformatics analysis to minimize non-target binding to the template DNA, to the probe, or to each other.
- The Tm of each PCR primer should be between 58–60°C, and the Tm of both primers to be used in the reaction should ideally be within 1–2°C of each other.
- For more information, see “Guidelines for primer design” on page 53.

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**Note:** We recommend using Primer Express™ Software to calculate the Tm the primers and the Tm of the probe. Alternatively, use the Custom Assay Design Tool, available at [Assay Design Tool](#). Other primer–design software may provide different Tm values due to different assumptions in the algorithm.

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## Probe design

- For gene expression experiments, the Tm of probes should be approximately 10°C higher than the Tm of the primers (approximately 68–70°C).
- For SNP genotyping, the Tm of the probes should be approximately 65–67°C and ideally within 1°C of each other.
- NFQ-MGB probe sequences are not interchangeable with TaqMan™ QSY™/QSY2™ probe sequences. The MGB-NFQ probe length is shorter.
- For custom–designed probes, see *Primer Express™ Software Version 3.0 Getting Started Guide* (Pub. No. 4362460).
- To have predesigned assays redesigned, email [specialty\\_oligos@thermofisher.com](mailto:specialty_oligos@thermofisher.com). Include the ID of the assay that you want redesigned from an MGB-NFQ probe to a QSY™/QSY2™ probe. You will receive ordering instructions for the QSY™/QSY2™ version of the assay.

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**IMPORTANT!** We recommend verification of the multiplex assay. Use samples that are representative of your experiment and confirm that the dynamic range of your multiplex assay meets the needs of your experiment. Contact Technical Support for assistance with validation.

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## Primer and probe selection

The guidelines and tools described in this section are for designing assays. They are not applicable to pre-designed assays.

- A multiplex reaction can contain up to twelve primers and six probes (to produce six amplicons). It is good practice to minimize conditions that result in primer-dimer formation or other unfavorable interactions by optimizing primer concentrations and avoiding complementary sequences.
- We recommend avoiding degenerate oligos. It is preferable to synthesize each oligo separately and mix them together in equal proportions.
- Confirm that amplicons do not overlap. If the amplicon coordinates are not known, map genomic assays to the genome, or gene expression assays to the transcriptome.
- Use the following tools to verify coordinates:
  - UCSC *In Silico* PCR tool: [genome.ucsc.edu/cgi-bin/hgPcr](http://genome.ucsc.edu/cgi-bin/hgPcr)
  - NCBI Basic Local Alignment Search Tool: [blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi)
- Confirm that amplicons are all approximately the same size, generally less than 150 bp, and that primer and probe dimers do not form (across all primer pairs).
- Use the Multiple Primer Analyzer tool at [thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html](http://thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html).

## Probe selection

For the available combinations of reporter dyes and quencher dyes for each assay, and ordering information, see “Overview of TaqMan™ Assays” on page 8.

- You can use predesigned assays containing FAM™ dye– or VIC™ dye–labeled MGB–NFQ probes (also referred to as MGB probes) in multiplex reactions.
- For the third and fourth dye, we recommend using ABY™ dye– and JUN™ dye–labeled probes made with either MGB–NFQ or QSY™ quencher.
- For the fifth dye, we recommend using a Cyanine 5 dye–labeled probe made with either MGB–NFQ or QSY2™ quencher.
- For the sixth dye, we recommend using a Cyanine 5.5 dye–labeled probe made with a QSY2™ quencher.
- Multiplex gene expression analysis can use gene expression assays with the following setup:
  - FAM™ dye with MGB–NFQ quencher or QSY™ quencher
  - VIC™ dye with MGB–NFQ quencher or QSY™ quencher
  - ABY™ dye with MGB–NFQ or QSY™ quencher
  - JUN™ dye with MGB–NFQ or QSY™ quencher
  - Cyanine 5 dye with MGB–NFQ or QSY2™ quencher
  - Cyanine 5.5 dye with QSY2™ quencher
- Multiplex SNP genotyping analysis can use SNP genotyping assays with the following setup:
  - FAM™ dye and VIC™ dye with MGB–NFQ or QSY™ quencher
  - ABY™ dye and JUN™ dye with MGB–NFQ or QSY™ quencher
- MicroRNA assays can be duplexed using a FAM™ dye– and VIC™ dye–labeled assay with MGB–NFQ.
- Custom probes can be ordered to help with concentration optimization. They can be ordered with QSY™ or QSY2™ quenchers. Order at [www.thermofisher.com/taqman-primers-probes](http://www.thermofisher.com/taqman-primers-probes).
- Probes with FAM™, VIC™, ABY™, and JUN™ dyes can be made with either a MGB–NFQ or QSY™ quencher. Changing the quencher requires a redesign of the probe to maintain correct Tm.
- Probes with Cyanine 5 dye can be made with either a MGB–NFQ or QSY2™ quencher.
- Probes with Cyanine 5.5 dye can be made only with QSY2™ quenchers.

## Dye selection

- Choose dyes that are compatible with the instrument and the other dyes used for the multiplex PCR. See the appropriate instrument user guide for dye compatibility.
- Match dye intensity with target abundance by pairing the brightest dye with low abundance targets, and the dimmest dye with high abundance targets (for example, an internal positive control).

## PCR reaction buffer

- All assays are amplified in the same tube. They compete for the same reagents (dNTPs, Mg<sup>2+</sup>, and polymerase).
- The more targets that are assayed in a multiplex reaction, the more likely it is that there is competition for reagents and inhibition between assays.
- Multiplex master mixes are recommended to make up for the effect of competition for reagents (see “Other materials and equipment not supplied” on page 25).

## Instrument calibration

- Calibrate the real-time instrument for the dyes being used in the assay.
- The instrument must be calibrated for MUSTANG PURPLE™ dye if it is used as the passive reference dye.
- Using more dyes in a reaction well increases the importance of high-quality spectral calibration.

## Passive reference dye

Do not use the default passive reference setting (ROX™ dye) when using MUSTANG PURPLE™ dye as a passive reference. They are read in different channels. Confirm that the passive reference is set to MUSTANG PURPLE™ dye when it is used as a passive reference. After adding a MUSTANG PURPLE™ custom dye calibration, it appears as an option in the menu. For information on the calibration process, see the appropriate guide for your instrument.

## Assignment of reporter dyes

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**IMPORTANT!** It is critical to calibrate the instrument for the dyes used in the experiment and to configure the instrument with the correct dyes in each well. If dyes are not assigned to their wells or dyes that are not used are assigned, the analysis will not be valid.

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Label the wells in the plate with the appropriate dyes because individual dye components overlap with adjacent dye channels.

**Table 16** Absorbance and emission wavelengths for recommended dyes

Dye <sup>[1]</sup>	Absorbance	Emission
FAM™ dye	496 nm	520 nm
VIC™ dye	532 nm	552 nm
ABY™ dye	568 nm	583 nm
JUN™ dye	606 nm	618 nm

**Table 16 Absorbance and emission wavelengths for recommended dyes (continued)**

Dye <sup>[1]</sup>	Absorbance	Emission
Cyanine 5	649 nm	667 nm
Cyanine 5.5	678 nm	695 nm

<sup>[1]</sup> Other dyes can function in multiplex PCR reactions but the dyes listed in this table are recommended.

Overlapping spectra from each dye in a well and across all filters generate the composite spectrum that represents a raw data fluorescent reading. The instrument distinguishes the contribution of each dye to the raw spectral data collected. This process is called multicomponenting. Refer to your instrument user guide to determine which dyes your instrument detects.

The instrument compares the raw spectra with a set of pure dye standards contained in the calibration file. When a plate document is saved after data analysis, the software stores the pure spectra information with the rest of the collected fluorescent data for that experiment.

After calibration of the instrument for each dye, the instrument can subtract the background from the dye that bleeds into the other dye. For example, the spectra for VIC™ dye and FAM™ dye overlap to some extent. If a probe with FAM™ dye is in the well and the software label is VIC™ dye, the instrument will read the fluorescence of the probe as VIC™ dye, which usually gives a value (though weaker than a FAM™ dye value). This is normal. On an instrument calibrated for both dyes, the algorithm accounts for the overlap.

The instruments allow the user to reassign the wells after the run is complete, provided that the data has been collected for the correct filter.

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## Overview of gene expression analysis

Gene expression analysis is 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 such as  $\beta$ -actin or 18S rRNA. In gene expression multiplexing experiments, the goal is to minimize the difference between the  $C_t$  value in singleplex and multiplex reactions.

TaqMan™ Gene Expression Assays can be multiplexed because both predesigned and custom assays are available with different fluorescent reporter dyes (see “Overview of TaqMan™ Assays” on page 8).

The most common duplex reaction is combining the target of interest and endogenous control assays in the same well. The most common duplex reaction uses FAM™ dye and VIC™ dye as reporter dyes with non-fluorescent MGB quencher. An endogenous control gene or set of genes is typically used to control for experimental variability due to RNA input and shows expression levels that are relatively constant and moderately abundant across tissues, cell types, and treatment protocols. For targets that are highly expressed (for example, 18S rRNA, a common endogenous control), we recommend choosing the VIC-primer-limited (VIC\_PL) assay format, which has a decreased amount of primers to limit the housekeeping gene from consuming the qPCR reagents before the target of interest can amplify.

See *TaqMan™ Gene Expression Assays User Guide—single-tube assays* (Pub. No. 4333458) and *Introduction to Gene Expression Getting Started Guide* (Pub. No. 4454239).

Up to six targets can be multiplexed in a single reaction, depending on the probes that are selected and the number of filters available on the real-time PCR instrument. In this case, optimization of primers and probes for each target is required.

# Procedural guidelines

## Target abundance

The amount of target and endogenous control in a sample can affect the outcome of PCR results when performing multiplex assays. Abundance can be characterized by the  $C_t$  range.

**Table 17** Target expression levels

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	$C_t > 35$

## Recommendations for target abundance

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

Target properties	Recommendations
Some targets more abundant than others	<ul style="list-style-type: none"><li>When multiplex PCR is performed on a sample in which one or more targets is more abundant than the others, the assays for the abundant species should be primer-limited. See “Primer and probe concentration” on page 34.</li><li>Housekeeping genes and endogenous controls are highly expressed.</li><li>Using primer-limited reaction conditions prevents consumption of reactants (dNTPs) before the less abundant target is amplified, by forcing the PCR reaction to plateau due to the lack of primers.</li><li>For very highly abundant transcripts or especially bright assays, probe concentration might need to be adjusted. See “Guidelines for primer design” on page 53.</li></ul>
Targets are of similar abundance	<ul style="list-style-type: none"><li>When all targets are present in approximately equal abundance, no single assay needs to be primer-limited.</li><li>Assay optimization is recommended to minimize the <math>C_t</math> difference between single and multiplex reactions.</li><li>Start with 900 nM for each primer and 250 nM for the probe in the final reaction mix.</li></ul>
Either target may be more abundant	<ul style="list-style-type: none"><li>If any of the targets could be more abundant than the others, all assays need to be primer-limited.</li><li>Establishing reaction conditions for extreme cases of low or high abundance is suggested for optimization.</li></ul>

## Primer and probe concentration

Optimization of the concentrations of primers and probe for each target is an important first step in assembling a multiplex reaction. The same considerations apply for both 1-step and 2-step RT-PCR.

In multiplex reactions, start with a standard condition. Optimization of the assay may be necessary.

- Forward primer—900 nM
- Reverse primer—900 nM
- Probe—250 nM

If one or more targets are more abundant than the others, the assays should be primer-limited.

Primer-limited TaqMan™ Assays have a final primer concentration of 150 nM each with 250 nM probe concentration. This is a suggested starting point to optimize for abundant targets.

If the required endogenous control target is available as a primer-limited assay, begin by verifying your duplex PCR. If not, you must limit the primer concentration in the assay. 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  $\Delta R_n$ . 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 and target assignments to balance fluorescence levels in the multiplex reaction.

- FAM™ dye and ABY™ dye can be used for targets with low to medium levels of expression.
- VIC™ dye, JUN™ dye, Cyanine 5 dye, and Cyanine 5.5 dye can be used for targets with high levels of expression.
- Because Cyanine 5 and Cyanine 5.5 are dimmer dyes, you should use them with higher levels of expression.

## Probe selection

- Up to six targets can be multiplexed in a single reaction using TaqMan™ QSY™ Probes or MGB probes (FAM™ dye, VIC™ dye, ABY™ dye, and JUN™ dye) and QSY2™ Probes (Cyanine 5 dye and Cyanine 5.5 dye).
- It is not recommended to add more than 800 nM total MGB probe in a single reaction when multiplexing with the master mixes listed in this guide.

# Methods

## Prepare singleplex PCR reactions

The goal of running singleplex PCR is to confirm the performance of the reaction components and PCR conditions. If you are using TaqMan™ Predesigned or Custom Assays, verification of performance under singleplex conditions is unnecessary.

For the available combinations of reporter dyes and quencher dyes, see “Overview of TaqMan™ Assays” on page 8.

Plan to run singleplex reactions in triplicate or quadruplicate.

1. Prepare an Assay Mix for FAM™ dye, VIC™ dye, ABY™ dye, JUN™ dye, Cyanine 5 dye, and Cyanine 5.5 dye, according to the expression level of the target.

Target expression level	Concentration			
	Assay Mix (Final)	Primer 1	Primer 2	Probe 1
High	20X	3 $\mu$ M	3 $\mu$ M	5 $\mu$ M
Medium	20X	6 $\mu$ M	6 $\mu$ M	5 $\mu$ M
Low	20X	18 $\mu$ M	18 $\mu$ M	5 $\mu$ M

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**Note:** Changes might be required for optimal performance.

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With a 20X Assay Mix, the concentrations of the primers and probes in the reaction will be as follows:

- High target expression level
  - Primers—150 nM
  - Probes—250 nM
- Medium target expression level
  - Primers—350 nM
  - Probes—250 nM
- Low target expression level
  - Primers—900 nM
  - Probes—250 nM

2. Prepare PCR Reaction Mix for each assay in an appropriately-sized microcentrifuge tube, according to one of the following tables.

**Table 18 96-well fast (0.1-mL) plate and 384-well (0.1-mL) plate**

Component	Volume per reaction <sup>[1]</sup>					
	Singleplex reaction 1	Singleplex reaction 2	Singleplex reaction 3	Singleplex reaction 4	Singleplex reaction 5	Singleplex reaction 6
Master Mix	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL
FAM™ dye Assay Mix (20X)	0.5 µL	—	—	—	—	—
VIC™ dye Assay Mix (20X)	—	0.5 µL	—	—	—	—
ABY™ dye Assay Mix (20X)	—	—	0.5 µL	—	—	—
JUN™ dye Assay Mix (20X)	—	—	—	0.5 µL	—	—
Cyanine 5 dye Assay Mix (20X)	—	—	—	—	0.5 µL	—
Cyanine 5.5 dye Assay Mix (20X)	—	—	—	—	—	0.5 µL
Nuclease-free water	3.5 µL	3.5 µL	3.5 µL	3.5 µL	3.5 µL	3.5 µL
<b>Total PCR Reaction Mix volume</b>	<b>9.0 µL</b>	<b>9.0 µL</b>	<b>9.0 µL</b>	<b>9.0 µL</b>	<b>9.0 µL</b>	<b>9.0 µL</b>

<sup>[1]</sup> Add 10% overage.

Table 19 96-well standard (0.2-mL) plate

Component	Volume per reaction <sup>[1]</sup>					
	Singleplex reaction 1	Singleplex reaction 2	Singleplex reaction 3	Singleplex reaction 4	Singleplex reaction 5	Singleplex reaction 6
Master Mix	10 µL	10 µL	10 µL	10 µL	10 µL	10 µL
FAM™ dye Assay Mix (20X)	1 µL	—	—	—	—	—
VIC™ dye Assay Mix (20X)	—	1 µL	—	—	—	—
ABY™ dye Assay Mix (20X)	—	—	1 µL	—	—	—
JUN™ dye Assay Mix (20X)	—	—	—	1 µL	—	—
Cyanine 5 dye Assay Mix (20X)	—	—	—	—	1 µL	—
Cyanine 5.5 dye Assay Mix (20X)	—	—	—	—	—	1 µL
Nuclease-free water	7 µL	7 µL	7 µL	7 µL	7 µL	7 µL
Total PCR Reaction Mix volume	18 µL	18 µL	18 µL	18 µL	18 µL	18 µL

<sup>[1]</sup> Add 10% overage.

3. Mix the components thoroughly, then centrifuge briefly to collect the contents at the bottom of each tube.
4. Transfer the appropriate volume of PCR Reaction Mix to each well of an optical reaction plate.
  - 96-well fast (0.1-mL) plate or 384-well (0.1-mL) plate: 9.0 µL
  - 96-well standard (0.2-mL) plate: 18 µL

5. Add cDNA template to each well.

Include a no-template control with water in place of cDNA.

- 96-well fast (0.1-mL) plate or 384-well (0.1-mL) plate: 1 µL (1–10 ng of cDNA)
- 96-well standard (0.2-mL) plate: 2 µL (2–20 ng of cDNA)

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**Note:** The amount of cDNA is calculated based on the amount of RNA input from the RT reaction.

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6. Seal the plate with an optical adhesive cover, vortex, then centrifuge briefly to collect the contents at the bottom of each well.

## Set up and run the real-time PCR instrument

See the appropriate instrument user guide for detailed instructions to program the thermal-cycling conditions or to run the plate.

**Note:** For more information see “Related documentation and software” on page 61.

**IMPORTANT!** Assign only the targets present in each reaction well of the plate to the corresponding well. Do not assign all the targets to a well.

1. Select the cycling mode appropriate for the master mix.

**IMPORTANT!** The cycling mode depends on the master mix that is used in the reaction. The cycling mode does not depend on a standard or fast plate format.

2. Set up the thermal protocol for your instrument.

**Table 20** ViiA™ 7, and QuantStudio™ Real-Time PCR Systems

Step	Temperature	Time	Cycles
Enzyme activation	95°C	20 seconds	1
Denature <sup>[1]</sup>	95°C	1 second	40
Anneal / Extend <sup>[2]</sup>	60°C	20 seconds	

<sup>[1]</sup> Denature time can be increased if needed for long amplicons.

<sup>[2]</sup> In some cases, increasing the anneal/extend time has been found to improve the performance of multiplex reactions.

**Table 21** 7500/7500 Fast Real-Time PCR Systems

Step	Temperature	Time	Cycles
Enzyme activation	95°C	20 seconds	1
Denature <sup>[1]</sup>	95°C	3 seconds	40
Anneal / Extend <sup>[2]</sup>	60°C	30 seconds	

<sup>[1]</sup> Denature time can be increased if needed for long amplicons.

<sup>[2]</sup> In some cases, increasing the anneal/extend time has been found to improve performance of multiplex reactions.

3. Set the reaction volume appropriate for the reaction plate.
  - 96-well fast (0.1-mL) plate and 384-well (0.1-mL) plate: **10 µL**
  - 96-well standard (0.2-mL) plate: **20 µL**
4. Load the plate into the real-time PCR instrument.
5. Start the run.

## 6. Analyze results of the singleplex real-time PCR reactions.

Use the Relative Quantification application on Thermo Fisher™ Connect to view the amplification curves.

- Replicates on 96- or 384-well plates with  $C_t$  values less than 30 should not be more than one-half of a  $C_t$  value apart.
- The amplification curves should be parallel to each other.

Perform a detailed analysis after collecting the multiplex real-time PCR data.

## Visual assessment of exponential PCR phase efficiency

The exponential phase of PCR is the first phase of PCR in which all the PCR reaction components are in excess and amplification efficiency is consistent. As PCR cycling progresses and the amplified product increases in amount, eventually the exponential phase transitions to linear phase in which amplification efficiency declines, caused by a limitation in one of the PCR reaction components.

For a number of reasons, it is important for all real-time PCR assays to have 100% exponential phase efficiency, which means the target sequence doubles each cycle. Thermo Fisher Scientific TaqMan™ Assays have 100% exponential phase efficiency when run with the recommended universal cycling and chemistry conditions.

Exponential phase efficiency can be quickly and easily determined by visually comparing exponential phase slopes of multiple single assays in real-time PCR amplification plots using a log fluorescence ( $\Delta Rn$ ) y-axis scale. See Figure 1. Parallel inter-assay exponential phase slopes indicate equivalent efficiencies, which equates to 100% efficiency, because assays with poor efficiency (<100%) have random efficiencies. Alternatively, exponential phase slopes of one assay can be compared to those from another assay with known 100% efficiency.

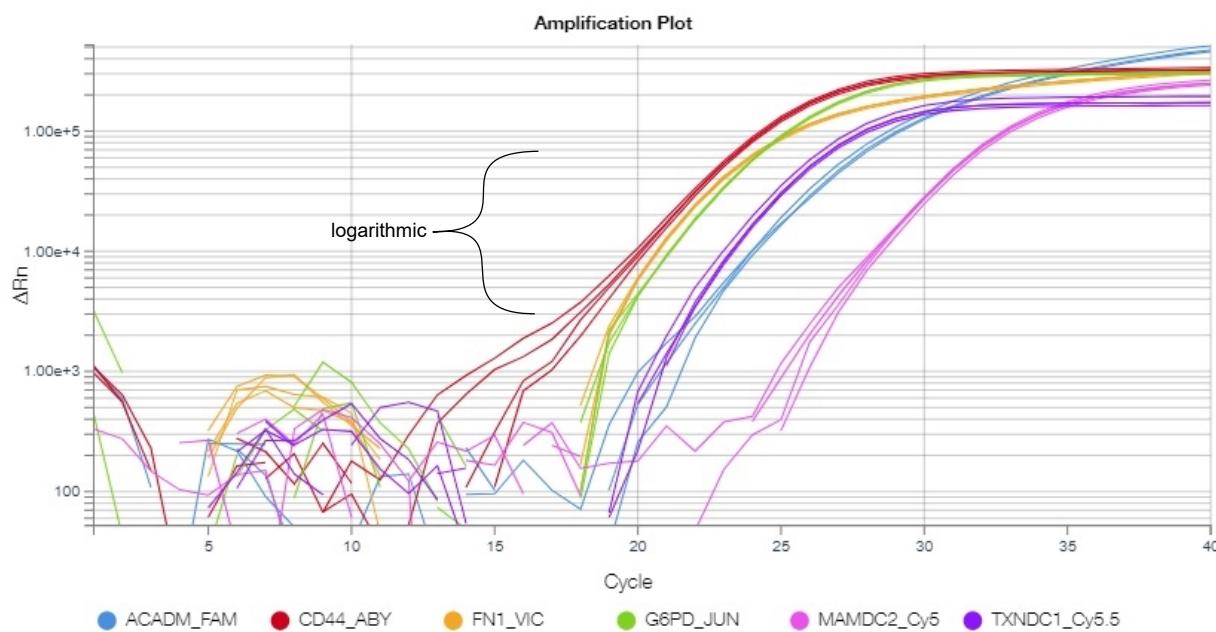


Figure 1 Visual comparison of 6 amplification plots

## Verification of multiplex assay performance

Multiplex real-time PCR assay performance is the degree to which a multiplex assay can detect the gene targets that are part of the multiplex with the same accuracy and sensitivity as when the assays are run in singleplex. Multiple factors can impact multiplex assay performance, such as dye discrimination, DNA polymerase saturation and adverse primer/probe interactions. While steps can be taken to minimize the risk of these problems, wet chemistry testing is needed to verify that such preventative steps were effective.

Multiplex verification effort increases with the number of assays in the multiplex. When considering whether to create a multiplex that contains more than 2 genes per well, validation time and effort should be weighed against the long-term benefits of the multiplex.

Multiplex verification requires individual assays or oligonucleotides. Once acceptable multiplex assay performance has been verified, all the individual assays can be formulated into a single assay tube by contacting [specialty\\_oligos@thermofisher.com](mailto:specialty_oligos@thermofisher.com). Use of a single-tube multiplex assay reduces labor, reduces error potential and improves primer/probe concentration consistency compared to using separate assays or oligonucleotides.

Exponential phase PCR efficiency is an issue independent of multiplexing, but the mixed standard curves used to verify multiplex performance may lead to questions about efficiency. A theoretical relationship exists between real-time PCR standard curve slopes and exponential efficiency ( $e$ ):  $e = 10^{-1/\text{slope}}$ . However, calculating exponential efficiency using standard curve slopes is problematic, because standard curve slopes fluctuate randomly due to a variety of errors related to pipetting, mixing and other factors that occur during preparation of the standard curve. Consequently, standard curve slopes usually do not accurately reflect the true exponential phase efficiency of the assay. Care should be taken in interpreting standard curve slopes.

### Multiplex verification method

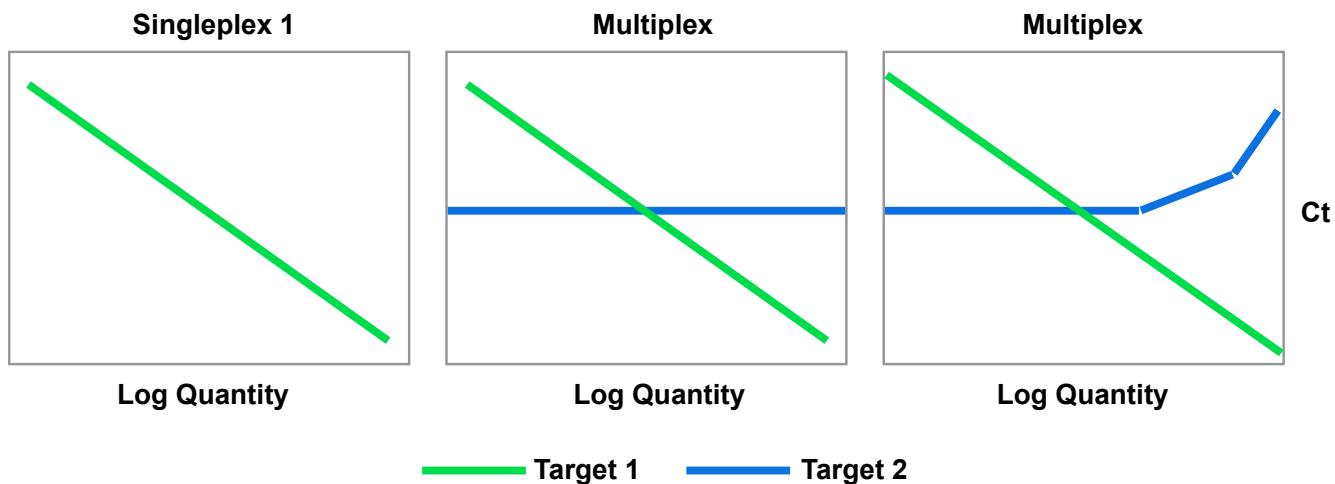
The most rigorous method to verify real-time PCR multiplex assay performance is to run mixed standard curves. Mixed standard curves consist of serially diluting one gene and mixing each dilution point with a fixed amount of a second gene.

Gene amounts tested by serial dilution should correspond to that expected in real samples. The standard curve should have a minimum of 5 dilution points. If the highest and lowest gene amounts expected in samples can be estimated, the dilution factor required to span that range with at least 5 dilution points can be calculated.

The mixed dilution series is run in real-time PCR with the assays in multiplex and singleplex for comparison.

GeneArt, a part of Thermo Fisher, can synthesize plasmids or DNA strings containing the desired target sequences, which could be used for multiplex verification.

Mixed standard curve results without assay interference are illustrated in Figure 2, middle, and results with assay interference are illustrated in Figure 2, right. A deviation from the expected Ct linearity of one gene when the other gene has greater abundance is a sign that the assays are interfering with each other in the multiplex. If interference is seen, the duplex reaction can be optimized by balancing the primer and probe concentrations to an ideal state. Contact [specialty\\_oligos@thermofisher.com](mailto:specialty_oligos@thermofisher.com) to order separate primers and probes.



**Figure 2 Illustration of possible mixed standard curve results** Mixed standard curves consist of serially diluting one gene template (Target 1) and mixing each dilution point with a fixed amount of a second gene template (Target 2).

Left: Singleplex standard curve for Target 1

Middle: Mixed standard curves for Target 1 (serial dilutions) and Target 2 (fixed amount), duplex. These curves show no interference in Target 2 Ct as Target 1 template increases.

Right: Mixed standard curves for Target 1 and Target 2, duplex, with interference. As the amount of Target 1 template increases, the Ct for Target 2 increases due to competition for reagents or other factors. Under laboratory conditions, the slope of Target 1 curve (green line) might also be distorted. When the kinetics of one reaction are more favorable than the other, the favorable reaction can be adjusted by reducing its primer concentration (primer limiting).

When verifying a triplex assay (3 assays per well), every possible duplex should be verified first before verifying the triplex. When verifying a quadruplex (4 assays per well), every possible duplex and triplex should be verified first before verifying the quadruplex assay. When verifying a pentaplex (5 assays per well), every possible duplex, triplex, and quadplex should be verified first before verifying the pentaplex assay. When verifying a hexaplex (6 assays per well), every possible duplex, triplex, quadplex, and pentaplex should be verified first before verifying the hexaplex assay. A mixed standard curve involving 3 genes or more can be performed by serially diluting a mixture of two or more genes and mixing each dilution point with a fixed amount of an additional gene.

A less rigorous way to verify real-time PCR multiplex assay performance is to run a representative collection of samples in singleplex and multiplex, comparing the quantity results, relative or absolute. Using this testing procedure, the user should take additional steps to monitor multiplex assay performance. For example, amplification plots should be examined for distortions or lack of exponential PCR phase data.

## Prepare mixed standard curve PCR reactions (two targets)

Mixed standard curves consist of serially diluting one cDNA template and mixing each dilution point with a fixed amount of a second cDNA template. In this example, a dilution series of Target 1 cDNA is run, while a constant amount of Target 2 cDNA is used.

The mixed dilution series is run in real-time PCR with the assays in multiplex and singleplex for comparison.

cDNA template amounts tested by serial dilution should correspond to that expected in real samples. The standard curve should have a minimum of 5 dilution points.

1. Prepare PCR Reaction Mix in appropriately-sized microcentrifuge tubes according to the following table.

Table 22 Example PCR Reaction Mix for mixed duplex standard curves

Component	Volume per singleplex reaction (Target 1)		Volume per duplex reaction <sup>[1]</sup>	
	96-well fast (0.1-mL) plate, 384-well (0.1-mL) plate	96-well standard (0.2-mL) plate	96-well fast (0.1-mL) plate, 384-well (0.1-mL) plate	96-well standard (0.2-mL) plate
Master Mix	5 µL	10 µL	5 µL	10 µL
FAM™ dye Assay Mix (20X) (Target 1)	0.5 µL	1 µL	0.5 µL	1 µL
VIC™ dye Assay Mix (20X) (Target 2)	—	—	0.5 µL	1 µL
Nuclease-free water	1.5 µL	6 µL	1.0 µL	5 µL
<b>Total PCR Reaction Mix volume</b>	<b>7 µL</b>	<b>17 µL</b>	<b>7 µL</b>	<b>17 µL</b>

<sup>[1]</sup> This represents a duplex reaction. Add assay mixes for triplex or quadruplex reactions (for example, ABY™ dye or JUN™ dye), reducing the amount of Nuclease-free water accordingly.

2. Mix the components thoroughly, then centrifuge briefly to collect the contents at the bottom of the tube.
3. Transfer the appropriate volume of PCR Reaction Mix to each well of an optical reaction plate.
  - 96-well fast (0.1-mL) plate or 384-well (0.1-mL) plate: 7 µL
  - 96-well standard (0.2-mL) plate: 17 µL
4. Prepare a 10-fold dilution series of Target 1 cDNA template, with a minimum of 5 dilution points. Prepare a sufficient volume of the Target 1 dilution series for both the singleplex and duplex reactions.
5. Prepare a dilution of Target 2 cDNA template that results in a Ct of 27–30. Prepare a sufficient volume of the Target 2 for all of the duplex reactions.
6. Add up to 3 µL (total volume Target 1 plus Target 2; 1–10 ng) of cDNA template to each well. Add the dilution series for Target 1, and a constant amount of Target 2 template in the duplex reactions.

If less than 3  $\mu$ L of cDNA is added, add nuclease-free water to achieve a total reaction volume of 10  $\mu$ L or 20  $\mu$ L.

Include a no-template control with water in place of cDNA.

7. Seal the plate with an optical adhesive cover, vortex, then centrifuge briefly to collect the contents at the bottom of each well.
8. Perform real-time PCR using the same thermal cycling conditions as for the singleplex PCR reactions (see “Set up and run the real-time PCR instrument” on page 38).

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## Overview of genotyping assays

Genotyping analysis is used to perform discrimination between two alleles containing single base differences. The discrimination is based on hybridization of the probe to the allele containing the complementary sequence.

It is recommended that duplex SNP reactions be performed using one SNP genotyping assay with FAM™ dye and VIC™ dye, and one SNP genotyping assay with ABY™ dye and JUN™ dye.

MGB-NFQ or QSY™ quencher can be used. We recommend redesigning TaqMan™ SNP Genotyping Assays when using a QSY™ quencher. The probes must be longer with a QSY™ quencher. This can affect specificity.

For the available combinations of reporter dyes and quencher dyes, see “Overview of TaqMan™ Assays” on page 8.

## Procedural guidelines

### Primer and probe concentration

The following assay concentrations are suitable for most ABY™ dye and JUN™ dye assays.

Assay	Concentration
Forward primer	900 nM
Reverse primer	900 nM
Allele 1 probe	200 nM
Allele 2 probe	200 nM

Individual primers and probes can be ordered. A higher probe concentration can increase the signal.

## Dye selection

- Pair FAM™ dye and VIC™ dye for SNP 1.
- Pair ABY™ dye and JUN™ dye for SNP 2.

## Probe selection

It is not recommended to add more than 800 nM total MGB probe in a single reaction when multiplexing with the master mixes listed in this guide.

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**IMPORTANT!** The use of non-MGB probes might reduce the performance of a SNP assay due to increased heterologous probe cleavage or for other reasons. Non-MGB probes are less sensitive to single base mismatches.

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## Methods

### Prepare singleplex PCR reactions

Confirm the performance of individual SNP reactions with ABY™ dye and JUN™ dye assays, and FAM™ dye and VIC™ dye assays, using at least one gDNA control sample for each expected genotype call and one no-template control (NTC).

1. *(Optional, if primers and probes are purchased separately.)* Prepare ABY™ dye and JUN™ dye Assay Mix, and FAM™ dye and VIC™ dye Assay Mix, according to the following table.

Concentration <sup>[1]</sup>				
Assay Mix (Final)	Primer 1	Primer 2	Probe 1	Probe 2
40X	36 µM	36 µM	8 µM	8 µM

<sup>[1]</sup> Using a 40X assay mix, the concentration of each primer in the reaction will be 900 nM and the concentration of each probe will be 200 nM.

---

**Note:** Slight changes in primer and probe concentration might be required for optimal performance.

---

2. Prepare the PCR Reaction Mix for each assay in an appropriately-sized microcentrifuge tube, according to one of the following tables.

**Table 23 96-well fast (0.1-mL) plate and 384-well (0.1-mL) plate**

Component	Volume per reaction	
	Singleplex reaction 1	Singleplex reaction 2
Master Mix	5 µL	5 µL
FAM™ dye and VIC™ dye Assay Mix (40X)	0.25 µL	—
ABY™ dye and JUN™ dye Assay Mix (40X)	—	0.25 µL
Nuclease-free water	2.25 µL	2.25 µL
<b>Total PCR Reaction Mix volume</b>	<b>7.5 µL</b>	<b>7.5 µL</b>

**Table 24 96-well standard (0.2-mL) plate**

Component	Volume per reaction	
	Singleplex reaction 1	Singleplex reaction 2
Master Mix	10 µL	10 µL
FAM™ dye and VIC™ dye Assay Mix (40X)	0.5 µL	—
ABY™ dye and JUN™ dye Assay Mix (40X)	—	0.5 µL
Nuclease-free water	6.5 µL	6.5 µL
<b>Total PCR Reaction Mix volume</b>	<b>17 µL</b>	<b>17 µL</b>

**Note:** Sample volumes can be up to 25% of the total reaction volume for crude lysates.

3. Vortex the components to mix, then centrifuge briefly to collect the contents at the bottom of each tube.
4. Transfer the appropriate volume of PCR Reaction Mix to each well of an optical reaction plate.
  - 96-well fast (0.1-mL) plate or 384-well (0.1-mL) plate: 7.5 µL
  - 96-well standard (0.2-mL) plate: 17 µL
5. Add gDNA template or no-template control to each well. For human assays we recommend 10–20ng DNA per PCR reaction.
  - 96-well fast (0.1-mL) plate or 384-well (0.1-mL) plate: 2.5 µL (1–10 ng of gDNA)
  - 96-well standard (0.2-mL) plate: 3 µL (2–20 ng of gDNA)
6. Seal the plate with an optical adhesive cover, vortex, then centrifuge briefly to collect the contents at the bottom of each well.

## Set up and run the real-time PCR instrument

See the appropriate instrument user guide for detailed instructions to program the thermal-cycling conditions or to run the plate.

1. Select the cycling mode appropriate for the TaqPath™ ProAmp™ Multiplex Master Mix.

**IMPORTANT!** The cycling mode depends on the master mix that is used in the reaction. The cycling mode does not depend on a standard or fast plate format.

2. Set up the thermal protocol for your instrument.

**Table 25 TaqPath™ ProAmp™ Multiplex Master Mix (with QuantStudio™ Real-Time PCR Systems, StepOne™ and StepOnePlus™ Real-Time PCR Instruments, ViiA™ 7 and 7500/7500 Fast Real-Time PCR Systems)**

Step	Temperature	Time	Cycles
Pre-read	60°C	30 seconds	1
Initial denature / Enzyme activation	95°C	5 minutes	1
Denature	95°C	15 seconds	40
Anneal / Extend	60°C	90 seconds	
Post-read	60°C	30 seconds	1

3. Set the reaction volume appropriate for the reaction plate.
  - 96-well fast (0.1-mL) plate or 384-well (0.1-mL) plate: **10 µL**
  - 96-well standard (0.2-mL) plate: **20 µL**

4. Load the plate into the real-time PCR instrument.

5. Start the run.

6. Analyze the results.

To help ensure that the control samples were set up correctly, use one of the following tools:

- The Genotyping application, available on Thermo Fisher™ Connect
- The TaqMan™ Genotyper Software, available for download at [thermofisher.com/qpcrsoftware](http://thermofisher.com/qpcrsoftware)
- The Genotyping Analysis Module in Diomi™ Design and Analysis (RUO) Software 3 (automatic calling only when more than one SNP assay is assigned to a well)

## Prepare duplex PCR reactions

Run a duplex (4-color) SNP reaction by combining the ABY™ dye and JUN™ dye assay with the FAM™ dye and VIC™ dye assay. Use at least one gDNA control sample for each expected genotype call, and one NTC.

1. Prepare PCR Reaction Mix in appropriately-sized microcentrifuge tubes according to the following table.

Component	Volume per multiplex reaction	
	96-well fast (0.1-mL) plate and 384-well (0.1-mL) plate	96-well standard (0.2-mL) plate
Master Mix	5 µL	10 µL
FAM™ dye and VIC™ dye Assay Mix (40X)	0.25 µL	0.5 µL
ABY™ dye and JUN™ dye Assay Mix (40X)	0.25 µL	0.5 µL
Nuclease-free water	2 µL	6.0 µL
<b>Total PCR Reaction Mix volume</b>	<b>7.5 µL</b>	<b>17 µL</b>

2. Mix the components thoroughly, then centrifuge briefly to collect the contents at the bottom of the tube.
3. Transfer the appropriate volume of PCR Reaction Mix to each well of an optical reaction plate.
  - 96-well fast (0.1-mL) plate or 384-well (0.1-mL) plate: 7.5 µL per well
  - 96-well standard (0.2-mL) plate: 17.0 µL per well
4. Add gDNA template to each well. For human assays we recommend 10–20ng DNA per PCR reaction. For a no-template control add water in place of gDNA.
  - 96-well fast (0.1-mL) plate or 384-well (0.1-mL) plate: 2.5 µL per well
  - 96-well standard (0.2-mL) plate: 3 µL per well
5. Seal the plate with an optical adhesive cover, vortex, then centrifuge briefly to collect the contents at the bottom of each well.
6. Perform real-time PCR using the same thermal cycling conditions as the singleplex PCR reactions. See “Set up and run the real-time PCR instrument” on page 47.

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**Note:** Do not use the StepOne™ and StepOnePlus™ systems for multiplexing more than two targets.

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## Analyze results

Use the Genotyping application to analyze and compare the singleplex and multiplex data. The application is available on Thermo Fisher™ Connect.

- Confirm that control sample calls are correct.
- Confirm that clusters are consistent between multiplex and singleplex and that clusters are well separated in both cases.
- Confirm that multiplex calls match those of singleplex calls.

The Genotyping Analysis Module in Diomni™ Design and Analysis (RUO) Software 3 can be used to analyze data. Two SNP assays can be assigned to one well. Only automatic calling can be performed. Manual calls cannot be performed when two SNP assays are assigned to one well.

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## Overview of microRNA analysis

MicroRNA assays are used to determine the relative expression levels between different microRNA targets within a single sample. Normalization between different samples is achieved by using a reference gene. Up to two targets can be multiplexed in a single reaction depending upon the probes that are selected. In microRNA duplexing experiments, the goal is to minimize the difference between the  $C_t$  value in singleplex and duplex reactions.

Before ordering assays for duplex microRNA experiments, contact Technical Support at [techsupport@thermofisher.com](mailto:techsupport@thermofisher.com) to request assistance with multiplex compatibility analysis. You will need to provide the two assay IDs that you wish to duplex.

## Procedural guidelines

### Target abundance

The amount of target and endogenous control in a sample can affect the outcome of PCR results when performing duplex assays. Abundance can be characterized by the  $C_t$  range.

**Table 26** Target expression levels

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.

**Note:** This information is provided as a general guideline and might require some optimization.

## Primer and probe concentrations

Optimization of the concentrations of primers and probe for each target is an important first step in assembling the duplex reaction. Guidelines for optimization will vary depending on the chemistry. For more information see the following documents:

- *TaqMan™ Advanced miRNA Assays User Guide—Single-tube Assays* (Pub. No. 100027897)
- *TaqMan™ Small RNA Assay User Guide* (Pub. No. 4364031)

## Dye selection

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

- FAM™ dye can be used with low to medium expressors (typically the microRNA of interest).
- VIC™ dye can be used with medium to high expressors (typically the endogenous control).

To obtain the assay with a probe labeled with VIC™ dye email [specialty\\_oligos@thermofisher.com](mailto:specialty_oligos@thermofisher.com). Include the following information:

- The assay ID of the assay you need with a probe labeled with VIC™ dye.
- The assay ID that will be duplexed with that assay. This allows us to check the sequences for multiplexing compatibility.

## Duplex guidelines for TaqMan™ Advanced miRNA Assays

The PCR concentrations of the primer and probe remain unchanged from the singleplex reaction.

If the required endogenous control target is much more abundant than the miRNA of interest, request a primer-limited assay with a primer concentration of 150 nM.

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**Note:** 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 should lower its  $\Delta Rn$  without affecting  $C_t$ .

---

Perform an experiment to confirm that  $C_t$  values are the same for singleplex and duplex reactions. Perform both reactions for each assay using the same dye and real-time PCR primer concentration. PCR for the singleplex and duplex reactions can be run on the same plate simultaneously.

For the duplex reaction, follow the cDNA template preparation procedure in the *TaqMan™ Advanced miRNA Assays User Guide—Single-tube Assays* (Pub. No. 100027897).

For the duplex reaction, follow the real-time PCR setup procedure in the *TaqMan™ Advanced miRNA Assays User Guide—Single-tube Assays*. Include two assays, FAM™ dye assay mix (20X) and VIC™ dye assay mix (20X), in the reaction mixture, and reduce the amount of water to account for the additional assay.

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**Note:** The concentration for each assay in the duplex reaction should be 1X.

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## Duplex guidelines for TaqMan™ MicroRNA Assays

Reverse transcription (RT) requires the RT stem loop primers to be mixed together and diluted to 0.05X concentration to avoid any interaction. See the *Custom Reverse Transcription Pools and Custom Preamplification Pools with TaqMan™ MicroRNA Assays User Bulletin* (Pub. No. 4465407).

If the required endogenous control target is much more abundant than the miRNA of interest, request a primer-limited assay of forward and reverse primers at 150 nM. If a primer-limited assay is used, it must be used in both singleplex and duplex reactions.

---

**Note:** 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 should lower its  $\Delta R_n$  without affecting  $C_t$ .

---

Perform an experiment to confirm that  $C_t$  values are the same for the singleplex and duplex reactions. Perform both reactions for each assay. PCR for the singleplex and duplex reactions can be run on the same plate simultaneously.

For the singleplex reaction, perform RT and real-time PCR as described in the *TaqMan™ Small RNA Assay User Guide* (Pub. No. 4364031). Use the same assay (dye and real-time PCR primer concentration) in the singleplex as in the duplex reaction.

For the duplex reaction, follow the same real-time PCR setup procedure as described in the *TaqMan™ Small RNA Assay User Guide*. The following updates are required.

- Two assay mixes are added to the reaction mixture:
  - FAM™ dye assay mix
  - VIC™ dye assay mix
- The amount of water is reduced because of the additional assays.
- The final concentration of each assay in the duplex reaction is 1X.



# Supplemental information

## Guidelines for primer and probe design

### Guidelines for target sequence and the amplicon

A target template is a DNA sequence that will be amplified. Target templates include the following:

- cDNA
- gDNA
- plasmid DNA

Design primers to amplify amplicons (segments of DNA) within the target sequence using Primer Express™ Software or the Custom Assay Design Tool, available at [Assay Design Tool](#). Consistent results can be obtained for shorter amplicons (50 to 150 bp).

### Guidelines for primer design

Use Primer Express™ Software to design primers. See the *Primer Express™ Software Version 3.0 Getting Started Guide* (Pub. No. 4362460).

- The primers should be specific for the target.
  - Perform a BLAST search of the amplicon at [blast.ncbi.nlm.nih.gov/Blast.cgi](https://blast.ncbi.nlm.nih.gov/Blast.cgi).
  - Perform *in silico* PCR using the UCSC Genome Browser at [genome.ucsc.edu/cgi-bin/hgPcr](https://genome.ucsc.edu/cgi-bin/hgPcr).
- The primer melting temperature (Tm) should be the same for all primers used in the multiplex reaction.
- The length of the amplicon should be 50–150 bp for optimal PCR efficiency. If longer amplicons cannot be avoided, it may be necessary to optimize the thermal cycling protocol and reaction components.
- Confirm that primers do not contain bases that are complementary to other bases within the primer (self-complementary), or have complementarity to other primers. Complementarity at the 3' ends should be avoided to minimize the formation of artifact products, for example, primer-dimers or primer-oligomers.

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**Note:** AutoDimer software can be used to check primers at <https://strbase.nist.gov//AutoDimerHomepage/AutoDimerProgramHomepage.htm>.

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- Do not overlap primer and probe sequences. The optimal primer length is approximately 20 bases, but may be longer or shorter depending on AT vs. GC content.
- Avoid runs of identical nucleotides. If repeats are present, there must be fewer than four consecutive G residues.
- Primers should not hybridize to regions of secondary structure within the target as these tend to have a higher melting point than the primer.

- Keep the GC content in the 40–60% range.
- Confirm that the last five nucleotides at the 3' end contain no more than two G and/or C bases.

## Guidelines for the amplicon site

Select an amplicon site that amplifies the target cDNA without co-amplifying the genomic sequence, pseudogenes, and related genes. Predesigned and custom TaqMan™ Assays (when bioinformatics analysis is selected) should have high specificity for the intended targets and species. For specificity considerations related to oligonucleotide interactions, consider using the Multiple Primer Analyzer Tool (see “Primer and probe selection” on page 28).

- The amplicon should span one or more introns to avoid amplification of the target gene in gDNA.
- The primer pair must be specific to the target gene. The primer pair should not amplify pseudogenes or other related genes.
- Design primers according to the guidelines in Primer Express™ Software.
- Test the amplicons, then select those that have the highest signal-to-noise ratio. Look for low  $C_t$  with cDNA and no amplification with no template control or gDNA.
- If a sequence that adheres to the guidelines is not available, you may need to examine the sequence and redesign the amplicon or screen for more sites.
- If the gene of interest does not have introns, then an amplicon that amplifies the mRNA sequence without amplifying the gDNA sequence cannot be designed. Use RT-minus controls.

# Optimize primer concentrations for real-time PCR

## Overview of primer concentration

Independently vary the forward and reverse primer concentrations to identify the primer concentrations that provide optimal assay performance. Optimal primer concentrations should use the following guidelines:

- Provide a low  $C_t$  and a high  $\Delta R_n$  for singleplex when run against the target template
- Provide a low  $C_t$  and a low  $\Delta R_n$  when the reaction is primer-limited
- Not produce nonspecific product formation with no-template controls (NTCs)

## Quantify the primers

1. Measure the absorbance of each primer oligonucleotide in TE Buffer.  
Absorbance of a 1:100 dilution is measured at 260 nm.
2. Calculate the sum of extinction coefficient contributions for each base within the primer.  
extinction coefficient contribution =  $\Sigma$ (extinction coefficient of a given base  $\times$  number of times that base appears in the oligonucleotide sequence)
3. Calculate the oligonucleotide concentration in  $\mu$ M for each primer.  
Use the following equation.

absorbance at 260 nm = sum of extinction coefficient contribution × cuvette path length × concentration/100

Solve for concentration.

concentration =  $100[\text{absorbance at } 260 \text{ nm} / (\text{sum of extinction coefficient contribution} \times \text{cuvette path length})]$

### Example calculation

The concentration of a primer with the sequence CGTACTCGTTCGTGCTGC, diluted 1:100, is calculated using the following values.

Chromophore	Extinction coefficient	Number of specific chromophores in example sequence	Extinction coefficient contribution
A	15,200	1	15,200
C	7,050	6	42,300
G	12,010	5	60,050
T	8,400	6	50,400
<b>Total</b>	—	—	<b>167,950</b>

measured absorbance at 260 nm = 0.13

sum of extinction coefficient =  $167,950 \text{ M}^{-1}\text{cm}^{-1}$  contributions for primer

cuvette path length = 0.3 cm

Absorbance (260 nm) = sum of extinction coefficient contributions × cuvette path length × oligonucleotide concentration/100

$0.13 = 167,950 \text{ M}^{-1}\text{cm}^{-1} \times 0.3 \text{ cm} \times C/100$

$C = 258 \mu\text{M}$

### Determine the optimal primer concentration

The objective of determining the optimal primer and probe concentrations for gene expression assays is to minimize the  $C_t$  while maintaining a  $\Delta R_n$  that is reasonable for the application.

The following table provides an example of a matrix used to determine optimal primer and probe concentrations for a single template when performing gene expression analysis. The number of conditions can be reduced to fit the constraints of the block type (96-well or 384-well) and the amount

of sample and reagents. Forward and reverse primers do not necessarily have to be at the same concentration; they can be changed in certain instances, for example, primer-limiting conditions.

Condition number	Concentration		
	Forward primer	Reverse primer	Probe
1	900 nM	900 nM	250 nM
2	450 nM	450 nM	250 nM
3	350 nM	350 nM	250 nM
4	150 nM	150 nM	250 nM
5	75 nM	75 nM	250 nM
6	900 nM	900 nM	200 nM
7	450 nM	450 nM	200 nM
8	350 nM	350 nM	200 nM
9	150 nM	150 nM	200 nM
10	75 nM	75 nM	200 nM
11	900 nM	900 nM	150 nM
12	450 nM	450 nM	150 nM
13	350 nM	350 nM	150 nM
14	150 nM	150 nM	150 nM
15	75 nM	75 nM	150 nM
16	900 nM	900 nM	100 nM
17	450 nM	450 nM	100 nM
18	350 nM	350 nM	100 nM
19	150 nM	150 nM	100 nM
20	75 nM	75 nM	100 nM

1. Prepare a reaction plate using the desired templates and single template concentrations.
2. Determine the concentration combinations to be evaluated and prepare the reactions accordingly.
3. Prepare the reaction plate.
4. Set up and run the real-time PCR instrument.
5. Analyze the results, and compile  $\Delta R_n$  and  $C_t$  values to select the concentrations that give high  $\Delta R_n$  values and low  $C_t$  values.

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**Note:** For SNP assay formulation, we recommend starting with the concentration of predesigned assays (900 nM for each primer and 200 nM for each probe).

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## Suggested 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 help 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.



**WARNING! GENERAL SAFETY.** Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, visit [thermofisher.com/support](http://thermofisher.com/support).

## Chemical safety



**WARNING! GENERAL CHEMICAL HANDLING.** To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the “Documentation and Support” section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's 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 necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

## Biological hazard safety



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

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



# Documentation and support

## Related documentation and software

Document	Pub. No.
<i>TaqMan™ SNP Genotyping Assays User Guide</i>	MAN0009593
<i>TaqMan™ Advanced miRNA Assays User Guide—Single-tube Assays</i>	100027897
<i>TaqMan™ Gene Expression Assays User Guide—single-tube assays</i>	4333458
<i>Introduction to Gene Expression Getting Started Guide</i>	4454239
<i>TaqMan™ Small RNA Assay User Guide</i>	4364031
<i>Custom Reverse Transcription Pools and Custom Preamplification Pools with TaqMan™ MicroRNA Assays User Bulletin</i>	4465407
Master mixes	Pub. No.
<i>TaqPath™ BactoPure™ Microbial Detection Master Mix User Guide</i>	MAN0025689
<i>TaqMan™ Multiplex Master Mix Quick Reference</i>	MAN0009605
<i>TaqPath™ ProAmp™ Master Mixes User Guide</i>	MAN0015758
<i>TaqPath™ DuraPlex™ 1-Step RT-qPCR Master Mix User Guide</i>	<a href="#">MAN0028387</a>
Software	SKU
<i>Diomni™ Design and Analysis (RUO) Software 3 Genotyping Analysis Module User Guide</i>	MAN0030164
<i>Primer Express™ Software Version 3.0 Getting Started Guide</i>	4362460
Custom Assay Design Tool	Available at <a href="#">Assay Design Tool</a>
<i>TaqMan™ Genotyper Software User Guide</i>	4448637

## Customer and technical support

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  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

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

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## Limited product warranty

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26 August 2025

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