

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

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C.0	18 January 2022	<ul style="list-style-type: none">• Added TaqMan™ MicroRNA Assays and TaqMan™ Advanced miRNA Assays.• Removed TaqMan™ Copy Number Assays.• Updated compatible Master Mixes, real-time PCR instruments, and calibration plates.• Updated for general style, formatting, and branding.
B.0	17 May 2014	Baseline for this revision history.

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

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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 TaqMan™ 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
- TaqMan™ custom probes with a QSY/QSY2 or MGB 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 TaqMan™ Assay primer and probe sets are available for gene expression, genotyping, and microRNA analysis.

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

Assay type	Reporter dye options	Quencher dye
TaqMan™ Gene Expression Assays, predesigned	FAM™ dye	Non-fluorescent MGB
TaqMan™ SNP Genotyping Assays, predesigned	VIC™ dye	
TaqMan™ MicroRNA Assays	FAM™ dye	Non-fluorescent MGB
TaqMan™ Advanced miRNA Assays		

Custom versions of the assays with VIC™, ABY™, and JUN™, and Cyanine 5 reporter dye and a non-fluorescent MGB quencher can be ordered (specialty_oligos@thermofisher.com).

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

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 capable of quenching the reporter dye. The absorbance range of the quencher must overlap sufficiently with the emission range of the reporter dye.

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

For gene expression assays and genotyping assays, ABY™ dye and JUN™ dye can be utilized with either the MGB or the QSY™ non-fluorescent quencher. Alternatively, you can also design assays with Cyanine 5 and/or Cyanine 5.5 dyes and the QSY2 quencher. If utilized with an MGB quencher, the probe sequence needs to be shorter than if utilized with a QSY™ non-fluorescent quencher to help ensure similar T_m.

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

TaqMan™ QSY/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 will maintain the same T_m. MGB probes must be redesigned to accommodate a QSY or QSY2 quencher.

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 2 Absorbance and emission wavelengths for recommended dyes

Dye ^[1]	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

^[1] Other dyes can function in multiplex PCR reactions but the dyes listed in this table are recommended.

These dyes are optimized to work together for performing multiplex PCR on the following instruments:

- QuantStudio™ Real-Time PCR Systems
- ViiA™ 7 Real-Time PCR Systems
- 7500/7500 Fast Real-Time PCR Systems

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

Table 3 TaqMan™ QSY and QSY2 Probes

Probe	Cat. No.	Amount
QSY	4482777	6,000 pmol
	4482778	20,000 pmol
	4482779	50,000 pmol
QSY2	A56899	6,000 pmol
	A56900	20,000 pmol
	A56901	50,000 pmol

Master mixes for multiplex PCR

When assays are amplified together, they compete for the same reagents, including dNTPs, Mg²⁺, 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 ^[1]	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	

^[1] 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^[1] (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^[2] 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™ Fast DNA Polymerase
- Heat-labile Uracil-DNA Glycosylase (UNG)
- dNTPs (with dUTP)
- Optimized buffer

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

Table 4 TaqPath™ BactoPure™ Microbial Detection Master Mix (No ROX™)

Cat. No.	Unit size	Number of 20- μ 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	

^[1] 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.

^[2] Pre-PCR stability is influenced not only by the master mix, but also by the target being analyzed. For maximum confidence, stability profiles of specific targets should be confirmed.

Table 5 TaqPath™ BactoPure™ Microbial Detection Master Mix

Cat. No.	Unit size	Number of 20-μL 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 6 TaqPath™ ProAmp™ Multiplex Master Mix

Cat. No.	Amount	Number of 20- μ 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 7 TaqPath™ ProAmp™ Master Mix

Cat. No.	Amount	Number of 20- μ 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™ DuraPlex™ 1-Step RT-qPCR Master Mix

TaqPath™ DuraPlex™ 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™ DuraPlex™ 1-Step RT-qPCR Master Mix (No ROX™)—Does not contain a passive reference dye
- TaqPath™ DuraPlex™ 1-Step RT-qPCR Master Mix—Contains ROX™ 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 maximum 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 8 TaqPath™ DuraPlex™ 1-Step RT-qPCR Master Mix (No ROX™)

Cat. No.	Amount	Number of 20-μL 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 9 TaqPath™ DuraPlex™ 1-Step RT-qPCR Master Mix

Cat. No.	Amount	Number of 20-μL 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

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.

-
- StepOne™ and StepOnePlus™ Real-Time PCR Systems

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.

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

Filter selection

Table 10 Filters for QuantStudio™ 3 and 5 Real-Time PCR Systems

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

^[1] Used as a passive reference dye.

^[2] The QuantStudio™ 5 384-well instrument has only five filters.

Table 11 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™ ^[1] or JUN™ dye	x4m4	x4m4	x4m4	x4m4
MUSTANG PURPLE™ ^[1] or Cyanine 5 dye	x5m5	x5m5	x5m5	x5m5
Cyanine 5.5 dye	x6m6	N/A	x6m6	x6m6

^[1] Used as a passive reference dye.

Table 12 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™ ^[1] or JUN™ dye	Filter D	Filter D
MUSTANG PURPLE™ ^[1] or Cyanine 5 dye	Filter E	Filter E

^[1] Used as a passive reference dye.



Table 13 Filters for StepOne™ and StepOnePlus™ Real-Time PCR Systems

Dye	System and filter	
	StepOne™ Real-Time PCR System	StepOnePlus™ Real-Time PCR System
FAM™ dye	Filter 1	Filter 1
VIC™ dye	Filter 2	Filter 2
ABY™ dye	N/A	Filter 3
ROX™ ^[1] 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™ dye and VIC™ dye plates using new file names.

Note: Cyanine 5.5 dye calibration material at 1X concentration can be ordered through specialty_oligos@thermofisher.com. For use with ViiA™ 7 Real-Time PCR Systems, QuantStudio™ 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.

IMPORTANT! Do not overwrite the original system FAM™ dye and VIC™ 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 ^[1]	QuantStudio™ 6 Pro and 7 Pro Real-Time PCR Systems and ViiA™ 7 Real-Time PCR Systems ^[2]
Standard 96-well plates (0.2 mL)			
QuantStudio™ 3/5 Spectral Calibration Plate 1 (FAM™, VIC™, ROX™, and SYBR™ dyes), 96-Well 0.2-mL	A26331	✓	✓
QuantStudio™ 3/5 Spectral Calibration Plate 2, 96-Well 0.2-mL (ABY™, JUN™, and MUSTANG PURPLE™ dyes)	A26332	✓	✓
QuantStudio™ 3/5 Spectral Calibration Plate 3, 96-Well 0.2-mL (TAMRA™, NED™, Cyanine 5 dyes)	A26333	✓	✓
Fast 96-well plates (0.1 mL)			
QuantStudio™ 3/5 Spectral Calibration Plate 1, (FAM™, VIC™, ROX™, and SYBR™ dyes), 96-well, 0.1 mL	A26336	✓	✓
QuantStudio™ 3/5 Spectral Calibration Plate 2 (ABY™, JUN™, MUSTANG PURPLE™ dyes), 96-well Fast (0.1-mL) Plate	A26337	✓	✓
QuantStudio™ 3/5 Spectral Calibration Plate 3 (TAMRA™, NED™ and Cyanine 5 dyes) 96-well Fast (0.1-mL) Plate	A26340	✓	✓
384-well plates			
QuantStudio™ 5 Spectral Calibration Plate 1, (FAM™, VIC™, ROX™ TAMRA™, and SYBR™ dyes), 384-well	A26334	✓	✓
QuantStudio™ 5 Spectral Calibration Plate 2 (ABY™, JUN™, MUSTANG PURPLE™, NED™, and Cyanine 5 dyes), 384-well	A26335	✓	✓

^[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 ^[1, 2]
Standard 96-well plates (0.2 mL)				
ABY™ Dye Spectral Calibration Plate for Multiplex qPCR, 96-well	A24738	✓	–	✓
JUN™ Dye Spectral Calibration Plate for Multiplex qPCR, 96-well	A24737	✓	–	✓
FAM™ Dye Spectral Calibration Plate, 96-well	4432327	✓	–	✓
VIC™ Dye Spectral Calibration Plate, 96-well	4432334	✓	–	✓
MUSTANG PURPLE™ Dye Spectral Calibration Plate for Multiplex qPCR, 96-well	4461599	✓	–	✓
Fast 96-well plates (0.1 mL)				
ABY™ Dye Spectral Calibration Plate for Multiplex qPCR, Fast 96-well	A24734	–	✓	✓
JUN™ Dye Spectral Calibration Plate for Multiplex qPCR, Fast 96-well	A24735	–	✓	✓
FAM™ Dye Spectral Calibration Plate, Fast 96-well	4432389	–	✓	✓
VIC™ Dye Spectral Calibration Plate, Fast 96-well	4432396	–	✓	✓
MUSTANG PURPLE™ Dye Spectral Calibration Plate for Multiplex qPCR, Fast 96-well	4457328	–	✓	✓
384-well plates				
ABY™ Dye Spectral Calibration Plate for Multiplex qPCR, 384-well	A24736	–	–	✓

(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 ^[1, 2]
JUN™ Dye Spectral Calibration Plate for Multiplex qPCR, 384-well	A24733	—	—	✓
FAM™ Dye Spectral Calibration Plate, 384-well	4432271	—	—	✓
VIC™ Dye Spectral Calibration Plate, 384-well	4432278	—	—	✓
MUSTANG PURPLE™ Dye Spectral Calibration Plate for Multiplex qPCR, 384-well	4457334	—	—	✓

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

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

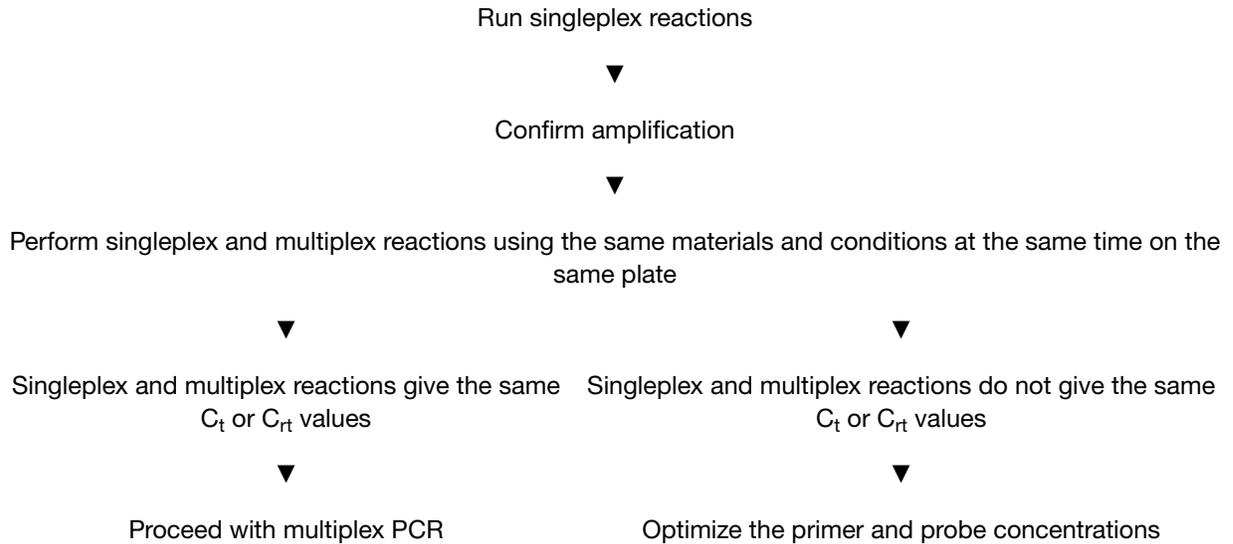
Other materials and equipment not supplied

For compatible instruments, see “Real-time PCR instruments” on page 16.

Table 14 Other materials and equipment required for the workflow

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

Workflow



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

2

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 T_m of each PCR primer should be between 58–60°C, and the T_m 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 50.

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

Probe design

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

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

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.
- Ensure 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
 - NCBI Basic Local Alignment Search Tool: blast.ncbi.nlm.nih.gov/Blast.cgi
- Ensure 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.

Probe selection

- 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. Preformulated assays made with ABY™ probes and JUN™ probes are available with either MGB–NFQ or QSY quenchers from specialty_oligos@thermofisher.com.
- For the fifth and sixth dye, we recommend Cyanine 5 and Cyanine 5.5 dye labeled probes with a QSY2 quencher.
- Multiplex gene expression analysis can use gene expression assays with FAM™ dye, VIC™ dye, Cyanine 5 dye, and Cyanine 5.5 dye combined with one assay containing a custom ABY™ probe and one assay containing a custom JUN™ probe with either an MGB–NFQ or QSY™ quencher. Alternatively, assays containing Cyanine 5 or Cyanine 5.5 labeled QSY2 probes can be used.
- For multiplex SNP genotyping analysis, an existing SNP genotyping assay with FAM™ dye and VIC™ dye can be combined with a custom ABY™/JUN™ assay with either an MGB–NFQ or QSY™ quencher.
- MicroRNA assays can be duplexed using a FAM™ dye– and VIC™ dye–labeled assay with MGB quencher. The VIC™ dye–labeled assay can be ordered through specialty_oligos@thermofisher.com.
- Custom probes can be ordered to help with concentration optimization. They can be ordered with MGB–NFQ quenchers and QSY/QSY2 quenchers. Order at www.thermofisher.com/taqman-primers-probes.
- Probes with FAM™, VIC™, ABY™, and JUN™ dye can be made with either a MGB–NFQ or QSY quencher. Changing the quencher requires a redesign of the probe to maintain correct T_m. Probes with Cyanine 5 and Cyanine 5.5 dyes can only be made 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 of the assays are amplified in the same tube. They compete for the same reagents (dNTPs, Mg²⁺, and polymerase).
- The more targets that are assayed in a multiplex reaction, the more likely it is that there will be competition for reagents and inhibition between assays.
- Multiplex Master Mixes are recommended to offset the effect of competition for reagents (see “Other materials and equipment not supplied” on page 23).

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. Ensure 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 will appear as an option in the menu. For information on the calibration process, refer to the appropriate guide for your instrument.

Assignment of reporter dyes

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.

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

Table 15 Absorbance and emission wavelengths for recommended dyes

Dye ^[1]	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

^[1] 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.



Gene expression analysis

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Overview of gene expression analysis

Gene expression analysis is performed 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 β -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 are easily duplexed, because both predesigned and custom assays are available with either FAM™ or VIC™ fluorescent reporter dyes. The most common duplex reaction is combining the target of interest and endogenous control assays in the same well. 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 probe 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 16 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"> 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 32. Housekeeping genes and endogenous controls are highly expressed. 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. For very highly abundant transcripts or especially bright assays, probe concentration might need to be adjusted. See “Guidelines for primer design” on page 50.
Targets are of similar abundance	<ul style="list-style-type: none"> When all targets are present in approximately equal abundance, no single assay needs to be primer-limited. Assay optimization is recommended to minimize the C_t difference between single and multiplex reactions. Start with 900 nM for each primer and 250 nM for the probe in the final reaction mix.
Either target may be more abundant	<ul style="list-style-type: none"> If any of the targets could be more abundant than the others, all assays need to be primer-limited. Establishing reaction conditions for extreme cases of low or 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 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 ΔR_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 ensure 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. Predesigned and Custom are available as FAM™ or VIC™ assays. If you need to use ABY™, JUN™ Cyanine 5, and Cyanine 5.5 dye assays or custom probe and primers using any dye, follow this procedure.

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 μM	3 μM	5 μM
Medium	20X	6 μM	6 μM	5 μM
Low	20X	18 μM	18 μM	5 μM

Note: Changes might be required for optimal performance.

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—300 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 17 96-well fast (0.1-mL) plate and 384-well (0.1-mL) plate

Component	Volume per reaction ^[1]					
	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
Total PCR Reaction Mix volume	9.0 µL	9.0 µL	9.0 µL	9.0 µL	9.0 µL	9.0 µL

^[1] Add 10% overage.

Table 18 96-well standard (0.2-mL) plate

Component	Volume per reaction ^[1]					
	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

^[1] 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)

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

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 59.

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 19 ViiA™ 7, and QuantStudio™ Real-Time PCR Systems

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

^[1] Denature time can be increased if needed for long amplicons.

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

Table 20 7500/7500 Fast Real-Time PCR Systems

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

^[1] Denature time can be increased if needed for long amplicons.

^[2] 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 (ΔRn) 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.

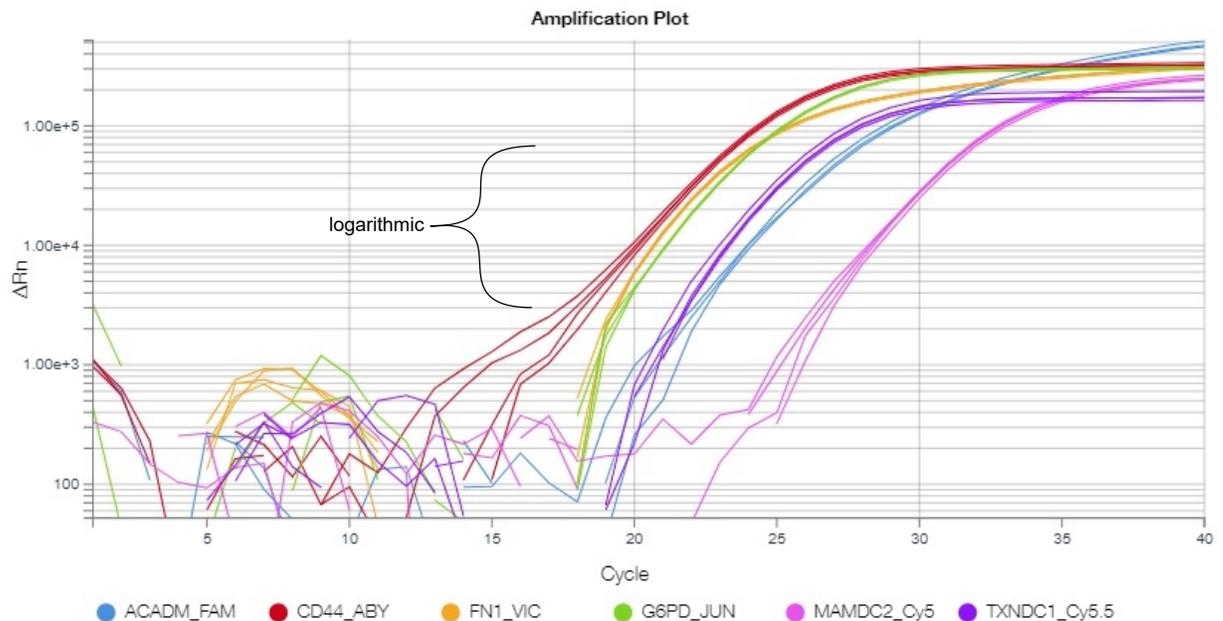


Figure 1 Visual comparison of 6 amplification plots

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. 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 to order separate primers and probes.

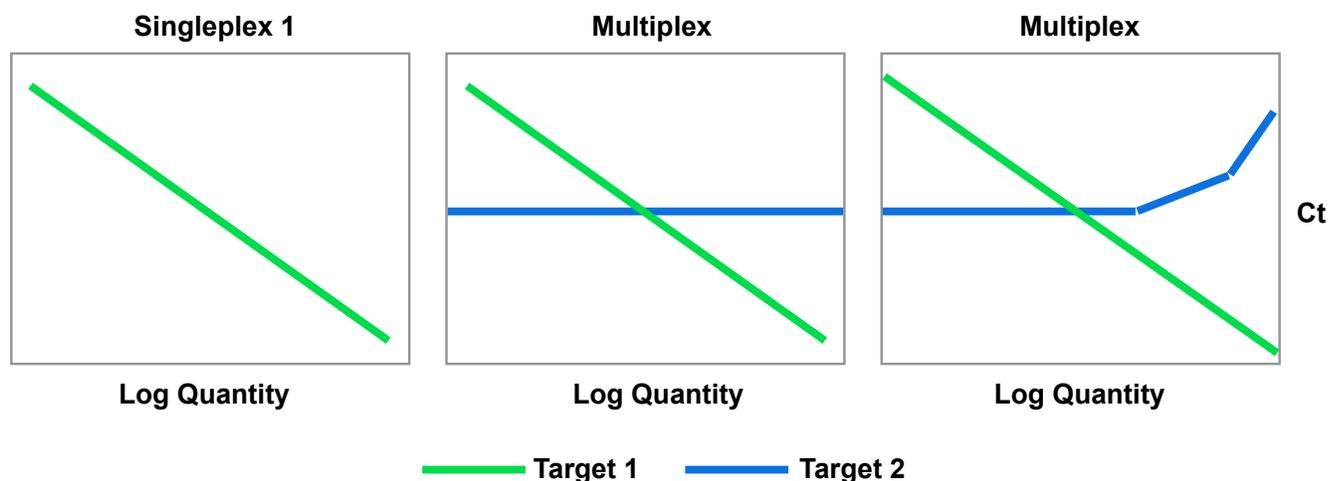


Figure 2 Illustration of possible mixed standard curve results

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 21 Example PCR Reaction Mix for mixed duplex standard curves

Component	Volume per singleplex reaction (Target 1)		Volume per duplex reaction ^[1]	
	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
Total PCR Reaction Mix volume	7 µL	17 µL	7 µL	17 µL

^[1] 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 μL of cDNA is added, add nuclease-free water to achieve a total reaction volume of 10 μL or 20 μL .

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 36).



TaqMan™ genotyping analysis

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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 (FAM™ dye and VIC™ dye) with the MGB–NFQ–quenched probe, and one custom SNP genotyping assay (ABY™ dye and JUN™ dye).

TaqMan™ SNP Genotyping Assays are available as predesigned assays or custom assays using the FAM™ dye and the VIC™ dye with MGB probes from the standard design pipeline. The assay with ABY™ dye and JUN™ dye can include MGB quenchers or be redesigned with QSY™-quenched probes.

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.

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.

Methods

Prepare singleplex PCR reactions

Ensure 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 ^[1]				
Assay Mix (Final)	Primer 1	Primer 2	Probe 1	Probe 2
40X	36 µM	36 µM	8 µM	8 µM

^[1] 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 22 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
Total PCR Reaction Mix volume	7.5 µL	7.5 µL

Table 23 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
Total PCR Reaction Mix volume	17 µL	17 µL

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

- Vortex the components to mix, then centrifuge briefly to collect the contents at the bottom of each tube.
- 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
- 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)
- 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.

- 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.

- Set up the thermal protocol for your instrument.

Table 24 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

- 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**

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

- Start the run.

- 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

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.

- 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
Total PCR Reaction Mix volume	7.5 µL	17 µL

2. Mix the components thoroughly, then centrifuge briefly to collect the contents at the bottom of the tube.
3. Transfer the appropriate volume of 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 44.

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

Analyze results

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

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



TaqMan™ microRNA analysis

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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 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 25 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. 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.

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 ΔR_n without affecting C_t .

Perform an experiment to ensure 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.

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

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 ΔR_n without affecting C_t .

Perform an experiment to ensure 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.
 - Perform *in silico* PCR using the UCSC Genome Browser at genome.ucsc.edu/cgi-bin/hgPcr.
- The primer melting temperature (T_m) 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.
- Ensure 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.

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

- 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.
- Ensure 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 ensures amplification of 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 26).

- 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 ΔR_n for singleplex when run against the target template
- Provide a low C_t and a low ΔR_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 = Σ (extinction coefficient of a given base \times number of times that base appears in the oligonucleotide sequence)
3. Calculate the oligonucleotide concentration in μM 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[absorbance at 260 nm / (sum of extinction coefficient contribution × 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
Total	—	—	167,950

measured absorbance at 260 nm = 0.13

sum of extinction coefficient = 167,950 M⁻¹cm⁻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 M⁻¹cm⁻¹ × 0.3 cm × C/100

C = 258 μM

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 ΔR_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	300 nM	300 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	300 nM	300 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	300 nM	300 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	300 nM	300 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 ΔR_n and C_t values to select the concentrations that give high ΔR_n values and low C_t values.



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).

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 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, 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 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
<https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf>
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
www.who.int/publications/i/item/9789240011311



Documentation and support

Related documentation 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 Pre-amplification 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>	MAN0028387
Software	SKU
<i>Primer Express™ Software Version 3.0 Getting Started Guide</i>	4362460
Custom Assay Design Tool	Available at Assay Design Tool
<i>TaqMan™ Genotyper Software User Guide</i>	4448637

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- 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.

