USER GUIDE

applied biosystems® by life technologies™

TaqMan® Multiplex PCR Optimization

For optimization of Multiplex PCR using 7500/7500 Fast, ViiA™ 7, and QuantStudio™ Real-Time PCR Systems

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About This Guide

Overview

This guide is designed to give guidance to researchers when performing multiplex PCR with TaqMan® Assays, and covers the following subjects:

- Guidance on optimizing multiplex PCR reactions
- Information on using products from Life Technologies 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 four probes using Life Technologies Real-Time PCR Systems and reagents

Product Information

Introduction

Life Technologies provides a variety of products to facilitate the performance of multiplex PCR and decrease the amount of time required to perform optimization, including TaqMan® QSY® probes, TaqMan® Multiplex Master Mix, and spectral calibration plates.

TaqMan® QSY® Probes

Introduction

QSY® is a new non-fluorescent quencher that can be used for designing Custom TaqMan® probes. QSY® custom probes can be substituted for 3′ TAMRA or 3′ BHQ probes without redesigning the probe sequence.

Custom TaqMan® QSY® Probes are available with FAM™, VIC®, ABY®, and JUN® dyes.

Dye	Absorbance (nm)	Emission (nm)
FAM™	496	520
VIC®	532	552
ABY®	568	583
JUN®	606	618

These dyes are optimized to work together for performing multiplex experiments on the QuantStudio™, ViiA™7, and 7500/7500 Fast Real-Time PCR Systems, and can be used to detect up to four targets in gene expression assays, two SNPs in SNP genotyping assays, and up to four copy number targets (e.g., two reference genes and two target genes) in copy number analysis.

TaqMan® QSY® Probes	Cat. No.
TaqMan® QSY® Probe 6,000 pmoles	4482777
TaqMan® QSY® Probe 20,000 pmoles	4482778
TaqMan® QSY® Probe 50,000 pmoles	4482779

TaqMan® Multiplex Master Mix

Introduction

The TaqMan® Multiplex Master Mix is formulated with optimized buffer components to accommodate multiplex amplification of up to four targets in a single reaction. It is supplied in a convenient 2X concentration premix to perform real-time PCR using TaqMan® probes. The master mix contains:

- AmpliTaq® DNA Polymerase, UP (Ultra Pure)
- Heat-labile Uracil-DNA Glycosylase (UDG)
- Mustang Purple® Passive Reference
- dNTP blend containing dUTP/dTTP

TaqMan® Multiplex Master Mix	Amount	Cat. No.
Mini-Pack	1-mL (100 × 20-µL reactions)	4461881
1-Pack	5-mL (500 × 20-µL reactions)	4461882
2-Pack	2 × 5-mL (1000 × 20-µL reactions)	4461884
5-Pack	5 × 5-mL (2500 × 20-µL reactions)	4484262
10-Pack	10 × 5-mL (5000 × 20-µL reactions)	4484263
Bulk Pack	50-mL (5000 × 20-µL reactions)	4486295

Storage

Store the TaqMan® Multiplex Master Mix at 2°C to 8°C.

Hot start

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

The polymerase is equipped with a proprietary hot start mechanism that provides improved specificity. The polymerase is re-activated after a 20 second incubation at 95°C.

UDG

TaqMan® Multiplex Master Mix contains heat-labile uracil-DNA glycosylase (UDG). UDG is also known as uracil-N-glycosylase (UNG).

Treatment with heat-labile UDG can prevent the reamplification of carryover PCR products by removing any uracil incorporated into single- or double-stranded amplicons (Longo et al., 1990). Heat-labile UDG prevents reamplification of carryover PCR products in an assay if all previous PCR for that assay was performed using a dUTP-containing master mix. See Appendix B (page 28) for more information about UDG.

PCR products are stable for up to 72 hours post-amplification using master mixes containing heat-labile UDG. Unlike standard UDG, heat-labile UDG is completely inactivated prior to amplification.

Mustang Purple® Passive Reference

TaqMan® Multiplex Master Mix contains Mustang Purple® dye as a passive reference (absorption 647 nm, emission 654 nm) to provide an internal reference for normalizing the reporter-dye signal during data analysis. Normalization corrects for fluorescence fluctuations due to changes in concentration or volume. Mustang Purple® dye is used in place of the typical ROX™ reference dye to allow use of the JUN® dye which is detected by the same channel.

dUTP/dTTP

A blend of dUTP/dTTP is included to enable UDG activity and maintain optimal PCR results.

Required Materials

Real-time instruments

TaqMan® Multiplex Master Mix can be used to run experiments on the following Life Technologies Real-Time PCR Systems:

- 7500 Real-Time PCR System
- 7500 Fast Real-Time PCR System
- ViiA™ 7 Real-Time PCR System
- QuantStudio™ 6 Flex, 7 Flex, and 12K Real-Time PCR Systems

Note: The software for the 7500 Fast Real-Time PCR Systems does not support assigning two SNP assays to one well.

Filters

Select the appropriate filter set for your dyes to maximize sensitivity and reduce signal crossover. The following filters are used for Life Technologies Real-Time PCR Systems:

Dye	7500/7500 Fast System	ViiA™7 System (384-well or 96-well)	QuantStudio™ Systems (384-well or 96-well)
FAM™	Filter A	x1m1	x1m1
VIC®	Filter B	x2m2	x2m2
ABY®	Filter C	x3m3	x3m3
JUN®	Filter D	x4m4	x4m4
Mustang Purple®	Filter E	x5m5	x5m5

Plates

Choose the plate appropriate for your real-time instrument.

Instrument	Plates‡	Cat. No.
ViiA™ 7 and QuantStudio™ 12K systems	MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode (0.1-mL)	
	• 20 plates	4346906
	• 200 plates	4366932
	MicroAmp® Optical 96-Well Reaction Plate with Barcode	
	• 20 plates	4306737
	• 500 plates	4326659
	MicroAmp® Optical 384-Well Reaction Plate with Barcode	
	• 50 plates	4309849
	• 500 plates	4326270
	• 1000 plates	4343814
7500 Fast systems	MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode (0.1-mL)	
	• 20 plates	4346906
	• 200 plates	4366932
7500 system	MicroAmp® Optical 96-Well Reaction Plate with Barcode	
	• 20 plates	4306737
	• 500 plates	4326659

Optical seals

Seal all plates with MicroAmp® Optical Adhesive Film.

Item	Cat. No.
MicroAmp® Optical Adhesive Film:	
• 25 covers • 100 covers	4360954 4311971

Spectral calibration plates

It is recommended that calibration and verification of your real-time instrument be performed at least every six months. The following spectral calibration plates are used for Life Technologies Real-Time PCR Systems.

Note: ABY® and JUN® are not currently recognized as system dyes, therefore a custom calibration will be required. For spectral calibration on 7500 and 7500 Fast Systems, set up custom calibration files for FAM™ and VIC® plates using new file names. **Do not** over-write the original system FAM™ and VIC® calibration files. Refer to your instrument maintenance guide for full instructions on use.

Calibration Plate	Real-Time PCR System			Cat. No.
	7500	7500 Fast	ViiA™7/ QuantStudio™	
Fast 96-Well Spectral Calibration Plate with ABY® Dye for Multiplex qPCR		X	X	A24734
Fast 96-Well Spectral Calibration Plate with JUN® Dye for Multiplex qPCR		X	X	A24735
Fast 96-well Spectral Calibration Plate (Mustang Purple® Dye)		X	X	4457328
384-Well Spectral Calibration Plate with ABY® Dye for Multiplex qPCR			X	A24736
384-Well Spectral Calibration Plate with JUN® Dye for Multiplex qPCR			X	A24733
384-well Spectral Calibration Plate (Mustang Purple® Dye)			X	4457334
96-Well Spectral Calibration Plate with ABY® Dye for Multiplex qPCR	X		X	A24738
96-Well Spectral Calibration Plate with JUN® Dye for Multiplex qPCR	X		X	A24737
96-well Spectral Calibration Plate (Mustang Purple® Dye)	X		X	4461599
Fast 96-well Spectral Calibration Plate (FAM™ Dye)		X	X	4432389
Fast 96-Well Spectral Calibration Plate (VIC® Dye)		X	X	4432396
96-well Spectral Calibration Plate (FAM™ Dye)	X		X	4432327
96-Well Spectral Calibration Plate (VIC® Dye)	X		X	4432334
384-well Spectral Calibration Plate (FAM™ Dye)			X	4432271
384-Well Spectral Calibration Plate (VIC® Dye)			X	4432278

Other kits

Item	Cat. No.
SuperScript® VILO™ cDNA Synthesis Kit:	
• 50 reactions • 250 reactions	11754-050 11754-250
High Capacity cDNA Reverse Transcription Kit:	
• 200 reactions • 200 reactions with RNase Inhibitor • 1000 reactions • 1000 reactions with RNase Inhibitor	4368814 4374966 4368813 4374967

Other consumables

Item	Source
Centrifuge with adapter for 96-well plates *or* Centrifuge with adapter for 384-well plates	Major laboratory supplier (MLS)
Disposable gloves	MLS
Microcentrifuge	MLS
Pipette tips, with filter plugs	MLS
Pipettors, positive-displacement or air-displacement	MLS
Polypropylene tubes	MLS
Tris-EDTA (TE) Buffer, pH 8.0	MLS
Vortexer	MLS

Strategies for Optimization

Introduction

The goal of multiplex PCR is the simultaneous amplification and accurate quantitation of multiple targets in a single reaction tube. Different reporters are used in multiplex PCR to track each individual amplification reaction. For applications using TaqMan® probes and probe-based assays, each specific probe is labeled with a unique fluorescent dye. The signal from each dye is detected by the real-time PCR instruments, and used to determine the amount of each target.

Factors that affect the reliability of multiplex PCR assays include:

- 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 dynamic range of their expression.

General considerations

Important considerations when optimizing multiplex assays include:

- Primer design
  Primers should not be able to bind elsewhere in 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 (see Appendix A, page 24).
- Probe design
  The Tm of TaqMan® probes should be ~10°C higher than the Tm of the primers (approximately 68–70°C). Please note, that MGB probe sequences are not interchangeable with QSY® probe sequences because the QSY® probe length will be too short. If you are using custom designed probes, please refer to the "Primer Express® Software Version 3.0 Getting Started Guide" (part number 4362460) for guidance on designing primers and probes for multiplex PCR. If you are using a TaqMan® predesigned assay contact Custom Services at **custom.solutions@lifetech.com** to have assays redesigned.
- Primer and probe selection for multiplex reactions
  A multiplex reaction can contain up to eight primers and four probes (to produce four amplicons), so it is good practice to minimize conditions that result in primer dimer formation or other unfavorable interactions.

  Make sure 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. Two web-based tools that can be used in verification of coordinates include the UCSC Genome Browser In Silico PCR utility at: **genome.ucsc.edu/cgi-bin/hgPcr**, or the NCBI e-PCR resource at: **www.ncbi.nlm.nih.gov/tools/epcr**.

  It is also important to make sure that amplicons are all approximately the same size, and that primer and probe dimers do not form (across all primer pairs).

General considerations, continued

- Probe selection

  If you are currently using TaqMan® predesigned assays containing MGB-NFQ probes (also referred to as MGB probes), you can continue to use these assays in a multiplex reaction. However, the multiplex reaction should not contain more than two MGB probes to ensure successful amplification.

- The ABY® and JUN® probes must be made with QSY®, but FAM™ and VIC® probes can be made with either QSY® or MGB. For example, multiplex gene expression analysis can use FAM™ and VIC® TaqMan® Gene Expression Assays combined with one assay containing a custom ABY®-QSY® probe and one assay containing a custom JUN®-QSY® probe. Also, for multiplex SNP genotyping analysis, an existing FAM™/VIC® TaqMan® SNP Genotyping assay can be combined with a custom ABY®-QSY®/JUN®-QSY® assay.
- Dye selection

  Choose dyes with little to no overlap in their emission spectra. Also, match dye intensity with target abundance by pairing the brightest dye with low abundance targets, and the dimmest dye with high abundance targets (e.g., an internal positive control).

- PCR reaction buffer

  Because all of the assays are amplified in the same tube, they compete for the same reagents (dNTPs, Mg^{2+}, 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. Master mixes specifically designed for performing multiplex PCR should be used to offset the effect of competition for reagents.

Experimental overview

In order to perform multiplex reactions, confirm that results obtained from multiplexing are the same as results obtained from singleplex reactions. The general procedure is as follows:

1. Run your singleplex reactions and confirm amplification.
2. After establishing conditions for performing singleplex reactions, set up conditions for multiplex reactions. Ensure amplification occurs and analyze data.
3. Determine whether the singleplex and multiplex reactions give the same Ct values.
4. If the singleplex and multiplex reactions do not give the same Ct values, optimize primer/probe concentrations if necessary to obtain the desired ΔCt.

Gene Expression Analysis

Gene expression assays

Gene expression assays are 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). In gene expression multiplexing experiments, the goal is to minimize the difference between the Ct value in singleplex and multiplex reactions.

Up to four targets can be multiplexed in a single reaction depending upon the probes that are selected.

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 Ct range as shown in the following table.

Target Expression Level	Ct Range
High	Ct ≤ 20
Medium	20 < Ct ≤ 27
Low	27 < Ct ≤ 35
No template control	Ct > 35

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

- **Some targets more abundant than others**
  When multiplex PCR is performed on a sample in which one (or more) target(s) is more abundant than the others, the assay(s) for the abundant species should be primer-limited. Typically, housekeeping genes/endogenous controls are high expressors. Using primer-limited reaction conditions prevents consumption of reactants (dNTPs) before the less abundant species begins to amplify. Primer-limited assays sold by Life Technologies have final primer concentration of 150 nM each with 250 nM probe concentration. This is a suggested starting point for customer optimization. Please note, in addition to limiting primers, for very highly abundant transcripts, probe concentration may need to be adjusted (see Appendix A, page 24).

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

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

Primer/probe concentration

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

In multiplexing start with a standard condition (900 nm/900 nm/250 µM for forward primer/reverse primer/probe), but optimization of the assay may be necessary.

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

Dye selection

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

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

Probe selection

- Up to four targets can be multiplexed in a single reaction using QSY® probes (FAM™, VIC®, ABY®, and JUN® dyes).
- Use no more than two probes that contain the MGB group. If the assays are from our Gene Expression Assay selection, contact Custom Services at **custom.solutions@lifetech.com** to order assays redesigned with a non-MGB probe.

Verifying singleplex reactions for gene expression analysis

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

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

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

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

2. Prepare the reaction mixtures as follows for PCR in a 384-Well Plate*:

Component	Singleplex reaction 1	Singleplex reaction 2	Singleplex reaction 3	Singleplex reaction 4
Master Mix	5 µL	5 µL	5 µL	5 µL
FAM™ assay mix (20X)	0.5 µL	—	—	—
VIC® assay mix (20X)	—	0.5 µL	—	—
ABY® assay mix (20X)	—	—	0.5 µL	—
JUN® assay mix (20X)	—	—	—	0.5 µL
Template	Up to 3 µL (1–10 ng of cDNA)			
Water	To total volume			
TOTAL	10 µL	10 µL	10 µL	10 µL

* Double all reaction volumes for performing validation in 96-Well Plates.

3. Mix the components thoroughly, and centrifuge briefly to spin down the contents and eliminate any air bubbles.
4. Transfer the appropriate volume of each reaction to each well of an optical plate.
5. Seal the plate with an optical adhesive cover, and centrifuge the plate briefly to spin down the contents and eliminate any air bubbles.
6. Perform PCR (Refer to “Run the PCR reaction plate”, page 23).
7. Analyze results etc.
8. Proceed to “Verifying multiplex reactions for gene expression analysis” (page 15).

Verifying multiplex reactions for gene expression analysis

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

1. Note the concentrations from "Verifying singleplex reactions for gene expression analysis" (page 14).
2. Combine validated singleplex concentrations in a multiplex reaction and confirm that they work together.

Component	Multiplex (4-plex) reaction	
	384-Well Plate (10 µL/well)	96-Well Plates (20 µL/well)
Master Mix	5 µL	10 µL
FAM™ assay mix (20X)	0.5 µL	1 µL
VIC® assay mix (20X)	0.5 µL	1 µL
ABY® assay mix (20X)	0.5 µL	1 µL
JUN® assay mix (20X)	0.5 µL	1 µL
Template	Up to 3 µL (1–10 ng of cDNA)	
Water	To total volume	
TOTAL	10 µL	20 µL

3. Mix the components thoroughly, and centrifuge briefly to spin down the contents and eliminate any air bubbles.
4. Transfer the appropriate volume of each reaction to each well of an optical plate.
5. Seal the plate with an optical adhesive cover, and centrifuge the plate briefly to spin down the contents and eliminate any air bubbles.
6. Perform PCR (Refer to "Run the PCR reaction plate", page 23).

Evaluation of gene expression PCR results

Evaluate multiplex qPCR results to determine that the reaction efficiency, ΔCt between singleplex and multiplex, and standard deviation of replicates are not compromised using the selected multiplex conditions. Ideally, there should be no difference between the results from single and multiplex reactions under the selected conditions.

- **Reaction efficiency**

  Make a dilution series of the sample containing seven 10-fold dilutions in triplicate. Run each assay individually and in multiplex using each dilution in the series.

  The standard curve method is recommended to evaluate (optimize) multiplex assays. Run as many 10-fold dilution points, in triplicate, as possible for the sample(s) and assays being investigated for each assay singly and in multiplex. A minimum of 3 logs should be used, but up to 6 logs is ideal. Ensure that the dynamic range of the standard curve is broad enough to encompass most of the experimental samples, bearing in mind that the expression levels of the target(s) of interest may vary widely between samples

  Take a careful look at the standard curve to verify that there is a good fit of the line to all the dilution points, and that the correlation coefficient (R^2) of the line is 0.98 or higher. A lower R^2 value indicates that some of the dilutions (usually the lowest, highest, or both) do not fall within the range of the standard curve. Refer to the document "Real-Time PCR: Understanding Ct" (Publication CO16926) at **www.lifetechnologies.com** for guidance on how to evaluate an experiment.

  - Results are analyzed in a plot of *log*[template amount]$_{(x\text{-axis})}$ against Ct value$_{(y\text{-axis})}$
  - The slope of the line is used to calculate the PCR efficiency using the formula:

    Efficiency = $10^{(-1/\text{slope})} - 1$

  The target efficiency for 5 to 6 logs should be 100% +/- 10% in both singleplex and multiplex. If there are significant differences, please re-optimize the primer and probe concentrations.

- **ΔCt between singleplex and multiplex**

  Using the dilution series, calculate ΔCt value between the target in singleplex and multiplex. The ΔCt values between multiplex and singleplex need to be <1.

  Differences in Ct between single and multiplex reactions can often be mitigated by using adjusting primer concentrations. Adjustments following the general guidelines provided under ''Verifying singleplex reactions for gene expression analysis" (page 14) may be required.

- **Standard deviation**

  A high standard deviation of the Ct indicates that other factors, such as competition or inhibition in the multiplex reaction, are contributing to the lack of reproducibility. In general, a Ct standard deviation variation of less than 3% indicates good reproducibility.

  Determine the standard deviations of samples assayed as single and multiplex reactions. High standard deviations of Ct values in multiplex reactions can often be minimized by adjustments to the cycling conditions. Increasing the anneal/extend times (to 30 to 45 seconds) is suggested if the standard deviations in multiplex reactions increase relative to singleplex.

Genotyping Analysis

SNP analysis

Genotyping assays are 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 TaqMan® Genotyping Assay (FAM™/VIC®) and one (ABY®/JUN®) SNP assay.

Primer/probe concentration

- In many, but not all, cases the Genotyping Assay concentrations of 900/900/200/200 nM (forward primer/reverse primer/allele 1 probe/allele 2 probe) are suitable for ABY®/JUN® assays. Individual primers and probes can be ordered if desired.
- Higher probe concentration can increase signal.

Dye selection

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

Probe selection

- Use no more than two probes that contain the MGB group. If the assays are from our Genotyping Assay Selection, a non-MGB probe can be designed and ordered.
- To convert a FAM™/VIC® SNP assays to ABY®/JUN® assays contact Custom Services at **custom.solutions@lifetech.com** to have assays redesigned.

Verifying individual SNP reactions for genotyping analysis

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

1. Prepare ABY®/JUN® and FAM™/VIC® assays using the concentrations in the table below as a starting point. Slight changes may be required to give optimal performance.

Concentration*				
Assay Mix (Final)	Primer 1	Primer 2	Probe 1	Probe 2
40X	36 µM	36 µM	8 µM	8 µM

* Using a 40X assay mix, the concentrations of primers and probes in the reaction will be 900 nM/900 nM/200 nM/200 nM, respectively (the same as used for TaqMan® FAM™/VIC® Genotyping Assays).

2. Prepare the reaction mixtures as follows for PCR in a 384-Well Plate*:

Component	SNP reaction 1	SNP reaction 2
Master Mix	5 µL	5 µL
FAM™/ VIC® assay mix (40X)	0.25 µL	—
ABY®/JUN® assay mix (40X)	—	0.25 µL
Template	Up to 3 µL (1–10 ng of gDNA)	
Water	To total volume	
TOTAL	10 µL	10 µL

* Double all reaction volumes for performing validation in 96-Well Plates.

3. Mix the components thoroughly, and centrifuge briefly to spin down the contents and eliminate any air bubbles.
4. Transfer the appropriate volume of each reaction to each well of an optical plate.
5. Seal the plate with an optical adhesive cover, and centrifuge the plate briefly to spin down the contents and eliminate any air bubbles.
6. Perform PCR (Refer to “Run the PCR reaction plate”, page 23). We suggest using 30 seconds as a starting point for the anneal/extend time. Longer amplicons may benefit from longer anneal/extend times.
7. Confirm that the results match controls
8. Proceed to “Verifying (4-color) 2 SNP reactions for genotyping analysis” (page 19).

Verifying (4-color) 2 SNP reactions for genotyping analysis

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

1. Note the concentrations from "Verifying individual SNP reactions for genotyping analysis" (page 18). For optimal clustering, quantification and normalization of gDNA concentration is recommended.
2. Combine validated singleplex concentrations in a multiplex reaction and confirm that they work together.

Component	Multiplex (2 SNP) reaction	
	384-Well Plate (10 µL/well)	96-Well Plates (20 µL/well)
Master Mix	5 µL	10 µL
FAM™/ VIC® assay mix (40X)	0.25 µL	0.5 µL
ABY®/JUN® assay mix (40X)	0.25 µL	0.5 µL
Template	Up to 3 µL (1–10 ng of gDNA)	
Water	To total volume	
TOTAL	10 µL	20 µL

3. Mix the components thoroughly, and centrifuge briefly to spin down the contents and eliminate any air bubbles.
4. Transfer the appropriate volume of each reaction to each well of an optical plate.
5. Seal the plate with an optical adhesive cover, and centrifuge the plate briefly to spin down the contents and eliminate any air bubbles.
6. Perform PCR (Refer to "Run the PCR reaction plate", page 23). We suggest using 30 seconds as a starting point for the anneal/extend time. Longer amplicons may benefit from longer anneal/extend times.

Copy Number Analysis

Copy number variation analysis

Germline or somatic alterations can result in a gene having one copy to three copies, more than three copies, or even no copies. Copy number variation (CNV) analysis is performed to detect such differences using DNA as a template.

Primer/probe concentration

Higher concentrations permit the addition of more template.

Probe selection

- Up to four targets can be multiplexed in a single reaction using QSY® probes (FAM™, VIC®, ABY®, and JUN® dyes).
- Use no more than two probes that contain the MGB group. If more than two of the assays are from our TaqMan® Copy Number Assays selection, they will need to be redesigned with a QSY® probe. Contact **custom.solutions@lifetech.com** for more information.
- Existing Copy Number Reference Assays (Cat. nos. 4403316 and 4403326) use the TAMRA™ quencher, and **cannot** be used with ABY® labeled assays. An ABY® RNaseP assay (Cat. nos. 4485714 and 4485715) can be used in place of Cat. no. 4403326.

Verifying singleplex reactions for copy number analysis

Verify the performance of the singleplex assays by running duplex reactions with a known reference assay (ABY®-RNaseP) against a panel of gDNA with known/previously determined copy numbers.

1. Prepare concentrated assay mix for assays using the values provided in the table below. Suggested concentrations are given below, but slight changes may be required to give optimal performance.

Concentration*			
Assay Mix (Final)	Primer 1	Primer 2	Probe 1
20X	18 µM	18 µM	5 µM

* With a 20X assay mix, the final reaction concentrations of primers and probe will be 900 nM/900 nM/250 nM, respectively. If a formulation greater than 20X is required, multiply the values accordingly.

2. Prepare the reaction mixtures as follows for PCR in a 384-Well Plate*:

   Note: This table assumes that the ABY® assay is being used for the reference assay. If the reference assay is labeled differently, the three duplex reactions should be adjusted so that the reference assay is contained in each of the three reactions.

Component	Assay and reference reaction		
	Duplex 1	Duplex 2	Duplex 3
Master Mix	5 µL	5 µL	5 µL
FAM™ assay mix (20X)	0.5 µL	—	—
VIC® assay mix (20X)	—	0.5 µL	—
JUN® assay mix (20X)	—	—	0.5 µL
ABY® assay mix (20X)	0.5 µL	0.5 µL	0.5 µL
Template	Up to 3 µL (1–10 ng of cDNA)		
Water	To total volume		
TOTAL	10 µL	10 µL	10 µL

* Double all reaction volumes for performing validation in 96-Well Plates.

3. Mix the components thoroughly, and centrifuge briefly to spin down the contents and eliminate any air bubbles.
4. Transfer the appropriate volume of each reaction to each well of an optical plate.
5. Seal the plate with an optical adhesive cover, and centrifuge the plate briefly to spin down the contents and eliminate any air bubbles.
6. Perform PCR (Refer to “Run the PCR reaction plate”, page 23). We suggest using 30 seconds as a starting point for the anneal/extend time. Longer amplicons (>120 bp) may benefit from longer anneal/extend times.
7. Proceed to “Optimizing multiplex reactions for copy number analysis” (page 22).

Verifying multiplex reactions for copy number analysis

After verifying duplex assays, assemble the multiplex copy number reaction using the volumes provided in the Table below.

1. Note the concentrations from "Optimizing singleplex reactions for copy number analysis" (page 21).
2. Combine validated assays in a multiplex reaction and confirm that they work together.

Component	Multiplex (4-plex) reaction	
	384-Well Plate (10 µL/well)	96-Well Plates (20 µL/well)
Master Mix	5 µL	10 µL
FAM™ assay mix (20X)	0.5 µL	1 µL
VIC® assay mix (20X)	0.5 µL	1 µL
ABY® assay mix (20X)	0.5 µL	1 µL
JUN® assay mix (20X)	0.5 µL	1 µL
Template	Up to 3 µL (1–10 ng of cDNA)	
Water	To total volume	
TOTAL	10 µL	20 µL

3. Mix the components thoroughly, and centrifuge briefly to spin down the contents and eliminate any air bubbles.
4. Transfer the appropriate volume of each reaction to each well of an optical plate.
5. Seal the plate with an optical adhesive cover, and centrifuge the plate briefly to spin down the contents and eliminate any air bubbles.
6. Perform PCR (Refer to "Run the PCR reaction plate", page 23). We suggest using 30 seconds as a starting point for the anneal/extend time. Longer amplicons (>120 bp) may benefit from longer anneal/extend times.

Run the PCR Reaction Plate

Before starting

- Make sure that the instrument is calibrated for the dyes (e.g. ABY® and JUN® dyes) being used in the assay.
- **Do not** use the default passive reference setting (ROX™) when using Mustang Purple® dye as a passive reference since they are read in different channels. Make sure the passive reference is set to Mustang Purple® when it is used as a passive reference.

Running the reaction plate

1. Place the reaction plate in the instrument.
2. Use the thermal cycling conditions specified in the following tables.

   Note: These are default conditions for ViiA™7, QuantStudio™, and 7500 Fast systems. If using the 7500 system, edit the thermal cycling program to correspond with the 7500 Fast thermal cycling protocol.

ViiA™ 7/QuantStudio™ Real-Time PCR Systems			
Step	Temperature	Duration	Cycles
AmpliTaq® DNA Polymerase, UP Activation	95°C	20 sec	Hold
Denature*	95°C	1 sec	40
Anneal/Extend	60°C	20 sec	

7500/7500 Fast Real-Time PCR Systems			
Step	Temperature	Duration	Cycles
AmpliTaq® DNA Polymerase, UP Activation	95°C	20 sec	Hold
Denature*	95°C	3 sec	40
Anneal/Extend**	60°C	30 sec	

   * Denature time can be increased if the amplicon size is long.
   ** In some cases, increasing the anneal/extend time has been found to improve performance of multiplex reactions.

3. Set the reaction volume being used for the PCR reaction.
4. Start the run.

Appendix A

Identify Target Sequences and Design Primers

Identify target sequence and amplicon size

A target template is a DNA sequence, including cDNA, genomic DNA, or plasmid DNA that you want to amplify.

Design primers to amplify amplicons (segments of DNA) within the target sequence using Primer Express® Software. Shorter amplicons work best. Consistent results are obtained for amplicon size ranges from 50 to 150 bp.

Guidelines for designing primers

Design primers using Primer Express® Software as described in the P*rimer Express® Version 3.0 Getting Started Guide* (PN 4362460) and *Online Help*.

- The primers should be specific for the target. Perform a BLAST search of the amplicon at **www.ncbi.nlm.nih.gov/blast**, or perform *in silico* PCR using the UCSC Genome Browser at **www.genome.ucsc.edu**.
- The primer 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.
- Makes sure 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 especially be avoided to minimize the formation of artifact products (i.e. primer-dimers or primer-oligomers).

  Note: The free AutoDimer software can be used to check your primers. This tool is located at: **www.cstl.nist.gov/strbase/AutoDimerHomepage/AutoDimerProgramHomepage.htm**
- Do not overlap primer and probe sequences. The optimal primer length is about 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.
- Make sure the last five nucleotides at the 3´ end contain no more than two G and/or C bases.

Select an amplicon site for cDNA

Selecting a good amplicon site ensures amplification of the target cDNA without co-amplifying the genomic sequence, pseudogenes, and related genes.

Guidelines

- The amplicon should span one or more introns to avoid amplification of the target gene in genomic DNA.
- The primer pair must be specific to the target gene; the primer pair does not amplify pseudogenes or other related genes.
- Design primers according to Primer Express® Software guidelines.
- Test the amplicons, then select those that have the highest signal-to-noise ratio (that is, low Ct with cDNA and no amplification with no template control or genomic DNA).
- If no good sequence is found, you may need to examine the sequence and redesign the amplicon or screen for more sites.

If the gene you are studying does not have introns, then you cannot design an amplicon that amplifies the mRNA sequence without amplifying the genomic sequence. In this case, you may need to run RT minus controls.

Optimize Primer Concentrations for PCR

Overview

By independently varying the forward and reverse primer concentrations, you can identify the primer concentrations that provide optimal assay performance. The primer concentrations you select should provide a low Ct and a high ΔR_n when run against the target template, but should not produce nonspecific product formation with NTCs.

Quantitate the primers

1. Measure the absorbance (at 260 nm of a 1:100 dilution) of each primer oligonucleotide in TE buffer.
2. Calculate the sum of extinction coefficient contributions for each base within the primer:

   extinction coefficient contribution = Σ(extinction coefficient of a given base × number of times that base appears in the oligonucleotide sequence)

   Refer to the following table for an example calculation.
3. Calculate the oligonucleotide concentration in µM for each primer:

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

   Rearrange to solve for concentration:

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

An example calculation of primer concentration

In this example, the concentration of a primer (in TE buffer, diluted 1:100), with the sequence CGTACTCGTTCGTGCTGC 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	7050	6	42,300
G	12,010	5	60,050
T	8400	6	50,400
Total	—	—	167,950

measured absorbance at 260 nm = 0.13

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

cuvette path length = 0.3 cm

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

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

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 Ct while maintaining a ΔR_n that is reasonable for the application.

The table below 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 block type (96-well or 384-well), and amount of sample and reagents.

	Forward Primer (nM)	Reverse Primer (nM)	Probe (nM)
1	900	900	250
2	450	450	250
3	300	300	250
4	150	150	250
5	75	75	250
6	900	900	200
7	450	450	200
8	300	300	200
9	150	150	200
10	75	75	200
11	900	900	150
12	450	450	150
13	300	300	150
14	150	150	150
15	75	75	150
16	900	900	100
17	450	450	100
18	300	300	100
19	150	150	100
20	75	75	100

1. Prepare a reaction plate using the desired template(s) and single template concentrations.
2. Determine the concentration combinations to be evaluated and prepare the reactions accordingly.
3. Prepare the reaction plate and load it into a Life Technologies real-time PCR system.
4. Program the thermal-cycling conditions according to the information in step 2 on page 23 and run the plate.
5. Analyze results, and compile ΔR_n and Ct values to select the concentrations that give high ΔR_n values and low Ct values.

Note: For SNP assay formulation, we recommend starting with the concentration of our predesigned assays (900 nm for each primer and 200 nM for each probe). Similarly for CNV assay formulation, we recommend starting with the concentration of our predesigned assays (900 nM for each primer and 250 nM for the probe).

Appendix B

Contamination Prevention

Overview

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

Using UDG to minimize reamplification carryover products

TaqMan® Multiplex Master Mix contains heat-labile uracil-DNA glycosylase (UDG). UDG is also known as uracil-N-glycosylase (UNG). Treatment with heat-labile UDG is useful in preventing the reamplification of carryover PCR products.

The heat-labile UDG used in the TaqMan® Multiplex Master Mix is a 26-kDa recombinant enzyme derived from the thermolabile UDG gene isolated from marine bacteria, and expressed in *E. coli*.

UDG acts on single- and double-stranded dU-containing DNA. It acts by hydrolyzing uracil-glycosidic bonds at dU-containing DNA sites. The enzyme causes the release of uracil, thereby creating an alkali-sensitive apyrimidinic site in the DNA. The enzyme has no activity on RNA or dT-containing DNA (Longo et al., 1990).

Using NTC controls

No Template Control (NTC) reactions can be used to identify PCR contamination. NTC reactions contain all reaction components (TaqMan® Multiplex Master Mix, primers, water) except sample, and therefore should not return a Ct value.

PCR good laboratory practices

When preparing samples for PCR amplification:

- Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation) and clean gloves.
- Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:

  Sample preparation

  PCR setup

  PCR amplification

  Analysis of PCR products
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes carefully. Try not to splash or spray PCR samples.
- Keep reactions and components capped as much as possible.
- Use a positive-displacement pipette or aerosol-resistant pipette tips.
- Clean lab benches and equipment periodically with a 10% bleach solution.

Appendix C

Safety

Chemical safety guidelines

To minimize the hazards of chemicals:

- 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.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the SDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the SDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

Chemical waste safety guidelines

To minimize the hazards of chemical waste:

- Read and understand the Safety Data Sheets (SDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Provide 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.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the SDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the SDS.
- Handle chemical wastes in a fume hood.
- After emptying the waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

Waste disposal

If potentially hazardous waste is generated when you operate the instrument, you must:

- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure the health and safety of all personnel in your laboratory.
- Ensure that the instrument 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. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

- U.S. Department of Health and Human Services guidelines published in “Biosafety in Microbiological and Biomedical Laboratories” (BMBL) at **www.cdc.gov/biosafety/publications/bmbl5**.
- U.S. Department of Labor regulations published in “Occupational Safety and Health Standards, Bloodborne Pathogens” (29 CFR§1910.1030). Search for 29 CFR 1910.1030 at **www.osha.gov**.
- Your company’s/institution’s Biosafety Program protocols for working with/handling potentially infectious materials.

Additional information about biohazard guidelines is available at: **http://www.cdc.gov**

Documentation and Support

Support documents

You can download the following documents from the Life Technologies website at: **www.lifetechnologies.com**

Document	Part number
All Systems	
High-Capacity cDNA Reverse Transcription Kit Protocol	4375575
Primer Express® Software Version 3.0 Getting Started Guide	4362460
Real-Time PCR Systems Chemistry Guide	4348358
TaqMan® Gene Expression Assays Protocol	4333458
7500 Fast System	
Applied Biosystems® 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Getting Started Guide	4347828
Applied Biosystems® 7300/7500/7500 Fast Real-Time PCR System Relative Quantification Getting Started Guide	4347824
Applied Biosystems® 7300/7500/7500 Fast Real-Time PCR System Absolute Quantification Getting Started Guide	4347825
ViiA™ 7 System	
Applied Biosystems® ViiA™ 7 Real-Time PCR System Getting Started Guides	4441434
Applied Biosystems® ViiA™ 7 Real-Time PCR System User Guide	4442661
QuantStudio™ 12K System	
Applied Biosystems® QuantStudio™ 12K Real-Time PCR System User Guide	4470050

Obtaining support

For the latest services and support information for all locations, go to **www.lifetechnologies.com**

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**techsupport@lifetech.com**)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at **www.lifetechnologies.com/support**.

Certificate of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to **www.lifetechnologies.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.

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 found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.

For support visit www.appliedbiosystems.com/support

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