2X Super Multiplex qPCR Master Mix-TaqMan Probe

Cat qPCR 159, 200/500/1,000 reactions x 20µL (No ROX, Low ROX or High ROX)

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Intended Use

The 2X Super Multiplex qPCR Master Mix is used for real-time qualitative and quantitative PCR amplifications with TaqMan probes.

Introduction

The kit is designed for multiplex qPCR with up to five pairs of primers and five TaqMan probes.

In multiplex qPCR amplification, a DNA-dependent DNA polymerase amplifies DNA templates and its 5´-3´exonuclease cleaves a TaqMan probes to generate fluorescent signals.

The kit contains Super *Taq*-Probe DNA polymerase, which is specially engineered for TaqMan probes and its increased 5’-3’ exonuclease activity generating S-shaped curves.

The kit has three formulations of No ROX, Low ROX or High ROX concentrations for your choice compatible with a variety of PCR instruments.

For larger volume requirements, customized and bulk packaging is available. Please contact: contact@genetictoolsllc.com for more information.

Characteristics

- The kit contains Super *Taq*-Probe DNA polymerase specially engineered for TaqMan probes, generating S-shaped curves.
- The preferable PCR product size is ≤150bp.
- The kit is formulated as No ROX, Low ROX or High ROX concentrations for your choice.

Components

2X Master Mix (2x1mL/5x1mL/10mL for 200/500/1000 reactions x 20µL) and an instruction for use are included in the kit.

The master mix is a premixed, 2X concentrated solution that has all the components, such as DNA polymerase and dNTPs, except for gene-specific primers, TaqMan probes and DNA templates.

Storage and Stability

The kit can be transported at below 4°C for up to 3 days. The kit should be kept stable in the dark at -20°C for ≤24 months with ≤10 times of freeze-thaw cycles. The kit can be stored at 4°C for a weak.

Required Materials Not Included

• Target-specific primers

• Fluorescently-labeled probes

• DNA template

• PCR strip tubes and microcentrifuge tubes

• PCR tubes or PCR plates and seals

• Real-time fluorescent PCR instrument

General Tips and Considerations

• Proper sterile technique and careful pipetting should be used to avoid cross-contamination between DNA samples and particularly carryover-contamination from one experiment to the next. For example, it is absolutely forbidden to open the cap of a tube or the film of a 96-well plate post PCR amplification.

• To exclude outlier amplifications, each DNA sample is recommended to run in triplicate for more consistency.

• Ensure that all components are thawed, mixed and centrifuged prior to use.

• When pipetting a positive or unknown DNA sample into the tube or PCR plate, it is advisable to avoid bubble formation so as to prevent cross-contamination due to the bubble breaking.

• Choose detection channels of your PCR instrument which are compatible to the fluorophore labelings of TaqMan probes.

• When using multichannel pipettes, care should be taken to ensure accuracy and consistency of pipetting volume.

2X Super Multiplex qPCR Master Mix-TaqMan Probe Kit Protocols

Before Use

• DNA samples should be extracted by a qualified silica-based kit and eluted with low EDTA TE buffer (10mM Tris-HCl, 0.1mM EDTA, pH 8.0-8.3). Determine its concentration by OD_{260} absorbance.

• Dilute standard DNA samples in either water or TE buffer freshly before experiment to avoid absorbance to tube surface.

• Check availability of your PCR instrument and compatibility with passive reference ROX dye.

Table 1. Compatible instruments

PCR instrument	ROX required by instrument	Passive reference ROX dye setup
Bio-Rad® iQ™5, CFX96, CFX384, Opticon, Roche Lightcycler®, Qiagen Rotor-Gene™, Eppendorf Mastercycler®, Cepheid® SmartCycler®	Not recommended	Not necessary
Applied Biosystems® 7500, 7500 Fast, QuantStudio™, ViiA7™, Agilent Mx™	Low ROX (50nM final concentration)	Turn on ROX passive reference dye button
Applied Biosystems® 5700, 7000, 7300, 7700, 7900, 7900HT, 7900HT Fast, StepOne™, StepOnePlus™	High ROX (500nM final concentration)	Turn on ROX passive reference dye button

Reaction Setup

For more consistency, we recommend that each standard and unknown DNA samples are performed in triplicate.

Table 2. Setting up a 20µL or 10µL reaction

Component	Volume per 20µL	Volume per 10µL	Final concentration
2X Master Mix	10µL	5µL	1X
10µM Primers [a]	0.4µL	0.2µL	Each 200nM
10µM TaqMan probes [a]	0.4µL	0.2µL	Each 200nM
DNA template [a]	Variable	Variable	≤500ng of human genomic DNA
H_2O	To 20µL	To 10µL	

[a] Typical optimal concentrations of well-designed primers and probes are exampled. However, the concentrations of primers and probes are variable from 150-900nM and from 100-500nM, depending on assay designs and thermo-cycling protocols. See Usage Notes for additional guidelines on primer/probe design and template preparation as well as concentrations.

1. Thaw 2X Super Multiplex qPCR Master Mix and other reaction components at room temperature, mix each component, centrifuge and then place on ice.

2. Determine the total volume for the number of reactions, add 5-10% extra volume, and prepare assay mix of all components except DNA template. Mix the assay mix, centrifuge and then place on ice.

3. Aliquot the assay mix into PCR tubes or plate. For best results, ensure accurate and consistent pipetting volumes.

4. Add DNA template to PCR tubes or plate, and avoid bubble formation so as to prevent cross-contamination due to the bubble breaking.

5. Seal tubes with flat, optically transparent caps or seal plates with optically transparent film.

6. Mix and then centrifuge the tubes or plate at 2,500rpm-3,000rpm.

7. Program PCR instrument with indicated thermo-cycling protocol (Tables 3 and 4).

Thermal Cycling Setup

Table 3. Standard thermo-cycling protocol [a]

Stage	Temperature	Period	Number of cycles
I	95°C	2min	1
II	95°C	10sec	35-40
	60°C, signal acquisition	60sec (+ plate read)	

[a] Each primer concentration used for the standard thermo-cycling protocol is around 0.2uM with well-designed primers.

Table 4. Fast thermo-cycling protocol [a]

Stage	Temperature	Period	Number of cycles
I	95°C	1min	1
II	95°C	5sec	35-40
	60°C, signal acquisition	30sec (+ plate read)	

[a] The product size for the fast thermo-cycling protocol is preferred to be less than 90bp. Each primer concentration used is typically between 0.4uM and 0.9uM.

Data Analysis and Expected Results

Analyze data according to the real-time PCR instrument manufacturer instruction. In addition, please refer to MIQE guidelines [Bustin, S.A., et al. (2009) The MIQE Guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem., 55(4): 611-622.] Briefly, this includes the following steps:

1. Determine the amplification efficiency of the standard curve by plotting log of the template concentration against the Ct. The linear equation of the standard curve should have a slope of -3.6 to -3.1, corresponding to an amplification efficiency of 90-110%.

2. Verify that the standard curve has a correlation coefficient (R^2) of ≥ 0.98.

3. Determine the reaction specificity by the difference between the template containing and non-template control reactions. A difference in Ct of 3 or greater should be observed.

4. Evaluate any unknown DNA samples with respect to the standard curve, taking any dilution factors into account.

Usage Notes

Primer and Probe Design

Design software such as primer3 is recommended for high efficiency and less non-specific amplification as well as less primer dimers in multiplex qPCR amplification.

T_m of a primer or probe is important for its efficiency which is correlated to its GC content and length. Because of 60-62°C in RT step, each primer's T_m should be designed ≥60°C, preferably between 62-65°C. The probe's T_m should be 8-10°C higher than the primers' T_m, preferably between 70-75°C.

A target with balanced GC content of 40–60% tends to be amplified most efficiently. When possible, enter sufficient flanking sequences and cross-reference sequences to avoid potential non-specific amplification. For cDNA targets, it is advisable to design the primer across known splicing sites in order to prevent amplification from genomic DNA.

Primer and Probe Concentration

The optimal concentration of a primer is variable from 150-900nM, depending on assay design and thermo-cycling protocol. With a well-designed primer and standard thermo-cycling protocol, the optimal concentration is around 200nM. However, with fast thermo-cycling protocol, the optimal concentration can be up to 900nM.

The optimal concentration of a probe can be various in the range from 100-500nM, commonly ranges from 150-250nM.

Multiplexing

When determining which fluorophores to include in a multiplex reaction, choose compatible reporter dyes and quenchers. For example, for Thermo Fisher/Applied Biosystems instruments, avoid ROX-labeled probes.

Each set of primers and probe should be tested individually for its efficiency and specificity before multiplexing.

In addition, for targets that differ significantly in abundance, use a lower primer concentration, such as 200nM, for the more abundant targets to prevent the more abundant targets from using up substrates such as dNTPs and DNA polymerase at too early stage of amplification.

Amplicon Length

The preferable PCR product size is ≤150bp, but it can exceed 200bp. Optimization is required for targets that exceed that range.

The product size for the fast thermo-cycling protocol is preferred to be less than 90bp.

DNA Template Preparation and Concentration

Note that the quality of DNA templates, particularly if a large amount, can greatly affect qPCR efficiency. The kit is compatible with DNA samples prepared by common nucleic acid extracted methods, such as silica-based.

Prepared DNA samples should be stored in TE buffer (10mM Tris-HCl, 0.1mM EDTA, pH 8.0-8.3) for long-term stability. DNA samples should be handled with appropriate precautions to prevent DNase contamination. Use of nuclease-free water is strongly recommended.

Generally, useful concentrations of DNA samples will be in the range of 10^6 copies to 1 copy. 1µg–10ng of human genomic DNA and cDNA produced from up to 1µg–0.1pg starting RNA can be used can be used in a reaction.

Note when a positive or unknown DNA sample is diluted into the range of single-digital copies of DNA template per reaction, some reaction will actually contain more copies and some will have fewer or even zero copies, as defined by Poisson distribution.

ROX Passive Reference Dye

Thermo Fisher/Applied Biosystems real-time PCR instruments use a passive reference dye (typically ROX) for normalization of well-to-well variations. Most other PCR instruments do not require such passive reference dye.

This kit is formulated to contain No ROX, Low ROX or High ROX concentrations in the master mix that is compatible with a variety of PCR instruments, including those that use no passive reference and those that use a low or high concentration of passive reference dye (ROX) (Table 1).

In addition, if you order a "No ROX" master mix but have a Thermo Fisher/Applied Biosystems instrument, please turn off ROX passive reference dye button when setup assays.

Carryover Contamination Prevention

In carryover contamination, a new qPCR assay contains products from previous amplification, causing a false positive result.

The best way to prevent this "carryover" contamination is to practice good laboratory procedures and never open reaction vessels post PCR amplification.

Reaction Setup and Cycling Conditions

For 96-well plates, a final reaction volume of 20µl is recommended.

For 384-well plates, a final reaction volume of 10µl is recommended.

When with SYBR Green dye, a melt curve analysis after cycling is added for product specificity.

Amplification for 40 cycles is sufficient for most applications, but for very low copies of DNA template 45 cycles may be used.

Troubleshooting Guide

Problem	Possible cause(s)	Solution(s)
	Cycling protocol is otherwise incorrectly setup	Refer to the proper thermo-cycling protocol in this user manual
	Reagent omitted from the PCR assay Reagent added improperly to the PCR assay	Verify all steps of the thermo-cycling protocol were followed correctly
	Incorrect channel selected on the PCR instrument	Verify correct instrument optical settings
	Incorrect passive reference ROX dye setting	Verify correct compatibility of your PCR instrument with passive reference ROX dye
	Reagents are contaminated or degraded	• Confirm correct amount of DNA template added • Confirm the expiration dates of the kit • Verify proper storage conditions of the kit • Rerun the PCR assay with fresh reagents
Inconsistent amplifications for triplicate	Improper pipetting	Ensure proper pipetting techniques
	PCR plate film has lost its seal, causing evaporation in the well, causing significant fluorescent difference relative to its replicates	• Ensure the PCR plate is properly sealed • Exclude problematic data from analysis
	Poor mixing of reagents during set-up	Make sure all reagents are properly mixed in every step
	Bubbles cause an abnormal amplification	• Avoid bubbles in the PCR plate • Centrifuge the PCR plate • Exclude problematic data from analysis
Standard curve has a poor correlation coefficient/amplification efficiency	Cycling protocol is incorrect	• Refer to the proper PCR cycling protocol in this user manual • Use a 1 minute 60°C annealing/extension step for more consistency
	Presence of outlier PCR amplification	Omit data that are clearly outliers caused by bubbles, plate sealing issues, or other experimental problems
	Improper pipetting	Ensure that proper pipetting techniques are used
	Reaction conditions are incorrect	Verify that all steps of the protocol were followed
	Bubbles cause an abnormal amplification	• Avoid bubbles in the PCR plate • Centrifuge the PCR plate
	Poor mixing of reagents	After thawing, make sure all reagents are properly mixed
	Threshold is improperly set	• Ensure the threshold is set in the early exponential phase of PCR amplification • Refer to the PCR instrument user manual to manually set an appropriate threshold
No template control shows amplification/NTC Ct is close to or overlapping lower copy standards	Reagents are contaminated with carryover products of previous PCR	• Replace all stocks and reagents • Clean equipment and setup area with a 10% chlorine bleach
	Primers produce non-specific amplification or primer dimers	Redesign primers with a T_m of ≥60°C or use PCR primer design software

Ordering Information

qPCR master mix

- 2X qPCR Master Mix-TaqMan Probe, Cat qPCR 153
- 2X Multiplex qPCR Master Mix-TaqMan Probe**,** Cat qPCR 156
- 2X Fast qPCR Master Mix-SYBR Green, Cat qPCR 157
- 2X Super Multiplex qPCR Master Mix-TaqMan Probe**,** Cat qPCR 159

RT-PCR master mix

- 1-Step 2X RT-PCR Master Mix-TaqMan Probe, Cat RT-PCR 143

- 1-Step 2X Multiplex RT-PCR Master Mix-TaqMan Probe, Cat RT-PCR 146
- 1-Step 2X RT-PCR Master Mix-SYBR Green, Cat RT-PCR 144
- 1-Step 2X Fast RT-PCR Master Mix-SYBR Green, Cat RT-PCR 147
- 1-Step 2X Super Multiplex RT-PCR Master Mix-TaqMan Probe, Cat RT-PCR 149

RT-PCR enzyme

- Thermophilic Reverse Transcriptase, Cat RT-PCR 140
- *Taq*-Probe Polymerase, Cat RT-PCR 145
- *Taq*-Fast Polymerase, Cat RT-PCR 148

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