HotStart™ 2X Probe qPCR Master Mix

Product description

Quantitative PCR (qPCR, also known as Real-time PCR) is a very versatile technique for accurately analyzing gene expression. According to different principles, it can be divided into two categories: dye-based method and probe-based method. Commonly used probes include Taqman probes, molecular beacons, dual hybridization probes, MGB probes, etc. Among them, the TaqMan probe is the most classic and widely used. Taqman probes can be hybridized specifically to the target sequence, with a 5' end labeled fluorescent reporter and a 3' end labeled quencher. When the probe is intact, the fluorescent reporter on the probe is quenched by the quencher due to fluorescence resonance energy transfer (FRET). When the primer-mediated extension reaches the probe position, Taq enzyme with 5'→ 3' exonuclease activity hydrolyzes the probe bound to the target sequence, lead to the separation of the fluorescent reporter at the 5' end and the quencher at the 3' end, apart more than 10 nm, beyond the distance of FRET, then the fluorescent reporter emits fluorescence with appropriate excitation light. With each PCR cycle, more fluorophores are released, and the fluorescence intensity is proportional to the number of newly synthesized fragments, enabling quantitative detection. Commonly used fluorescent reporters include FAM, HEX, TET, JOE, NED, Cy3, TAMRA, Cy5, Cal Fluor Gold540, Cal Fluor Orange560, etc., quenchers BHQ1, BHQ2, BHQ3, TAMRA, Dabcyl, NFQ-MGB, etc. When designing probes, pay attention to different models of the qPCR machine (different qPCR machines have different fluorescent channels), various abundance of target genes, and the types of experiments.

The HotStart™ 2X Probe qPCR Master Mix is a high-quality master mix used in probe-based real-time PCR. It's a 2X PreMix using a mutant hot-start Taq DNA polymerase with improved template affinity, resistance to PCR inhibitory components such as blood, serum IgG, humic acid, etc. This product is particularly suitable for quantitative or qualitative detection of low-abundance or high-specificity genes of interest.

This product is a 2X master mix that requires only additional primers, probes, and templates, which is convenient to use and compatible with fast procedures, that means shortened time to generate the result, and enables accurate, highly repeatable quantification of target gene over a wide dynamic range. This product can be used for multiplex qPCR and supports up to quadruplex qPCR.

Components and storage conditions

Components	5 mL 1000 rxn with 10 μL reaction 500 rxn with 20 μL reaction 200 rxn with 50 μL reaction	25 mL 5000 rxn with 10 μL reaction 2500 rxn with 20 μL reaction 1000 rxn with 50 μL reaction	50 mL 10000 rxn with 10 μL reaction 5000 rxn with 20 μL reaction 2000 rxn with 50 μL reaction
HotStart™ 2X Probe qPCR Master Mix	1 mL X 5	1 mL X 25	5 mL X 10
50X ROX Reference Dye (low concentration)	0.2 mL	1 mL	1 mL X 2
50X ROX Reference Dye (high concentration)	0.2 mL	1 mL	1 mL X2
Store all components at -20°C and protect from light for 12-24 months. Avoid repeated freeze/thaw cycles as possible.			

Experimental manipulation

1. ROX dye selection

ROX dye	qPCR instruments
No ROX dye required	Bio-Rad: CFX96™, CFX384™, iCycler iQ™, iQ™5, MyiQ™, Opticon®, Opticon 2, Chromo4™, MiniOpticon™ Cepheid: SmartCycler® Eppendorf: Mastercycler® eprealplex, realplex 2s llumina: Eco™ qPCR Qiagen: Corbett Rotor-Gene@ Q, Rotor-Gene@ 3000, Rotor-Gene® 6000 Roche: LightCycler@ 480, 96, Nano, 1.5/2.0** Thermo Scientific: PikoReal Cycler
Use 50X ROX Reference Dye (low concentration)	Applied Biosystems: 7500, 7500 Fast, ViA™7, QuantStudio 6 and 7 Flex System, QuantStudio 3 and 5. Agilent Stratagene: MX4000™, MX3005P™, MX3000P™
Use 50X ROX Reference Dye (high concentration)	Applied Biosystems: 5700, 7000, 7300, 7700, 7900, 7900HT, 7900HT Fast; StepOne™, StepOne Plus™

2. Establishment of a qPCR reaction system (ABI 7500 as an example).

Template DNA can be obtained by reverse transcription from RNA, DNA extraction and purification or

other approaches. For best results, we recommend at least three replicates per sample.

Thaw the HotStart™ 2X Probe qPCR Master Mix, template on ice, ROX dye and primers at room temperature and then place on ice. After complete thawing, invert the tube or pipet for homogeneity, then centrifuge to prevent air bubbles. If using a multichannel pipette, take care to ensure consistency of pipetting volume across all channels.

The singleplex qPCR reaction system is shown in the following table (ABI 7500 as an example):

Components	20 μL Reaction	Final Concentration
HotStart™ 2X Probe qPCR Master Mix	10 μL	1X
Forward Primer (10 μM)	0.6 μL	0.3 μM
Reverse Primer (10 μM)	0.6 μL	0.3 μM
TaqMan Probe (10 μM)	0.4 μL	0.2 μM
Template DNA	Variable	< 100 ng
50X ROX Reference Dye (low concentration)	0.4 μL	1X
Nuclease-free Water	Add to 20 μL	

***Note:**

- Generally, a final concentration of primers of 0.3 μM in the reaction system may generate a good amplification effect. If the yield is poor, the primer concentration can be adjusted in the final concentration range of 0.1 - 1 μM.
- The recommended probe final concentration range is between 0.05 – 0.5 μM.
- Probe-based qPCR reaction is extremely sensitive, and the accuracy of the adding amount of template when set up the reaction will greatly affect the accuracy of results.
- When using the reverse transcription product directly as a template, its volume should not exceed 10% of the final reaction mixture.

Multiplex qPCR reaction system (ABI 7500 as an example)

Components	20 μL Reaction	Final Concentration
HotStart™ 2X Probe qPCR Master Mix	10 μL	1X
Forward Primer (10 μM)	X	0.1 - 1 μM
Reverse Primer (10 μM)	X	0.1 - 1 μM
TaqMan Probe (10 μM)	X	0.05 - 0.5 μM
Template DNA	Variable	< 100 ng
50X ROX Reference Dye (low concentration)	0.4 μL	1X
Nuclease-free Water	Add to 20 μL	

***Note:**

- Up to quadruplex qPCR can be performed.
- When performing multiplex qPCR, take care to adjust the primer concentrations. Especially for highly abundant gene of interest, reducing the primer concentration to an appropriate concentration is recommended.
- When designing multiplex qPCR probes, attention should first be paid to determining fluorophores according to different models of qPCR instruments and reducing the interference of fluorescence emission spectra between different probes.
- The primer concentration can be adjusted in a range of 0.1 - 1 μM.
- The recommended probe final concentration range is between 0.05 - 0.5 μM.
- Probe-based qPCR reaction is extremely sensitive, and the accuracy of the adding amount of template when set up the reaction will greatly affect the accuracy of results.
- When using the reverse transcription product directly as a template, its volume should not exceed 10% of the final reaction mixture.

After thorough mixing, centrifuge all reactants to the bottom of the wells with a centrifuge (a few minutes, at 2500-3000 rpm) to eliminate air bubbles (which can interfere with signal acquisition).

3. Set the qPCR reaction.

When setting up the qPCR instrument program, choose the detection channel of the qPCR instrument that corresponds with the fluorophore label and quencher of the target-specific probe present in the array, and ensure that the fluorescence signal is acquired at the end of the extension step.

Standard qPCR procedure:

Stage	Cycles	Procedure	Temperature	Time
Stage1: Hot-Start Taq Polymerase Activation	Hold (1 cycle)	Initial Denaturation	95°C	1 min
Stage2: PCR	CYCLE (40-45 cycles)	Denaturation	95°C	15 sec
		Annealing/Extension	60°C	30-34 sec

***Note:**

1. The extension time should be adjusted according to the data acquisition time of the qPCR machine used.

   For example, when using ABI 7500 Fast / 7700 / 7900HT Fast/ 7900HT / ViiA 7 / StepOne / StepOnePlus / QuantStudio™ 3, 5, 6 Flex, 7 Flex, 12k Flex, Biorad CFX96, Roche LightCycler / LightCycler 480, set the extension time to 30 s; When using ABI 7000 and 7300, set the extension time to 31 s; When using the ABI 7500, set the extension time to 34 s.

Fast qPCR procedure:

Some qPCR instruments generate fast mode (such as ABI 7900HT Fast, QuantStudio series, StepOne, StepOnePlus, ViiA™ 7, 7500 Fast, etc.), choose the fast mode if you need to generate the result with a shortened time.

Stage	Cycles	Procedure	Temperature	Time
Stage1: Hot-Start Taq Polymerase Activation	Hold (1 cycle)	Initial Denaturation	95°C	20 sec
Stage2: PCR	CYCLE (40-45 cycles)	Denaturation	95°C	1-3 sec
		Annealing/Extension	60°C	20-30 sec

***Note:**

a. Please set the program according to the fast mode of the qPCR machine. Minimal extension time is not less than 20 sec.

Notes

1. Primer design

Primer design for probe-based qPCR can follow these design guidelines:

1.1 Primer length: 17-25 bp is recommended. Primers that are too short may lead to reduced amplification efficiency; Primers that are too long can increase the chance of the formation of primer secondary structure. Both will affect the accuracy of qPCR.

1.2 GC content: 40-60% (45-55% is ideal).

1.3 Tm: There should be no significant difference in Tm values between forward and reverse primers, preferably no more than 2°C. The optimal Tm value is around 60°C.

1.4 A suitable amplicon length is 70-200 bp.

1.5 Primer sequences: A, T, C, G even distribution is preferred, avoid areas with particularly high GC or AT content, especially at the 3' end, and avoid areas with uneven GC content.

1.6 3' end sequence: Avoid more than 2 G or C nucleotides in the last 5 nucleotides at the 3' end to reduce the risk of non-specific amplification.

2. TaqMan probe design guide

2.1 Probe sequence: design it close to the forward or reverse primer, but not have overlapping regions with primers.

2.2 Probe length: 20 - 40 bp.

2.3 GC content: 30-60%.

2.4 Probe sequence characteristics: the appearance of consecutive same bases should be avoided, especially the appearance of GGGG or more consecutive G. If the sequence contains polymorphic sites, it should be placed in the middle of the probe sequence.

2.5 Probe 5' end: Exclude G at the 5' end of the probe. Because the 5' end is connected to the fluorophore, the G base quenches the fluorescence signal of the fluorophore, and the quenching effect persists even only 1 G base remains attached to the reporter group after hydrolysis.

2.6 Probe annealing temperature: In the range of 65 ~ 70 °C. It is recommended to be 5-10 °C higher than the Tm value of the primer.

3. Template preparation and concentration

3.1 For long-term storage, to ensure stability, template DNA should be stored in EDTA-containing buffer (e.g., 1X TE), and the diluted solution for qPCR experiments should be freshly prepared and diluted using TE or water.

3.2 CDNA can be derived from RNA reverse transcription product ranging from 1 μg to 0.1 pg. Though cDNA can be added to the qPCR reaction without purification, a dilution at 1:10 ratio is recommended because too high concentration of reverse transcriptase will inhibit taq activity. For extremely low expression genes, template can be added without dilution, be careful not to exceed a tenth of the total reaction volume.

4. Reaction conditions and cyclic conditions

4.1 For 96-well plates, we recommend using a 20 μL reaction volume. A reaction volume of 10 μL is recommended for 384-well plates.

4.2 Amplifying 40 cycles is sufficient for most experiments, but 45 cycles can be used for target genes with very low copy in the sample.

Common problems and solutions

1. Significant amplification occurred in negative controls.

Possible causes	solution
The reagent used or Nuclease-free Water is contaminated	Using new reagents, Nuclease-free water, and primers, experiments is recommended to performed on a clean bench. Avoid opening the qPCR plate after amplification (aerosol contamination of previously amplified reaction products will cause a variety of problems in subsequent qPCR assays).

2. Ct value is abnormal.

Possible causes	solution

Low amplification efficiency	Make sure the primers and template are not degraded. Optimize the reaction system, such as adjusting primer concentration, probe concentration, annealing temperature, and time. Also, try redesigning the primers. For templates with high GC content, the initial denaturation time can be appropriately extended.
The template concentration is low	Increase the template concentration. If a diluted template is used, reduce the dilution ratio, or use gradient dilution to determine the optimal amount of template added.
Template degradation	Use the new template
The target sequence is too long	In general, the target fragment length is between 70-200 bp

3. The amplification curve is unusually shaped.

Possible causes	solution
The amplification curve is not smooth	When the signal is too weak, system calibration activates and causes this condition. In this case, increase the template concentration
Shape of the amplification curve is fractured or descending	When the template concentration is too high, the baseline endpoint value is higher than the CT value, reduce the baseline endpoint value (Ct value minus 4) and reanalyze the data
Individual amplification curves drop suddenly	There are bubbles in the reaction tube, and when the temperature rises, the bubbles will suddenly burst, causing the curve to drop suddenly. It is recommended to centrifuge before the reaction and check that no air bubbles are present in the reaction system

4. No amplification curve appears at the end of the reaction.

Possible causes	solution
The number of cycles set is not enough	Usually, the number of cycles is set to 40, but it should be noted that too many cycles increase the background and reduce the reliability of the data
Signal acquisition is not set up correctly	In the two-step method, signal detection should be set in the annealing and extension phases
Primer degradation	Confirm primer integrity using PAGE electrophoresis and use new

	primers if degradation occurs
The template concentration is too low	If the template is diluted, reduce the dilution ratio (for target sequences with unknown expression levels, it is recommended to use the template without dilution for the first time); If the template is not diluted, remake the template, or concentrate the sample.
Template degradation	Prepare a new template

5. Poor data repeatability

Possible causes	solution
Sampling error	It is important to use a more accurate pipettor
The template concentration is too low	It is possible to reduce the dilution factor of the template and increase the amount of template added
The sample purity is low	Re-extract or purify the sample
Differences between primers synthesized in different batches	Use the same primers as possible.
Instrument failure	If the temperature or detection of each hole varies, calibrate or repair the instrument.

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