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Protocol

Cat. No. K1171

HotStart™ 2X FAST Green 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 methods, it can be divided into two categories: dye-based qPCR and probe-based qPCR, of which the dye-based method is more popular, convenient, and less costly. Dye-based qPCR enables indirect measurement of DNA amplification at each cycle of PCR by monitoring the fluorescence emitted by the dye bound to double-stranded DNA in real time. When the detected fluorescence signal significantly exceeds the background at a certain time point, the Ct value (Cq value) can be determined. The obtained Ct values can be used to assess the relative abundance of the gene of interest, or to obtain absolute numbers based on appropriate standard curve calculations.

Our product, HotStart™ 2X FAST Green qPCR Master Mix, offers short extension times, superior specificity, robust amplification efficiency, ideal reproducibility and stability for quantifying target DNA or cDNA. It's a 2X PreMix using a mutant hot-start fast Taq DNA polymerase that is more tolerant to Green I inhibition, as well as EDTA-treated blood and heparin-treated blood. The ideal Taq polymerase and suitable buffer guarantee superior specificity and high amplification speed. Green I in Mix interacts within the minor groove of double-stranded DNA, and emits green fluorescence, thus the amplification product can be indirectly quantified in real time by monitoring the fluorescence with an instrument.

Designed and developed for fast real-time PCR, this new product improves durability, specificity, and fast elongation speed, as well as improvements in signal-to-noise ratio (fluorescence), cyclic domain value (Cq), linearity, and sensitivity.

Dye-based qPCR has certain limitations, i.e., Green I can insert any double-stranded DNA, such as primer dimers or other non-specific products, resulting in fluorescence of non-specific products, to confirm product specificity, after amplification, melt curve analysis is necessary. In the analysis of the melting curve, a spike near the primer annealing temperature is an ideal result.

Components and Storage

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 FAST Green 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 X 2
Store the components away from light at -20°C for 12-24 months.			

Experimental manipulation

1. ROX dye selection

ROX dye selection	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. Establish a qPCR reaction system.

Template DNA can be obtained by reverse transcription from RNA, DNA extraction and purification or other approaches. *Note: For best results, we recommend at least three replicates per sample.*

1) Thaw the HotStart™ 2X FAST Green qPCR Master Mix and template on ice, the ROX dye and primers can be thawed at room temperature and then placed on ice. After complete thawing, invert the tube or pipet for homogeneity, then centrifuge to prevent air bubbles.

2) Determine the total volume according to the number of reactions and the volume of each reaction, plus 10% overage, and prepare a mixture of all components except the corresponding template. Always

include a no template control (NTC).

3) Aliquot the mixture into qPCR tubes or plates. Ensure accurate and consistent dosing volumes and minimize air bubbles. Then, add the template.

4) Seal the qPCR tubes using an optically transparent caps and seal the qPCR plates with an optically adhesive film. Pay attention to seal the corners of the qPCR plate to prevent evaporation.

5) After mixing, centrifuge to collect all contents to the bottom of the wells and eliminate air bubbles (which will interfere with signal acquisition).

***Note:**

No template control (NTC) is used to identify contamination. The control well contains all reaction components except the template (HotStart™ 2X FAST Green qPCR Master Mix, primers, Rox, nuclease-free water) and this well should not return a significant Ct value.

Use diluted cDNA or DNA as a standard curve, dilution should be prepared before experiment.

The qPCR reaction system is shown in the following table: (If using a different reaction volume, scale all components proportionally.) Reaction systems smaller than 10 µL are not recommended.

Components	20 µL Reaction	50 µL Reaction	Final Concentration
HotStart™ 2X FAST Green qPCR Master Mix	10 µL	25 µL	1X
Forward Primer (10 µM)	0.4 µL	1 µL	0.2 µM
Reverse Primer (10 µM)	0.4 µL	1 µL	0.2 µM
Template DNA	Variable	Variable	1-100 ng
ROX Reference Dye	0.4 µL	1 µL	1X
Nuclease-free Water	Add to 20 µL	Add to 50 µL	

***Note:**

a. In most reactions, a final primer concentration of 0.2 µM can be used. When the reaction performance is poor, the optimal primer concentration can be adjusted between 0.2-1 µM.

b. When using the reverse transcription product directly as a template, its volume should not exceed 10% of the final reaction mixture. The quantity of templates added to the reaction depends on the number of target genes copies. In general, 1-10 ng single-stranded cDNA or 10-100 ng gDNA is used per reaction. The optimal amount of template can be determined by gradient dilution. The cDNA template should generally contain <10^9 copies of the target to ensure that the quantitative reaction remains linear. For low-abundance targets, the template should be appropriately increased. Due to the high sensitivity of qPCR, the accuracy of the template volume will have a great impact on the result.

It is recommended to dilute the template for improving the reproducibility of the experiment. For example, when used as a qPCR template, a 10-fold dilution of a reverse transcription product (10 μl reverse transcription system) of 1 μg RNA is recommended.

c. Since the mixture contains Green I dye, direct light should be avoided when preparing the reaction mixture.

3. Start the qPCR reaction.

Set up the qPCR using SYBR or SYBR®/FAM mode to ensure that the fluorescence signal is acquired at the end of the extension step. A two-step qPCR procedure is preferred. If poor results occur, such as nonspecific amplification or low amplification efficiency, the reaction components and conditions can be adjusted.

Two-step qPCR procedure:

Stage	Cycles	Procedure	Temperature	Time
Stage1: Hot-Start Taq Polymerase Activation	Hold (1 cycle)	Initial Denaturation	95°C	2 min
Stage2: PCR	CYCLE (40-45 cycles)	Denaturation	95°C	3 sec
		Annealing/Extension	60°C	30-34 sec
Stage3: Melt Curve	CYCLE (1 cycle)		95°C	15 sec
			60°C	60 sec
			95°C	15 sec

***Note:**

a. In general, predenaturation is performed at 95℃ for 2 min to activate DNA polymerase. For target sequences with higher GC content, the time for predenaturation can be appropriately extended.°

b. The extension time should be adjusted according to the minimum data acquisition time depending on the thermal cycler used.

For example, when using ABI 7500 Fast / 7700 / 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 seconds; When using ABI 7000 and 7300, set the extension time to 31 seconds; When using the ABI 7500, set the extension time to 34 seconds.

Some qPCR machines can use shorter extension times, ABI 7900HT Fast can set to 20 sec, and the minimum setting time should not be lower than 20 sec.

c. Different instrument types require different melt curve procedures, and the melt curve program is determined according to the instrument used.

Notes

1. Primer design

The primers designed for qPCR experiments require superior amplification and few non-specific products, the following design guidelines can be followed:

1) **Target sequence length**: 80-200 bp is appropriate. It can be extended up to 300 bp on practical requirements.

2) **Primer length**: 17-30 bp.

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

4) **Tm**: There must be no significant difference in the Tm values of forward and reverse primers, and Tm values can be calculated using software.

5) **Primer sequences: A, T, C,** G even distribution is preferred, avoiding areas with particularly high GC or AT content (especially at the 3' end), and avoiding polypyrimidine (T/C continuous structure) and polypurine (A/G continuous structure).

6) **3' end sequence**: The GC and AT content of the 3' end of the primer should not be too high. We recommend choosing sequences with G or C at the 3' end (avoid T at the 3' end). Complementary sequences of more than 3 bases cannot be present in primers or between primer pairs (resulting in hairpin structures or primer dimers, respectively). Primer pairs should not have more than two complementary sequences on each 3' end to avoid primer dimers.

7) Specificity: Confirm the specificity of the primers by software. When designing primers, enter enough sequence around the region of interest. Use search criteria that allow cross-referencing of relevant sequence databases (to avoid potential amplification off-targeting). For cDNA targets, primers with known splicing sites can optionally be designed to prevent genomic DNA amplification. In contrast, primers designed for the intron region ensure amplification from genomic DNA only.

2. Template preparation and concentration

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.

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.

3. Reaction conditions and cyclic conditions

1) For 96-well plates, we recommend using 20 µl of reaction volume. A 384-well plate recommends a reaction volume of 10 µl.
2) When setting up the cycling program, ensure that a signal detection routine is included at the end of the extension step and a melt curve analysis is performed at the end to determine product specificity.
3) Amplifying 40 cycles is sufficient for most experiments, but 45 cycles can be used for target genes with very low copy loads 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 were performed on an ultra-clean bench. Avoid opening qPCR plates after amplification (aerosol contamination of previously amplified reaction products can cause various problems in new qPCR assays).
Primer dimers	According to the results of the melt curve analysis, it is normal for the negative control to have a slight amplification after 35 cycles.

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, annealing temperature, and time. Besides, try to redesign the primers. For templates with high GC content, the initial denaturation time can be appropriately extended.
The stencil 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 a new template.
The target sequence is too long	In general, the target fragment length is between 80-200 bp.
PCR inhibitors are present in the reaction system	Try diluting or repreparing the template (the template can be purified) as inhibitors are usually in the template.

$Mg2^{+}$ concentration is not enough	For some qPCR reactions, it may be necessary to increase the final concentration of Mg^{2+}. When optimizing the Mg^{2+} final concentration, it is recommended to increase the concentration by 0.5 mM each time.

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. Centrifuge 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 loops is set to 40, but it should be noted that too many loops 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 rate (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. Multiple peaks appear in the melting curve.

Possible causes	solution

Inappropriate primer design	Primer dimers or undesired amplification product will lead to heterozygous peak in the melt curve. Redesign the primers. The peaks of primer dimers often occur at about 75℃.
The primer concentration is too high	Reduce primer concentration appropriately.
cDNA contains contamination of genomic DNA	Reprepare the cDNA template (Use DNase when extracting RNA).
Low annealing temperature	Increase the annealing temperature.
The reaction volume is too small	It is not recommended that the reaction system is less than 10 µl, if the reaction volume is too small, the detection accuracy will be reduced, and it is recommended to increase the reaction volume.

6. Poor data repeatability

Possible causes	solution
Sampling error	It is important to use a more accurate pipettor; A larger reaction system can be used, or the dilution ratio and reaction volume of the template can be increased at the same time.
The template concentration is too low	Reduce template dilution or increase the volume.
The sample purity is low	Re-extract or purify the sample.
Differences between primers of 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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