HotStart™ Universal 2X Green qPCR Master Mix

Product description

Quantitative PCR (qPCR, also called Real-time PCR) is a popular technology for precise analysis of gene expression. It can be classified into two categories according to different methods, dye-based and probe-based, in which, dye-based method is more popular, convenient, and less costly. Dye-based qPCR monitors real-time fluorescence of the dye binding to the double-stranded DNA to measure DNA amplification indirectly during each cycle. At a point where the fluorescence signal is distinctly detected over the background, Ct value (Cq value) can be determined. The obtained Ct values can be used to evaluate relative target abundance or calculate absolute target quantities in reference to an appropriate standard curve.

Our product HotStart™ Universal 2X Green qPCR Master Mix provides superior specificity, robust amplification efficiency, ideal reproducibility and stability in quantifying target DNA or cDNA. It's a 2X premix, taking advantage of a hot-start Taq polymerase combined with the antibody. Ideal Taq polymerase and suitable buffer will guarantee preferable specificity and high amplification speed. Green I in the mix which is a DNA intercalator will emit fluorescence when bounds to the double-stranded DNA amplified in each cycle and monitoring the fluorescence by the instrument allows for the indirect quantification of amplification products at real time. This reagent has special Specific ROX Reference Dye which is suitable for all qPCR instruments. Instead of adjusting the concentration of the ROX on different instruments, you can simply add primers and templates to amplify when formulating the reaction system.

However, dye-based qPCR has some limitations. Green I can intercalate into any double-stranded DNA, such as primer dimer or other undesired products, causing nonspecific products to emit fluorescence. To confirm the specificity of the product, following amplification, performing a melt curve analysis is necessary. A sharp peak around the annealing temperature in the melt curve analysis is ideal.

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™ Universal 2X Green qPCR Master Mix	1 mL X 5	1 mL X 25	5 mL X 10
Store all components at -20°C and protect from light. Avoid repeated freeze/thaw cycles as possible.			

Experimental manipulation

1. Set up the qPCR Reaction System.

Prepare cDNA by reverse transcription or genomic DNA by DNA extraction and purification. For best results, we recommend running each sample in triplicate at least.

1) Thaw 2X Green qPCR Master Mix, ROX Reference Dye, template, primers at room temperature, then place them on ice. After thawing completely, briefly mix each component by inversion or pipetting (spin down if necessary to avoid bubbles).

2) Determine the total volume depending on the number of reactions, plus 10% overage and prepare mix of all components except the corresponding template.

3) Aliquot the mix into qPCR tubes or plate. Ensure accurate and consistent pipetting volumes and minimize bubbles. Then, add templates to qPCR tubes or plate.

4) Seal tubes with optically transparent caps, seal plates with optical adhesive cover. Take care to properly seal plate edges and corners to prevent evaporation.

5) Mix thoroughly and centrifuge (several minutes at 2,500–3,000 rpm) briefly to spin down all contents to the bottom of the tube or plate, eliminate any air bubbles (which will disturb signal acquisition).

***Note:**

a. No template control (NTC) should be used to identify PCR contamination. The control contains all reaction components (HotStart™ 2X Green qPCR Master Mix, primers, Nuclease-free Water) except sample, and therefore should not return a Ct value.

b. Make dilutions of cDNA or DNA to be used for the standard curve. These should be prepared fresh before each experiment.

The qPCR reaction system is shown in the table below, if taking different reaction volumes, scale all components proportionally. Reaction volume less than 10 µl is not recommended.

Components	20 µL Reaction	50 µL Reaction	Final Concentration
HotStart™ Universal 2X Green qPCR Master Mix	10 µL	25 µL	1X
Forward Primer (10 µM)	0.5 µL	1.25 µL	0.25 µM
Reverse Primer (10 µM)	0.5 µL	1.25 µL	0.25 µM
Template DNA	Variable	Variable	1-100 ng
Nuclease-free Water	Add to 20 µL	Add to 50 µL	

***Note:**

a. In most reactions, 0.25 μM final concentration of primers will lead to an approving outcome. When the reaction performance is poor, try to find the optimal primer concentration between 0.2-1 μM.

b. When the reverse transcription product is directly used as a template, it should not exceed 10% volume of the final mixture. The quantity of template added into the system varies depending on the number of target genes copies. Generally, use 1–10 ng single-stranded cDNA or 10-100 ng gDNA per reaction. Gradient dilution is preferred to determine the optimum template addition. The cDNA template should typically contain<109 copies of the target to ensure that quantitation remains linear. For low abundance targets, the template should be increased properly. Because of the high sensitivity of qPCR, the accuracy of the template quantity will greatly affect the final results. It is recommended to dilute the template to improve the reproducibility of the experiment. For example, to serve as a template, the reverse transcription product of 1 μg RNA (10 μl reverse system) is recommended to be diluted 10 times.

c. Because of the contained Green I dye in the mix, avoid strong light when preparing the reaction mix.

2. Start the qPCR reaction.

Use the SYBR® or SYBR/FAM scan mode setting on the qPCR instrument. Ensure a fluorescence signal acquisition is included at the end of the extension step. It is recommended to use the two-step PCR procedure. If poor performance appeared, such as overmuch non-specific amplification, or poor amplification efficiency, reproducibility, you can adjust the reaction mix, program or proceed to the three-step procedure.

1) Two-step 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 cycles)	Denaturation	95°C	15 sec
		Annealing/Extension	60°C	30-60 sec
Stage3: Melt Curve	CYCLE (1 cycle)		95°C	15 sec
			60°C	60 sec
			95°C	15 sec

***Note:**

a. Generally, heat for 2 min at 95°C to activate the heat-activated DNA polymerase in the initial denaturation step. For target sequence rich in GC content, you can increase the time for initial denaturation.

b. The extension time should be adjusted according to the minimum time limit for data collection required by the Real-time PCR instrument used.

When using ABI 7500 Fast / 7700 / 7900HT / 7900HT Fast / ViiA 7 / StepOne / StepOnePlus, set the extension time to 30 sec. When using ABI 7000 and 7300, set the extension time to 31 sec.

When using the ABI 7500, set the extension time to 34 sec.

Some qPCR instrument need less time, such as 10 sec for ABI StepOnePlus™, or 20 sec for Roche LightCycler / LightCycler 480, you can adjust the extension time according to your target sequence length and the instrument requirement.

c. Different melt curve procedures are need for different instrument types, follow real-time instrument recommendations for melt curve step.

2) Three-step Procedure

Stage	Cycles	Procedure	Temperature	Time
Stage1: Hot-Start Taq Polymerase Activation	Hold (1 cycle)	Initial Denaturation	95℃	2 min
Stage2: PCR	CYCLE (40 cycles)	Denaturation	95℃	15 sec
		Annealing	50-60℃	30 sec
		Extension	72℃	30 sec
Stage3: Melt Curve	CYCLE (1 cycle)		95℃	15 sec
			60℃	60 sec
			95℃	15 sec

Notes

1. Primer Design

It is crucial to design primers that allow good reactivity and minimize the formation of non-specific products. Please follow the guidelines below.

1) Target sequence size: 80-200 bp is recommended. It is possible to amplify a target up to 300 bp in size, in such case, you can extend the extension time or proceed to the three step procedure.

2) Primer length: 17-30 bp.

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

4) Tm: Tm values of forward and reverse primers must not be significantly different. Tm values can be calculated with softwares.

5) Sequence of primer: The chosen sequence should not be partially rich in any base in the whole sequence. Avoid including regions that have high GC or AT content, (especially at the 3' end). Avoid including polypyrimidine (serial T/C sequence) and polypurine (serial A/G sequence).

6) Sequence of 3’ end: The 3’ terminus region of the primers should not have a high GC or AT content. We recommend that you choose a sequence with G or C at the 3' end (Avoid T at the 3' end). A complementary sequence of more than 3 bases should not exist within a primer or even between primer pairs (causing hairpin structure or primer dimer, respectively). A primer pair should not have a complementary sequence of more than two bases at each 3’ end to avoid primer dimer.

7) Specificity: Specificity of primers should be confirmed through softwares.

When design primers, enter sufficient sequence around the area of interest. Take search criteria that permit cross-reference against relevant sequence databases (to avoid potential off-target amplification). For cDNA targets, you can choose designing primers across known splicing sites in order to prevent amplification from genomic DNA. Conversely, primers designed to target’s intron region can ensure amplification exclusively from genomic DNA.

2. Template Preparation and Concentration.

a) Template DNA should be stored in an EDTA-containing buffer (e.g., 1X TE) for long-term stability, and dilutions should be freshly prepared for a qPCR experiment either into TE or water.

b) cDNA can be derived from a starting 1 µg - 0.1 pg RNA reverse transcription reaction product. cDNA can be added to the qPCR reaction without purification, but since too high a concentration of reverse transcriptase can inhibit taqase activity, a dilution of at least 1:10 is generally recommended, although it can be left undiluted for very low expression genes, when the amount of template added should not exceed one tenth of the total volume. One tenth of the total volume of template should not be added.

3. Reaction Setup and Cycling Conditions

a) For 96-well plates, we recommend a final reaction volume of 20 µl. For 384-well plates, a final reaction volume of 10 µl is recommended.

b) When setting instrument cycling procedures, ensure a plate read is included at the end of the extension step, and a melt curve after cycling should be conducted to analyze product specificity.

c) Amplification for 40 cycles is sufficient for most applications, but for target gene of very low copy in samples, 45 cycles may be used.

Troubleshooting

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 Mg2+. When optimizing the Mg2+ 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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