2X SYBR Green qPCR Master Mix

Introduction

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 2X SYBR 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. SYBR 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 is supplied together with two ROX Reference Dye of different concentration which should be used to normalize the fluorescent signal intensity between reactions for instruments with the corresponding requirements. Use the SYBR® or SYBR/FAM scan mode setting on the real-time instrument.

However, dye-based qPCR has some limitations. SYBR 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

Components	5mL 1000 rxn with 10 μL reaction 500 rxn with 20 μL reaction 200 rxn with 50 μL reaction	25ml 5000 rxn with 10 μL reaction 2500 rxn with 20 μL reaction 1000 rxn with 50 μL reaction	50ml 10000 rxn with 10 μL reaction 5000 rxn with 20 μL reaction 2000 rxn with 50 μL reaction
2X SYBR Green qPCR Master Mix	1 ml × 5	1 ml × 25	5 ml × 10
50X ROX Reference Dye (low concentration)	0.2 ml	1 ml	1 ml × 2
50X ROX Reference Dye (high concentration)	0.2 ml	1 ml	1 ml × 2

Store all components at –20°C and protect from light. Avoid repeated freeze/thaw cycles as possible.

Reference for Selection of ROX Dye

No need for ROX Dye	**Bio-Rad**: CFX96™, CFX384™, iCycler iQ™, iQ™5, MyiQ™, Opticon®, Opticon 2, Chromo4™, MiniOpticon™ **Cepheid**: SmartCycler® **Eppendorf**: Mastercycler® eprealplex, realplex 2s **Illumina**: 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, ViiA™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™

Guidelines

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 SYBR 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 (2X SYBR 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
2X SYBR 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
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, 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<10^9 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 SYBR 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°C	2 min
Stage2: PCR	CYCLE (40 cycles)	Denaturation	95°C	15 sec
		Annealing	50-60°C	30 sec
		Extension	72°C	30 sec
Stage3: Melt Curve	CYCLE (1 cycle)		95°C	15 sec
			60°C	60 sec
			95°C	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

1) 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.

2) For cDNA, use the product of a reverse transcription reaction containing 1 µg-0.1 pg starting RNA. cDNA doesn't need to be purified before addition to the qPCR reaction but should be diluted at least 1:10 before addition.

3. Reaction Setup and Cycling Conditions

1) 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.

2) 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.

3) 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. Amplification Appears in the Negative Control Obviously

Probable Cause	Corresponding Suggestion
The reagent or Nuclease-free Water used is contaminated	Use new reagents, deionized water and primers, and operate the experiment in a clean laboratory bench. Avoid opening the reaction vessel post amplification (Contamination in new qPCR assays with products from previous amplification reactions can cause a variety of issues).
Primer dimers	Analysis the results according to the melt curves. It is normal to generate amplification curves in the negative control after 35 cycles.

2. Abnormal Ct Value Appears

Probable Cause	Corresponding Suggestion
Low amplification efficiency	Make sure the primers and templates are not degraded. Optimize the reaction system, such as adjusting the concentration of primers, annealing temperature and time. Besides, try three-step method or redesign primers. For templates with high GC content, the initial denaturation time can be extended appropriately.
Low template concentration	Increase the concentration of template. If the template is diluted, use reduced dilution ratio, or you can take gradient dilution to determine the optimum template addition.
Template degradation	Prepare fresh template
Too long target sequence	Generally, the length of target fragments is between 80-200 bp.
PCR inhibitors exist in reaction system	Try to dilute or re-prepare the template (you can repurify the template), because inhibitors were usually added with the template.
The concentration of Mg^{2+} is not enough	For some qPCR reaction, the final concentration of Mg^{2+} may need to be increased. When optimizing Mg^{2+} final concentration, it is recommended to add 0.5 mM each time.

3. No Amplification Curve After the Reaction

Probable Cause	Corresponding Suggestion
Cycle number set is insufficient	In general, the cycle number is set to be 40. However, it should be noted that too many cycles will increase the background and reduce the reliability of the data.
Signal detection didn’t set on correctly	In two-step procedure, signal detection should be set at annealing and extension stage; for three-step procedure, signal detection should be positioned at 72°C extension stage.
The primer degradation	Confirm the integrity of primers by PAGE gel, if degradation occurred, take new primers.
Too low concentration of templates	If the template is diluted, decrease the dilution ratio (For target sequences with unknown levels of expression, the template is recommended to be used without dilution for the first time). If the template is not diluted, remake the sample or concentrate the sample.
The template degradation	Prepare fresh template.

4. Abnormal Amplification Curves

Probable Cause	Corresponding Suggestion
Amplification curve is not smooth	When the signal is too weak, the system calibration will active and lead to this result. In such situations, 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. Decrease the baseline endpoint value (Ct value minus 4) and re-analysis the data.
Several amplification curves suddenly fall	The bubbles in the reaction system will burst suddenly when the temperature rises. A sudden drop will appear in the curve. Centrifuge and check that no bubbles show in the plate.

5. Heterozygous Peak in the Melt Curve

Probable Cause	Corresponding Suggestion
Unreasonable primer design	Primer dimers or undesired amplification product will lead to heterozygous peak in the melt curve. You should redesign the primers. The peaks of primer dimers often occur at about 75°C.
The primer concentration is too high	Decrease primer concentration properly.
The contamination of genomic DNA in the cDNA	Prepare cDNA template again (Use DNase when extract RNA).
Low annealing temperature	Increase the annealing temperature.
Reaction system is too small	Reaction system less than 10 μl is not recommended. If the reaction volume is too small, the detection accuracy will decrease. Increase the reaction volume.

6. Poor Stability of the Data

Probable Cause	Corresponding Suggestion
Sampling error	Using better pipettes is important. Larger reaction system can be taken, or you can increase the dilution ratio and volume of templates simultaneously.
The template concentration is too low	Reduce template dilution or increase sample volume added.
Poor sample purity	Re-extract or purify the samples.
Differences between batches of synthesized primers	Use the same primers as possible.
Instrument malfunction	The temperature or detection of each hole varies, calibrate or repair the instrument.

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