TOOLS 2xSYBR qPCR Mix

For fast quantitative real-time PCR using SYBR Green

Contents

Introduction

The TOOLS 2xSYBR qPCR Mix Kit is specially designed to perform real-time PCR in SYBR Green I fluorescent-based detection assays. The 2× premixed solution included in this kit provides an optimum concentration of SYBR Green I solution, which facilitates the preparation of a qPCR reaction mixture. The TOOLS 2xSYBR qPCR Mix adopts a unique dual hot-start enzymes system (chemically modified HotStar Taq DNA polymerase and antibody-modified Anti-*Taq* DNA Polyerase), which, in addition to the preoptimized buffer solution, provides a convenient format for performing highly sensitive and specific qPCR amplification.

Important Notes

1. The initial denaturation conditions must be 95°C for 15 min to activate the hot-start enzymes.
2. The TOOLS 2xSYBR qPCR Mix includes SYBR Green I. Store the reagent in the dark and avoid direct exposure to strong light during the preparation of PCR reaction mixtures.
3. Gently mix the reagents by inverting the tubes and centrifuge briefly prior to use. Do NOT vortex and avoid producing bubbles.
4. The purity of primers is important for the specificity of PCR. Therefore, primers purified by PAGE or more superior methods are recommended.
5. Typically, the best amplification results can be obtained using a primer concentration of 0.3 μM. However, for individual determination of optimal primer concentration, a primer titration from 0.2–0.5 μM can be performed.
6. In a 20-μL reaction volume, the amount of genome DNA or cDNA template is usually less than 100 ng. The reverse transcription products, if used as a template, should not comprise more than 20% of the total PCR reaction volume.

Kit Contents

Contents	FPT-BB05 (50 rxn)
TOOLS 2xSYBR qPCR Mix	1.25mL
50×ROX Reference Dye	250μL

Storage

The TOOLS 2xSYBR qPCR Mix Kit should be stored immediately upon receipt at –20°C and protected from light. Thaw the TOOLS 2xSYBR qPCR Mix and 50×ROX Reference Dye and mix thoroughly before use. If the TOOLS 2xSYBR qPCR Mix is thawed and not used, it is important to thoroughly mix it prior to refreezing. The layering of salts during the thawing process and subsequent crystallization during freezing will damage the enzyme and decrease product performance. For frequent use, the TOOLS 2xSYBR qPCR Mix can be stored at 2ºC–8°C for 3 months. Repeated freeze–thaw cycles should be avoided.

Protocol

1. Set Up the Real-Time PCR System

Note: The TOOLS 2xSYBR qPCR Mix and 50×ROX Reference Dye should be stored and protected from light.

a. Thaw the TOOLS 2xSYBR qPCR Mix (if stored at –20°C), 50×ROX Reference Dye, template, primers, and RNase-free ddH_2O. Completely mix and equilibrate the reagents to room temperature before use.

b. Prepare a reaction solution according to the following table. All steps should be executed on ice.

Component	50μL volume	25μL volume	20μL volume	Final concentration
TOOLS 2xSYBR qPCR Mix	25μL	12.5μL	10μL	1×
Forward Primer (10μM)	1.5μL	0.75μL	0.6μL	0.3 μM[*1]
Reverse Primer (10μM)	1.5μL	0.75μL	0.6μL	0.3 μM[*1]
cDNA template	X μL	X μL	X μL	-
50×ROX Reference Dye[*2]	-	-	-	-
RNase-free ddH_2O	Up to 50μL	Up to 25μL	Up to 20μL	-

Note

*1. A final primer concentration of 0.3 μM is optimal for most applications. Higher concentrations can be used when the amplification efficiency is not favorable. If nonspecific amplification is observed, however, the primer concentration should be reduced. For further optimization, a primer titration from 0.2–0.5μM can be performed.

*2. The optimal concentration of the ROX Reference Dye for commonly used real-time PCR instruments is as follows:

Instrument	Final concentration
ABI PRISM 7000/7300/7700/7900HT/Step one	5× (e.g. 5μL ROX/50μL volume)
ABI 7500, 7500 Fast; Stratagene Mx3000P, Mx3005P and Mx4000	1× (e.g. 1μL ROX/50μL volume)
Instruments of Roche, Bio-Rad and Eppendorf	No need to add

2. Real-Time Amplification

Typically, the best results are obtained using two-step PCR. However, if two-step PCR does not yield favorable results (e.g., nonspecific amplification caused by low template concentration or reduced amplification efficiency induced by low temperature), three-step PCR is recommended.

Two-Step PCR

Stage	Cycle	Temperature	Time	Step	Signal Collection
Initial denaturation	1×	95°C	15min	Initial denaturation	N
PCR	40×	95°C	10s	Denaturation	N
		60-66°C *1	20-32s*3	Annealing/ Extension	Y
Melting/Dissociation Curve Stage					

Three-Step PCR

Stage	Cycle	Temperature	Time	Step	Signal Collection
Initial denaturation	1×	95°C	15min	Initial denaturation	N
PCR	40×	95°C	10s	Denaturation	N
		50-60°C *2	20s	Annealing	N
		72°C	20-32s*3	Extension	Y
Melting/Dissociation Curve Stage					

Note:

- An annealing/extension temperature of 60°C (for 20, 30, 31, or 32 s) is optimal for most applications. However, if further optimization is required, a temperature of 60°C–66°C is acceptable.
- Normally, the annealing temperature would be 5°C lower than the temperature of a primer. If primers are relatively short, the annealing temperature can be increased to improve specificity. Otherwise, the opposite treatment should be performed.
- For a certain real-time instrument, the extension time should be set according to its instruction manual. For the guidelines for commonly used instruments, please see the list below.

Roche LightCycler	20s
ABI 7700/7900HT/7500 Fast	30s
ABI 7000/7300	31s
ABI 7500	32s

3. Close the tubes and mix samples gently. Brief centrifugation can be performed to collect residual liquid from the walls of the tubes.
4. Place the PCR tubes in the thermal cycler and begin the PCR cycle.
5. Taking an ABI 7500 real-time PCR instrument as an example, the optimization strategies to improve amplification efficiency are as follows:

Basic program			Optimized program 1 (extending the elongation time)	Optimized program 2 (using three-step PCR)	
Cycle	Temperature	Time	Time	Temperature	Time
1×	95°C	15 min	15 min	95°C	15 min
40×	95°C	10s	10s	95°C	10s
	60°C	32s	32-60s	55°C	30s
	NA			72°C	32s

Optimization strategy to improve specificity in an ABI 7500 real-time PCR instrument.

Basic program			Optimized program 1 (increasing annealing temperature)	
Cycle	Temperature	Time	Temperature	Time
1×	95°C	15 min	95°C	15 min
40×	95°C	10s	95°C	10s
	60°C	32s	60-64°C	32s

Troubleshooting

No signal, signal detected late in PCR, or only primer–dimers detected

Comments	Suggestions
Inhibitors in template	Reduce the amount of the template. If necessary, perform the purification procedure again.
Mg^{2+} concentration not optimal	The Mg^{2+} concentration provided in the TOOLS 2xSYBR qPCR Mix is 2 mM. For a few targets, an increase up to 5 mM Mg^{2+}may be helpful. Perform the titration in 0.5-mM steps.
Pipetting error or missing reagent	Check the concentrations and storage conditions of the reagents, including primers and template nucleic acids. Repeat the PCR.
Hot-Start Taq DNA Polymerase not activated	Ensure that the cycling program includes the initial denaturation step (5 min at 95°C) to activate the hot-start enzymes.
PCR programs or primer concentration not optimal	Use optimal primer concentrations and check for possible degradation of primers. Modify the PCR thermal cycling according to the information provided in this handbook. If necessary, redesign the primers.
Problems with starting template	Check the concentration, storage conditions, and quality of the starting template. If necessary, make new serial dilutions of template nucleic acids from the stock solutions. Repeat the PCR using the new dilutions.

High fluorescence in “No Template” control

Comments	Suggestions
Contamination of reagents	Discard reaction components and repeat PCR with new reagents.
Contamination during reaction setup	Take appropriate safety precautions (e.g., use filter tips).
Primer degradation	Check for possible degradation of primers on a denaturing polyacrylamide gel.

Primer–dimers and/or nonspecific PCR products

Comments	Suggestions
Mg^{2+} concentration not optimal	The Mg^{2+} concentration provided in the TOOLS 2xSYBR qPCR Mix is 2 mM. For a few targets, an increase up to 5 mM Mg^{2+} may be helpful. Perform the titration in 0.5-mM steps.
Annealing temperature too low	Increase annealing temperature in increments of 2°C.

Primer design not optimal	Review primer design.
PCR product too long	For optimal results, PCR products should be between 100 and 150 bp. PCR products should not exceed 500 bp.
Primers degraded	Check for possible degradation of primers on a denaturing polyacrylamide gel.
Metering inaccuracies	A too-small reaction volume may reduce the accuracy of detection. Use the volume recommended in the instruction manual and repeat the PCR.

No linearity in ratio of CT value/crossing point to log of the template amount

Comments	Suggestions
Instrument malfunction	Operate the real-time PCR instrument according to the instruction manual.
Contamination of templates	Contamination of templates may result in poor linearity.
Long-stored dilutions of template	Make new serial dilutions of template nucleic acids from the stock solutions. Repeat the PCR using the new dilutions.
PCR programs or primer concentration not optimal	Use optimal primer concentrations and check for possible degradation of primers. Modify the PCR thermal cycling according to the information provided in this handbook. If necessary, redesign the primers.
Metering inaccuracies	A too-small reaction volume can reduce the accuracy of detection. Use the volume recommended in the instruction manual and repeat the PCR.