TOOLS Easy SYBR qPCR Mix

Cat. no.: FPT-BB01-4

Storage:

The TOOLS Easy SYBR qPCR Mix should be stored immediately at −20°C upon receipt and should be protected from light. Thaw the 2× SYBR PreMix, 40× Dilution Buffer (optional), and 50× ROX Dye and mix thoroughly before use. If the 2× SYBR PreMix is thawed and not used, it is important to thoroughly mix prior to re-freezing. The layering of salts during the thawing process and subsequent crystallization during freezing will damage the enzyme and reduce product performance. For frequent use, the TOOLS 2× SYBR PreMix can be stored at 2°C–8°C for 3 months. Repeated freeze–thaw cycles should be avoided.

Product Size:

Contents	Volume
2× SYBR PreMix (blue)	1.25ml x 4
50×ROX Dye	250µl x 4
40× Dilution Buffer (Yellow)	1.25 ml
RNase-Free ddH_2O	1 ml x 4

Introduction

The TOOLS Easy SYBR qPCR Mix Kit is specially designed to perform real-time polymerase chain reaction (PCR) in SYBR Green I fluorescent-based detection assays. The Real-Time PCR Reaction Buffer, a 2× premixed solution included in this kit, provides an optimum concentration of SYBR Green I solution, which greatly facilitates the preparation of the quantitative PCR (qPCR) reaction mixture. The TOOLS 2× SYBR PreMix adopts a unique dual hot-start enzyme system (chemically modified HotStar Taq DNA polymerase and antibody-modified Anti-Taq DNA Polymerase); the PreMix plus the preoptimized buffer solution provides convenient, 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 2× SYBR PreMix 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 before use. Do NOT vortex and avoid producing bubbles.
4. The purity of primers is important for the specificity of PCR. Primers purified using polyacrylamide gel electrophoresis (PAGE) or more superior methods are recommended.
5. Typically, the optimal amplification results can be obtained using a primer concentration of 0.3 µM. However, for individually determining the optimal primer concentration, primer titration in the primer

concentration range from 0.2 to 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. Reverse transcription products, if used as template, should not comprise more than 20% of the total PCR reaction volume.

Protocol

A. Set up the real-time reaction system

Note: The 2× SYBR PreMix and 50×ROX Dye should be stored and protected from light.

1. Thaw the 2× SYBR PreMix (if stored at −20°C), 50× ROX Dye, template, primers, and RNase-free ddH_2O. Completely mix and equilibrate the reagents to room temperature before use.
2. Prepare a reaction solution according to the following table. All the steps should be performed on ice.

Component	**50μl volume**	**25μl volume**	**20μl volume**	**Final concentration**
2xSYBR PreMix	25 μL	12.5 μL	10 μL	1×
Forward Primer (10μM)	1.5 μL	0.75 μL	0.6 μL	0.3 μM[*1]
Reverse Primer (10μM)	1.5 μL	0.75 μL	0.6 μL	0.3 μM[*1]
cDNA template[*2]	-	-	-	-ng–pg
50×ROX Dye[*3]	-	-	-	-
RNase-free ddH_2O	Up to 50 μL	Up to 25 μL	Up to 20 μL	-

Note:

a. 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, the primer concentration should be reduced. For further optimization, primer titration in the primer concentration range from 0.2 to 0.5 μM can be performed.

b. Optional: The cDNA template can be diluted with 40× Dilution Buffer (Yellow). The color of the reaction solution turns green after adding 40× Dilution Buffer (the final concentration of 40× Dilution Buffer should be 1×).

c. The optimal concentration of ROX Dye for commonly used real-time PCR instruments:

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

B. Real-time PCR amplification

Typically, the optimal results are obtained using two-step PCR. However, if two-step PCR does not yield favorable results (e.g., nonspecific amplification caused by a low template concentration or reduced amplification efficiency induced by a low Tm value), then 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:

a. An annealing/extension temperature of 60℃ (20, 30, 31, and 32 s) is optimal for most applications. However, if further optimization is required, the temperature from 60°C to 66°C can be applied.

b. Normally, the annealing temperature would be 5°C lower than primer′s Tm value. If primers are relatively short, the annealing temperature can be increased to improve specificity. Otherwise, the opposite treatment should be conducted.

c. For a particular real-time PCR 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 then start the PCR cycle.

Take the ABI 7500 Real-Time PCR Instrument as an example. The following table presents the optimization strategies to improve amplification efficiency for this instrument:

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 for improving specificity for the 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 Guide

No signal or signal detected late in PCR, or only primer-dimers detected.

Comments	**Suggestions**
Inhibitors in the 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 2× SYBR PreMix is 2 mM. For a few targets, an increase in the Mg^{2+} concentration of up to 5 mM 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 PCR.
HotStarTaq DNA Polymerase not activated	Ensure that the cycling program includes the initial denaturation step (15 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 PCR

	thermal cycling according to the information provided in this handbook. If necessary, redesign the primers.
Problems with the 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 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 2× SYBR PreMix is 2 mM. For few targets, an increase in the Mg^{2+} concentration of up to 5 mM 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	Too small a reaction volume may reduce the accuracy of detection. Use the volume recommended in the instruction manual and repeat 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 lead to poor linearity.
Long stored dilutions of template	Make new serial dilutions of template nucleic acids from the stock solutions. Repeat PCR using the new dilutions.
PCR programs or primer concentration not optimal	Use optimal primer concentrations and check for possible degradation of primers. Modify PCR thermal cycling according to the information provided in this handbook. If necessary, redesign the primers.
Metering inaccuracies	Too small a reaction volume can reduce the accuracy of detection. Use the volume recommended in instruction manuals and repeat the PCR.