SUPRA
SYBR MASTER MIX

Master mix (2X)

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SUPRA Sybr Master mix (2x)

Cat. no. EC05

Kit Contents

Contents	EC05-01 25 µl × 100 rxns	EC05-02 25 µl × 400 rxns
2× Supra Sybr Master Mix (with SYBR Green I)	1.25 ml	4 × 1.25 ml
50× ROX Reference Dye	250 µl	1 ml
RNase-free ddH_2O	1 ml	5 × 1 ml
Handbook	1	1

Storage

The Supra Sybr Master Mix (SYBR Green) Kit should be stored immediately upon receipt at -20ºC, protected from light. Thaw the 2× Supra Sybr Master Mix and 50× ROX Reference Dye and mix thoroughly before use. If the 2× Supra Sybr Master Mix and 50× ROX Reference Dye are thawed but not used, it is important to thoroughly mix them prior to re-freezing. The layering of salts during the thawing process and subsequent crystallization during freezing will damage the enzyme and decrease product performance. For frequent use, Supra Sybr Master Mix can be stored at 2-8ºC for 3 months. Repeated freeze-thaw cycles should be avoided.

Introduction

Supra Sybr Master Mix (SYBR Green) Kit is specially designed to perform Real-time PCR in SYBR Green I fluorescent-based detection assays. The Real-Time PCR reaction buffer, a 2× pre-mixed solution included in this kit, provides an optimum concentration of SYBR Green I solution, which greatly facilitates the preparation of qPCR

reaction mixture. Supra Sybr Master Mix adopts a unique dual hot-start enzymes system (chemically modified HotStar *Taq* DNA polymerase and antibody modified Anti *Taq* DNA Polyerase), which, plus the pre-optimized buffer solution, provides a convenient format for highly sensitive and specific qPCR amplification.

Important Notes

1. The initial denaturation conditions must be 95ºC 15 min, in order to activate the hot start enzymes.
2. Supra Sybr Master Mix includes the SYBR Green I. Store the reagent in dark and avoid direct exposition 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 bubble.
4. The purity of primers is important for the specificity of PCR. Primers purified by PAGE or more superior methods are recommended.
5. Typically, 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 µM to 0.5 µM can be performed.
6. In a 20 µl reaction volume, the amounts of genome DNA or cDNA template is usually less than 100 ng. The reverse transcription products, if used as template, should not comprise more than 20% of the total PCR reaction volume.

Protocol

<1> Set up the Real-Time reaction system

Note: 2× Supra Sybr Master Mix and 50× ROX Reference Dye should be stored protected from light.

1. Thaw 2× Supra Sybr Master Mix (if stored at -20℃), 50× ROX Reference Dye, template, primers and RNase-free ddH_2O. Completely mix and equilibrate reagents to room temperature before use.

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

Component	50 µl volume	25 µl volume	20 µl volume	Final concentration
2× Supra Sybr Master Mix	25 µl	12.5 µl	10 µl	1×
Forward Primer (10 µM)	1.5 µl	0.75 µl	0.6 µl	0.3 µM*[1]
Reverse Primer (10 µM)	1.5 µl	0.75 µl	0.6 µl	0.3 µM*[1]
cDNA template	-	-	-	-ng-pg
50× ROX Reference Dye*[2]	-	-	-	-
RNase-free ddH_2O	Up to 50 µl	Up to 25 µl	Up to 20 µl	-

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

*[2] The optimal concentration of ROX Reference Dye for commonly used Real-Time PCR instruments:

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

<2> Real-Time Amplification

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

Two-step PCR

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

Three-step PCR

Stage	Cycle	Temperature	Time	Step	Signal Collection
Initial denaturation	1×	95℃	15min	Initial denaturation	N
PCR	40×	95℃	10 sec	Denaturation	N

		50-60°C*[2]	20 sec	Annealing	N
		72°C	20-32 sec*[3]	Extension	Y
Melting/Dissociation Curve Stage					

*1 An Annealing/Extension temperature of 60ºC is optimal for most applications. However, if further optimization is required, temperature from 60ºC to 66ºC can be performed.

*2 Normally, annealing temperature would be 5ºC lower than primer's Tm value. If primers are relatively short, the annealing temperature can be increased to improve the specificity. Otherwise, the opposite treatment should be taken.

*3 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	20 sec
ABI 7700/7900HT/7500 Fast	30 sec
ABI 7000/7300	31 sec
ABI 7500	32 sec

3. Close the tubes and mix samples gently. Briefly 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 ABI 7500 Real Time PCR Instrument as an example. Optimization strategies to improve amplification efficiency in 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	15min	15min	95°C	15min
40×	95°C	10 sec	10 sec	95°C	10 sec
	60°C	32 sec	32-60 sec	55°C	30 sec
	NA			72°C	32 sec

Optimization strategy to improve specificity in ABI 7500 Real Time PCR Instrument:

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

Troubleshooting Guide

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

Comments	**Suggestions**
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 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.

2. 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.

3. Primer–dimers and/or non-specific PCR products

Comments	**Suggestions**
Mg^{2+} concentration not optimal	The Mg^{2+} concentration provided in 2x Supra Sybr Master Mix is 2 mM. For few targets, an increase up to 5 mM Mg2+ 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 reaction volume may reduce the accuracy of detection. Use the volume recommended in instruction manual and repeat the PCR.

4. 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 the 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	Too small reaction volume can reduce the accuracy of detection. Use the volume recommended in instruction manuals and repeat the PCR.