TIANGEN®

SuperReal PreMix Plus (SYBR Green)

For fast, quantitative, real-time PCR using SYBR Green

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SuperReal PreMix Plus (SYBR Green)

Cat. no. 4992214/4992215/4992248

Kit Contents

Contents	4992214 20 µl × 125 rxn	4992215 20 µl × 500 rxn	4992248 20 µl × 5000 rxn
2× SuperReal PreMix Plus (SYBR Green)	1.25 ml	4 × 1.25 ml	10 × 4 × 1.25 ml
50× ROX Reference Dye	250 µl	1 ml	10 × 1 ml
RNase-Free ddH_2O	2 × 1 ml	5 × 1 ml	10 × 5 × 1 ml
Handbook	1	1	10 × 1

Storage

The SuperReal PreMix Plus (SYBR Green) Kit should be stored immediately upon receipt at -20°C, protected from light. Thaw the 2×SuperReal PreMix Plus and 50× ROX Reference Dye and mix thoroughly before use. If the 2×SuperReal PreMix Plus 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, SuperReal PreMix Plus can be stored at 2-8°C for 3 months. Repeated freeze-thaw cycles should be avoided.

Introduction

SuperReal PreMix Plus (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. SuperReal PreMix Plus adopts a unique dual hot-start enzymes system (chemically modified HotStart 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 should be 95°C 15 min to activate the hot start enzymes.
2. SuperReal PreMix Plus 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 amount of genomic 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× SuperReal PreMix Plus and 50× ROX Reference Dye should be stored protected from light.

1. Thaw 2× SuperReal PreMix Plus (if stored at -20°C), 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× SuperReal PreMix Plus	25 µl	12.5 µl	10 µl	1×
Forward Primer (10 µM)	1.5 µl	0.75 µl	0.6 µl	0.3 µM*
Reverse Primer (10 µM)	1.5 µl	0.75 µl	0.6 µl	0.3 µM*
cDNA template	-	-	-	-ng-pg
50× ROX Reference Dye △	-	-	-	-
RNase-free ddH_2O	Up to 50 µl	Up to 25 µl	Up to 20 µl	-

* 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.

△ The optimal concentration of ROX Reference Dye for commonly used Real-Time PCR instruments:

Instrument	Final Concentration
ABI PRISM 7000/7300/7700/7900HT /StepOne	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

<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°C	15 min	Initial denaturation	N
PCR	40×	95°C	10 sec	Denaturation	N
		60-66°C △1	20-32 sec*	Annealing/ Extension	Y
Melting/Dissociation Curve Stage					

Three-step PCR

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

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

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

* 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/LightCycler 480	20 sec
ABI 7500Fast/7900HT/7900HT Fast/ ViiA 7/StepOne/StepOnePlus	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 program1 (extending the elongation time)	Optimized program2 (using three-step PCR)	
Cycle	Temperature	Time	Time	Temperature	Time
1×	95°C	15 min	15 min	95°C	15 min
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	15 min	95°C	15 min
40×	95°C	10 sec	95°C	10 sec
	60°C	32 sec	60-64°C	32 sec

Troubleshooting Guide

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

Comments	Suggestions
Inhibitor in the DNA template	Purify the DNA template further or lower the amount of DNA template.
Improper Mg^{2+} concentration	The final Mg^{2+} concentration is 2 mM in SuperReal PreMix Plus. It could be increased to 5 mM for some specific system. If needs further optimization, please start with 0.5 mM Mg^{2+} concentration of increase each time.
Pipetting error or missing reagent	Check the concentrations and storage conditions of the reagents, including primers and template nucleic acid. Repeat the PCR.
PCR programs or primer sequence not optimal	Make sure that primers have not been degraded, primer concentration and PCR program are correct. Reduce annealing temperature, increase annealing time and primer concentration should be tried out first, sometimes, increase of annealing temperature, extension time and lower temperature increase rate could be considered. For high GC content template, the denaturation time could be properly prolonged. If no improvement, primers should be redesigned.
Problems with starting template	Check the concentration, storage conditions, and quality of the starting template. If necessary, Repeat PCR using new serial dilutions of template nucleic acids from the stock solutions. Increase the amount of the template.

2. Positive signal in no-template control (NTC)

Comments	Suggestions
Contamination of reagents	Discard all the reagents, use newly prepared reagents to perform PCR amplification
Contamination in PCR setup	Review setup procedure and use aerosol-barrier pipette tips if possible
Primers degraded	Check for possible degradation of primers on a denaturing polyacrylamide gel.

3. Primer-dimers and/or nonspecific amplification products

Comments	Suggestions
Improper Mg^{2+} concentration	The final Mg^{2+} concentration is 2 mM in SuperReal PreMix Plus. It could be increased to 5 mM for some specific system. If needs further optimization, please start with 0.5 mM Mg^{2+} concentration of increase each time.
Annealing temperature too low	Increase the annealing temperature in increments of 2°C.
Primer design not optimal	Re-design the primer sequence.
PCR product too long	For optimal results, PCR products should be between 100-150 bp, 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. Poor repeatability of CT value

Comments	Suggestions
Instrument malfunction	Thermal cycling is not suitable or performs in improper temperature or has poor repeatability. Check the Real-Time PCR instrument according to the manual.
Impurities in templates	Impurities in templates may lead to the poor repeatability.
Long term stored dilutions of template	PCR reaction should use the new dilutions immediately.
PCR programs or primer sequence not optimal	Make sure that primers have not been degraded, primer concentration and PCR program are correct. Reduce annealing temperature, increase annealing time and primer concentration should be tried out first, sometimes, increase of annealing temperature, extension time and lower temperature increase rate could be considered. For high GC content template, the denaturation time could be properly prolonged. If no improvement, primers should be redesigned.
Metering inaccuracies	Too small reaction volume may reduce the accuracy of detection. Use the volume recommended in instruction manual and repeat the PCR.