TIANGEN®

SuperReal PreMix Color (SYBR Green)

Considerate and specific dye-based fluorescence quantitative reagent

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SuperReal PreMix Color (SYBR Green)

Cat. No. 4992777/4992876/4992902

Kit Contents

Contents	4992777 20 μl×125 rxn	4992876 20 μl×500 rxn	4992902 20 μl×5000 rxn
2× SuperReal Color PreMix (SYBR Green, blue)	1.25 ml	4×1.25 ml	10×4×1.25 ml
50× ROX Reference Dye	250 μl	1 ml	10× 1 ml
40× Dilution Buffer (yellow)	1.25 ml	1.25 ml	10×1.25 ml
RNase-Free ddH_2O	2×1 ml	5×1 ml	10×5×1 ml
Handbook	1	1	10× 1

Storage

The product can be stored at -30~-15°C for 1 year. After receiving this product, please immediately place it at -30~-15°C and keep it away from light. When taking out from -30~-15°C for use, dissolve the frozen 2× SuperReal Color PreMix and 50× ROX Reference Dye, then gently inverse the tube to mix them evenly. Please use them after the solution is completely uniform. If it is not used after thawing, it must be thoroughly mixed and then frozen again (salt will be delaminated during thawing; if it is not mixed before freezing, the precipitation of salt crystals will cause damage to enzymes). If it needs to be used frequently for a period of time, it can be stored at 2 - 8°C for 3 months. Avoid repeated freezing and thawing.

Introduction

This product is a special reagent for real-time PCR using SYBR Green I chimeric fluorescence. The reaction reagent is a 2× concentration PreMix with SYBR Green I at the optimum concentration required for real-time PCR. The indicator is additionally added to the product, which is convenient for adding a large number of samples and reduces the probability of misoperation.

SuperReal Color PreMix adopts a unique two-component HotStart DNA polymerase (chemically modified HotStart *Taq* DNA polymerase and antibody modified Anti *Taq* DNA polymerase). Cooperating with a carefully optimized buffer system, this product has high amplification efficiency and high specificity and wider credible range. The improved reagent has prominent advantage in amplification specificity after composition adjustment.

Features

1. SuperReal Color PreMix uses a unique two-component HotStart DNA polymerase (chemically modified HotStart *Taq* DNA polymerase and antibody modified Anti *Taq* DNA polymerase) to form an enzyme activity automatic regulation system, which is characterized by high amplification efficiency, high amplification specificity and wide credible range in cooperation with a carefully optimized buffer system.
2. The Buffer system of this product balances the ratio of K^+ and NH_4^+, and specially adds the original H-Bond factor, which can coordinate to adjust the hydrogen bond force in the reaction system, so that the annealing conditions of primers and templates are more rigorous, the specificity of the reaction is enhanced, and the repeatability is better.
3. SuperReal Color PreMix is pre-mixed with SYBR Green I. When preparing PCR reaction solution, the Real Time PCR reaction can be carried out by simply adding templates, primers and RNase-Free ultrapure water. The operation is simple and convenient.
4. This product comes with ROX Reference Dye, which is used to eliminate the signal background and to correct the fluorescence signal error produced between holes. The ROX Reference Dye is convenient for users to select the corresponding concentration for different types of fluorescence quantitative PCR instruments.
5. The addition of color indicator makes sample addition easier and effectively reduces the probability of misoperation.

Principle

The product uses a unique two-component HotStart DNA polymerase for PCR amplification, and the purpose of detecting the amplification amount of PCR products is achieved by detecting the fluorescence intensity of SYBR Green I in the reaction process.

The dual HotStart enzyme in this product forms a unique enzyme activity automatic regulation system. The automatic regulation system of enzyme activity consists of chemically modified HotStart *Taq* DNA polymerase and antibody modified Anti *Taq* DNA polymerase. HotStart *Taq* DNA polymerase accounts for the vast majority of the total. The activation of its polymerase activity is a slow-release process that strictly depends on the high temperature of 95°C, while Anti *Taq* DNA polymerase is fully activated at the high temperature of 95°C. After incubation at 95°C for 15 min, most HotStart *Taq* DNA polymerases are activated. After entering the PCR cycle, some HotStart *Taq* DNA polymerases can be reactivated after each round of denaturation at 95°C. HotStart *Taq* DNA polymerase has a unique slow release mechanism of enzyme activity, which enables it to form a unique automatic regulation system of enzyme activity with Anti *Taq* DNA polymerase. At the beginning of the PCR reaction, the fully activated Anti *Taq* DNA polymerase can cooperate with the already activated HotStart *Taq* DNA polymerase to reach the optimal enzyme activity state, and in the whole PCR reaction process, each round of newly released HotStart Taq DNA polymerase activity can just make up for some enzyme activity losses caused by thermal denaturation. Therefore, SuperReal Color PreMix keeps the optimum DNA polymerase activity throughout the whole PCR reaction process with careful optimization of buffer system, high amplification efficiency, high amplification specificity and more extensive template adaptability can be obtained.

Important Notes Before Starting

1. The final concentration of each color indicator in the PCR reaction solution should be 1×. Please calculate the dosage of Dilution Buffer according to the amount of template added.
2. The initial denaturation condition for PCR reaction must be set at 95°C for 15 min to fully activate the HotStart enzyme.
3. This product contains fluorescent dye SYBR Green I, so strong light should be avoided when storing this product or preparing PCR reaction solution.

4. If the reagents are not mixed evenly, the reaction performance will be decreased. When using, please gently mix it up and down, and do not use vortex to mix. Try to avoid foam, and use it after instantaneous centrifugation.
5. The final concentration of primer is 0.3 μM, and good amplification results can be obtained in most systems. If further optimization is needed, the primer concentration can be adjusted in the range of 0.2-0.5 μM.
6. In a 20 μl reaction system, the usage amount of genomic DNA or cDNA template is generally less than 100 ng. When reverse transcription product is used as template, the usage amount should not exceed 20% of the final volume of PCR system.

Protocol

<1> Establish Real-Time PCR reaction system:

Please note that 2× SuperReal Color PreMix and 50× ROX Reference Dye should be stored away from light.

1. Thaw 2× SuperReal Color PreMix (if stored at -30~-15°C), 50× ROX Reference Dye, template, primer, 40× Dilution Buffer and RNase-Free ddH_2O, balance at room temperature and thoroughly mix all reagents.
2. It is suggested to prepare the Real Time PCR reaction solution on ice.

   40× dilution buffer is yellow and 2× SuperReal Color PreMix is blue. If the template needs to be diluted, the final reaction system should be green. Otherwise, it should be blue. If the colors do not match, please check whether the relevant components are correctly added into the system.

   Reaction system:

Components	50 μl system	25 μl system	20 μl system	Final concentration
2× SuperReal Color PreMix	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 (with Dilution Buffer)▲	-	-	-	-ng-pg
50× ROX Reference Dye**	-	-	-	-
RNase-free ddH_2O	To 50 μl	To 25 μl	To 20 μl	-

* Primer with the final concentration of 0.3 μM can obtain good amplification results in most systems. When the amplification efficiency is not high, the primer concentration in the PCR reaction system can be increased. When non-specific amplification occurs, the primer concentration in the PCR reaction system can be appropriately reduced. If the concentration of primer needs to be further optimized, it can be adjusted in the range of 0.2 - 0.5 μM.

▲ If the template needs to be diluted, the cDNA template and 40× Dilution Buffer should be mixed into a diluted template according to a certain proportion. The dilution ratio is as follows:

Table of cDNA dosage and corresponding 40× Dilution Buffer content in 20 μl reaction system:

Volume of diluted template in 20 μl PCR System	1 μl	2 μl	2.5 μl	3 μl	4 μl	5 μl	6 μl
Concentration of Dilution Buffer in diluted template	20×	10×	8×	6.7×	5×	4×	3.3×
Volume of 40×Dilution Buffer in the 100 μl diluted template	50 μl	25 μl	20 μl	16.7 μl	12.5 μl	10 μl	8.4 μl
cDNA amount in the 100 μl dilution template	50 μl	75 μl	80 μl	83.3 μl	87.5 μl	90 μl	91.6 μl

Table of cDNA dosage and corresponding 40× Dilution Buffer volume in 50 μl reaction system:

Volume of diluted template in 50 μl PCR System	2 μl	2.5 μl	3 μl	4 μl	5 μl	6 μl	8 μl
Concentration of Dilution Buffer in diluted template	25×	20×	16.7×	12.5×	10×	8.3×	6.25×
Volume of 40×Dilution Buffer in the 100 μl diluted template	62.5μl	50 μl	41.7μl	31.3μl	25 μl	20.8 μl	15.6 μl
cDNA amount in the 100 μl diluted template	37.5μl	50 μl	58.3μl	68.7μl	75 μl	79.2 μl	84.4 μl

** The optimum ROX Reference Dye concentrations for several common instruments are shown in the following table:

Instrument	Final concentration
ABI PRISM 7000/7300/7700/7900HT/ StepOne and so on	5× (e.g. 5 μl ROX/50 μl system)
ABI 7500, 7500 Fast; Stratagene Mx3000P, Mx3005P andMx4000 and so on	1× (e.g. 1 μl ROX/50 μl system)
Roche instruments, Bio-Rad instruments, Eppendorf instruments, etc.	No need

<2> Carry out Real time PCR reaction

It is suggested to adopt a two-step PCR reaction flow for the reaction. When non-specific amplification occurs due to low template concentration, or low amplification efficiency or poor reproducibility of amplification curve due to low Tm value of primer, etc., it is suggested to try to carry out three-step PCR amplification reaction.

Two-step reaction program:

Phase	Cycle	Temperature	Time	Content	Fluorescence signal acquisition
Initial denaturation	1×	95°C	15 min	Initial denaturation	No
PCR reaction	40×	95°C	10 sec	Denaturation	No
		60-66°C▲1	20-32 sec*	Annealing /extension	Yes
Melting/Dissociation Curve Stage					

Three-step reaction program:

Phase	Cycle	Temperature	Time	Content	Fluorescence signal acquisition
Initial denaturation	1×	95°C	15 min	Initial denaturation	No
PCR reaction	40×	95°C	10 sec	Denaturation	No
		50-60°C▲2	20 sec	Annealing	No
		72°C	20-32 sec*	Extension	Yes
Melting/Dissociation Curve Stage					

▲1 First use 60°C 32 sec (20 sec, 30 sec, 31 sec) for amplification. If further optimization is needed, it can be set in the range of 60-66°C.

▲2 Usually, the annealing temperature of the primer is 5°C lower than the melting temperature (Tm) of the primer. If the number of primer bases is small, the annealing temperature can be appropriately increased, which can increase the specificity of PCR. If the number of bases is large, the annealing temperature can be appropriately reduced.

* When using different types of instruments to set the time, please carry out the experimental operation according to the requirements of the instrument operating instructions. See the following table for the time setting of several common instruments:

Set Roche LightCycler/ LightCycler 480 at 20 sec.
Set ABI 7500 Fast/7900HT/7900HT Fast/ViiA 7/ StepOne/StepOnePlus at 30 sec.
Set ABI 7000 and 7300 at 31 sec.
Set ABI 7500 at 32 sec.

3. Cover the reaction tube and mix gently. Centrifuge briefly to ensure that all components are at the bottom of the tube.
4. Place the reaction tubes in a real time PCR instrument, and start the program.

   Taking ABI 7500 Real Time PCR instrument as an example, when optimizing the amplification efficiency, the reaction can be carried out according to the reaction conditions of Optimization Scheme 1 or Optimization Scheme 2 shown in the following table:

Basic Reaction Procedure			Optimization Scheme 1 (Optimization by increasing extension time of two-step method)	Optimization Scheme 2 (PCR reaction using three-step method)	
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

Taking ABI 7500 Real Time PCR instrument as an example, the specific optimization reference scheme is as follows:

Basic Reaction Procedure			Specific Optimization Scheme (Increase annealing temperature in two-step method)	
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

Operation suggestions for RT-qPCR reaction

When performing RT-PCR reaction, there are two kinds of cDNA first strand synthesis kits available, namely FastKing RT Kit (With gDNase) (4992223/4992224/4992250) and TIANScript II RT Kit (4992910/4992911). The FastKing RT Kit is specially prepared for the first step experiment of the two-step RT-PCR and has highly sensitivity, which can synthesize the first strand cDNA from extremely low amount of total RNA or poly $(A)^+$ RNA. The reverse transcriptase King Reverse Transcriptase used in the kit is different from the commonly used M-MLV derived from Moloney murine leukemia virus and AMV derived from bird myeloblast virus. It is a brand-new high-efficiency reverse transcriptase recombinantly expressed by *Escherichia coli* engineering bacteria. It can efficiently transcribe various RNA templates and transcribe RNA into the first strand of cDNA to the maximum extent.

Taking FastKing RT Kit (With gDNase) (4992223/4992224/4992250) as an example, the following operation steps are applicable to total RNA with template quantity of 50 ng-2 μg.

1. Thaw template RNA on ice. Thaw 5× gDNA Buffer, FQ-RT Primer Mix, 10× King RT Buffer, RNase-Free ddH_2O at room temperature (15-30°C), and quickly place on ice after thawing. Before use, mix each solution by vortex and centrifuge briefly to collect the liquid remaining on the tube wall.

   Please perform the following steps on ice. In order to ensure the accuracy of the preparation of the reaction solution, a mastermix should be prepared before each reaction, and then aliquot into each reaction tube.

2. Prepare the mixed solution according to the genomic DNA removal system shown in Table 1 and mix thoroughly. Centrifuge briefly and incubate at 42°C for 3 min. Then place it on ice.
3. Prepare the mixture according to the reverse transcription reaction system in Table 2.
4. Add the reverse transcription reaction mastermix to the reaction solution in the gDNA removal step and fully mix the solution.
5. Incubation at 42°C for 15 min, then incubate at 95°C for 3 min.

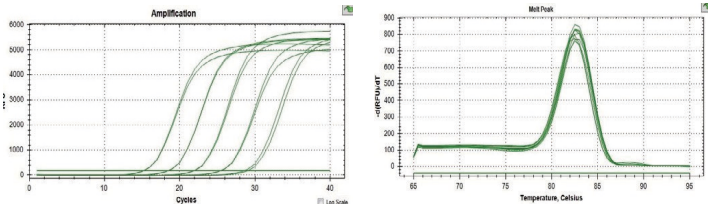
6. Set up PCR reaction

Table 1 gDNA Removal Reaction System

Component	Volume
5× gDNA Buffer	2 μl
Total RNA	-
RNase-Free ddH_2O	To 10 μl

Table 2 Reverse Transcription Reaction System

Component	Volume
10× King RT Buffer	2 μl
FastKing RT Enzyme Mix	1 μl
FQ-RT Primer Mix	2 μl
RNase-Free ddH_2O	To 10 μl



This experiment adopts two-step quantitative RT-PCR to quantitatively detect Hsc mRNA of human Jurkat cell. The fluorescence quantitative PCR is performed by SuperReal PreMix Color (SYBR Green) (4992777/4992876/4992902). cDNA synthesis is performed using FastKing RT Kit (With gDNase) (4992223/4992224/4992250).

The amount of cDNA is equivalent to 100 ng -10 pg of total RNA.

The amplification curve (left) and melting curve (right) show good amplification efficiency and specificity.

Primer Design Instructions

The design of PCR primers is very important in real-time PCR reaction. To design primers with high PCR amplification efficiency and strong reaction specificity, please refer to the following requirements.

◆The requirements for primer design are as follows:

Primer length	18-30 bases
GC contents	40-60%
Tm value	Primer software can give Tm, which is also related to primer length, base composition and ionic strength of buffer used by primer. Tm values of upstream and downstream primers should be as close as possible. The simple Tm calculation formula is Tm = 4°C(G+C)+ 2°C(A+T). Generally, 5°C lower than Tm value of primer is used as PCR annealing temperature. Increasing annealing temperature can increase the specificity of PCR reaction.
Sequences of primers and PCR product	The length of PCR amplification product is preferably between 80-200 bp. Try to avoid designing primers in the secondary structure area of the template. Avoid the formation of 2 or more complementary bases between the 3' ends of upstream and downstream primers to reduce the formation of primer dimer. The 3' end base of primer cannot have more than 3 consecutive G or C. The primer itself should not have complementary sequences, otherwise the primer itself will fold into a hairpin structure. Avoid T at the 3' end of the primer. A, T, G and C in the primer sequence should be evenly distributed as far as possible.

Trouble Shooting

1. No amplification signal, late peak of amplification curve, or only primer dimer was detected

Possible reasons	Suggestions
Inhibitors are present in DNA templates	Repurify templates or reduce template usage
Inappropriate Mg^{2+}concentration	When 2× SuperReal Color PreMix is used, the final concentration of Mg^{2+} in PCR reaction system is 2 mM. For some amplification systems, the final concentration of Mg^{2+} can be increased to 5 mM. When optimizing the final concentration of Mg^{2+}, it is recommended to increase the concentration of Mg^{2+} by 0.5 mM each time.
Sample addition error or reagent problem	Check reagent concentration and storage conditions, including that of primers and templates. Repeat the experiment.
The hotstart enzyme is not activated	When using reagents, please ensure that the initial denaturation condition is 95°C for 15 min to effectively activate hotstart DNA polymerase.
Improper PCR conditions, primer sequence or concentration	Please confirm that the primer has not been degraded. Check the primer concentration and PCR conditions. If the amplification efficiency is not good, first try to reduce the annealing temperature, prolong the annealing time and increase the primer concentration. Sometimes the annealing temperature can be increased, the extension time can be increased, and the heating rate can be reduced. For templates with high GC content, denaturation time can be appropriately prolonged. If the amplification is still not good, please redesign the primer.
Initial template problem	Check the concentration, storage conditions and quality of the initial template. Carry out linear gradient dilution of the template again, and experiment with the new diluted template. Increase initial template usage.

2. NTC has high fluorescence value

Possible reasons	Suggestions
Reagent contamination	It is suggested to use new reagents for experiments.
Biological pollution occurs during PCR reaction set up	Take necessary anti-pollution strategies (e.g. using pipette tips with filter).
Primers degradation	Denatured polyacrylamide gel can be used to detect primer degradation.

3. Primer dimer and/or nonspecific amplification

Possible reasons	Suggestions
Inappropriate Mg^{2+} concentration	The final concentration of Mg^{2+} in the reaction system using 2×SuperReal Color PreMix is 2 mM. For some amplification systems, the final concentration of Mg^{2+} can be increased to 5 mM. It is suggested to increase the concentration of Mg^{2+} by 0.5 mM each time for optimization.
PCR annealing temperature is too low	It is suggested to optimize the annealing temperature by increasing 2°C each time.
Inproper design of primers	It is suggested to redesign the primer sequence.
PCR product is too long	The length of the fluorescent quantitative PCR product is preferably between 100 and 150 bp and should not exceed 500 bp.
Degradation of primers	Denatured polyacrylamide gel can be used to detect primer degradation.
Measurement error	If the reaction volume is too small, the detection accuracy will decrease. Please repeat the experiment according to the reaction volume recommended by the real time PCR instrument.

4. Poor reproducibility of quantitative values

Possible reasons	Suggestions
Instrument failure	Due to the inapplicability of the instrument, the reproducibility of the products produced during temperature management or detection is poor. Please check according to the instructions of the corresponding instruments.
Poor sample purity	The impure sample will lead to poor reproducibility of the experiment.
The diluted template has been left for too long	The template diluted by gradient is preferably prepared and used right before use.
The quality of primers declines	Try to avoid the difference between batches of newly synthesized primers, and use the original primers with good quality as the control.
Inappropriate PCR reaction conditions, primer concentration, sequence, etc.	PCR with poor amplification efficiency is easy to produce with poor reproducibility. Adjust the PCR reaction conditions by changing the concentration of primers. If the amplification efficiency is poor, it can be optimized by lower the annealing temperature, increase the primer concentration, or prolong the extension time. If the GC content of the template is high, the denaturation time can be prolonged. It is suggested to redesign the primer when the improvement is still not achieved.
Measurement error	If the reaction volume is too small, the detection accuracy will decrease. Please repeat the experiment according to the reaction volume recommended by the quantitative PCR instrument.