TIANGEN®

SuperReal PreMix Color (Probe)

Stable and efficient probe-based fluorescence quantitative reagent

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SuperReal PreMix Color (Probe)

Cat. No. 4992883/4992884

Kit Contents

Contents	4992883 20 μl×125 rxn	4992884 20 μl×500 rxn
2× SuperReal Color PreMix (Probe)	1.25 ml	4×1.25 ml
50× ROX Reference Dye	250 μl	1 ml
40× Dilution Buffer (yellow)	1.25 ml	1.25 ml
RNase-Free ddH_2O	2×1 ml	5×1 ml
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Storage

The product can be stored at -30~-15°C for one year. Please store the product at -30~-15°C immediately after receiving it. When taking out from -30~-15°C for use, dissolve the frozen 2× SuperReal Color PreMix and 50× ROX Reference Dye, then gently mix by inverting up and down 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 probe method. It is in the form of PreMix with a concentration of 2× and the preparation of reaction solution is very simple and convenient. 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.

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 product is optimized to achieve the optimum effect of fluorescence signal release, and at the same time fully degrades the signal release of probe fluorescence quenching group, and stronger signals will be obtained with the same amount of templates.
3. SuperReal Color PreMix is in the form of 2× concentration PreMix. 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 error of fluorescence signal generated between holes, and is convenient for customers to select corresponding concentrations 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 adopts a unique two-component HotStart DNA polymerase for PCR amplification, and achieves the purpose of detecting the amplification amount of PCR products by adding a fluorescent probe into the PCR reaction solution and then detecting the fluorescence intensity 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.

The optimized product is especially beneficial for *Taq* polymerase to exert its 5'-3' exonuclease activity, so as to achieve the best effect of fluorescence signal release. In addition, SuperReal Color PreMix also fully degrades the signal release of the probe fluorescence quenching group. After the above optimization, a stronger signal will be obtained with the same amount of templates.

Important Notes Before Starting

1. The initial denaturation condition for PCR reaction must be set at 95°C for 15 min to fully activate the HotStart enzyme.
2. The final concentration of each color indicator in PCR reaction solution should be 1×. Please calculate the dosage of Dilution Buffer according to the amount of template added.
3. 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.
4. This product does not contain fluorescent probes, etc.
5. Good amplification results can be obtained in most systems with the final concentration of primer 300 nM and the final concentration of probe 200 nM.
6. If the concentration of primer needs to be further optimized, it can be adjusted within the range of 50-900 nM. If the probe concentration needs to be further optimized, it can be adjusted in the range of 100-500 nM.

Protocol

<1> Establish Real-Time PCR reaction system:

1. Thaw 2× SuperReal Color PreMix (if stored at -30~-15°C), 50× ROX Reference Dye, templates, primers and RNase-Free ddH_2O, and balance all reagents at room temperature and thoroughly mix.
2. It is suggested to prepare Real-Time PCR reaction solution on ice.
   The 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, the system should be blue. If the colors do not match, please check whether the relevant components are correctly added into the system.

Reaction system:

Component	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	300 nM[*1]
Reverse Primer (10 μM)	1.5 μl	0.75 μl	0.6 μl	300 nM[*1]
Probe (10 μM)	1.0 μl	0.5 μl	0.4 μl	200 nM[*2]
DNA template (with Dilution Buffer) ▲	-	-	-	≤200 ng
50×ROX Reference Dye[*3]	-	-	-	-
RNase-Free ddH_2O	To 50 μl	To 25 μl	To 20 μl	-

[*1] 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 50-900 nM.

[*2] The concentration of the probe is related to the Real-Time PCR instument, the type of probe, and the type of fluorescent labeling substance used. Please refer to the instrument instructions or the specific use instructions of each fluorescent probe. Generally, a probe with a final concentration of 200 nM can obtain good amplification results in most systems. If the probe concentration needs to be further optimized, it can be adjusted in the range of 100-500 nM.

[*3] 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/Step One, etc.	2.5× (e.g. 2.5 μl ROX /50 μl system)
ABI 7500, 7500 Fast; Stratagene Mx3000P, Mx3005P and Mx4000, etc.	0.5× (e.g. 0.5 μl ROX /50 μl system)
Roche, Bio-Rad, Eppendorf instruments, etc.	No need

▲ 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 volume in 20 μl Reaction System:

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

<2> Carry out Real-time PCR reaction

It is suggested to adopt a two-step PCR reaction procedure for the reaction. The denaturation time can be adjusted in the range of 1-3 sec, and the annealing/extension time can be adjusted in the range of 20-32 sec.

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	3 sec[*4]	Denaturation	No
		60°C	20-32 sec[*5]	Annealing/ extension	Yes

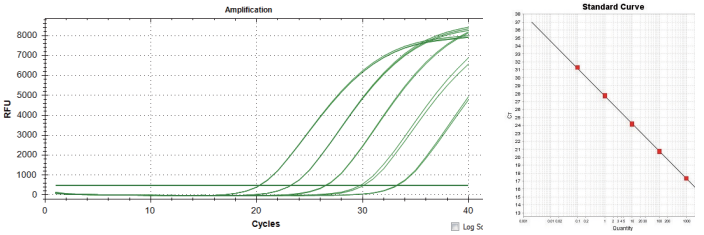
[*4] For time-setting on different types of instruments, please carry out the experimental operation according to the requirements of the instrument's operating instructions. Set to 1 sec when using ABI 7900HT/7900HT Fast/ViiA 7/StepOne/StepOnePlus.

*5 For time-setting on different types of instruments, please carry out the experimental operation according to the requirements of the instrument's 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. Centrifuge briefly to ensure that all components are at the bottom of the tube.

Reaction Example (Applied Biosystems 7500 Fast Real-time PCR System)



Human whole blood RNA was extracted and GAPDH was detected after reverse transcription using SuperReal PreMix Color (Probe) (4992883/4992884). FastKing RT Kit (With gDNase) (4992223/4992224/4992250) was used for reverse transcription, with cDNA amount equivalent to Total RNA 1 pg-10 ng. Negative Control: The negative control was set using ddH_2O as the template. The amplification curves (left) and the standard curves (right) show good amplification efficiency and sensitivity.

Primer design instructions

The design of PCR primers is very important in Real-time PCR. The design of 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 100-150 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.

Probe design instructions

Please prepare a fluorescent probe suitable for the target gene sequence. For the design of probe sequence, please refer to the design guide of each probe. In addition, please use purified probe above HPLC level, otherwise the residual unbound fluorescent dye will increase the fluorescent baseline and reduce the detection sensitivity.

Operation of RT-PCR 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). When used in combination with this product, highly reliable PCR reaction results can be obtained.

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 Mix according to the genomic DNA removal system shown in Table 1 and thoroughly mix it. Centrifuge briefly and incubate at 42°C for 3 min. Then place it on ice.

Table 1 gDNA Removal Reaction System

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

3. Prepare the mixture according to the reverse transcription reaction system in Table 2.

Table 2 Reverse Transcription Reaction System

Reagent	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

4. Add the reverse transcription reaction mastermix to the reaction solution in the gDNA removal step and fully mix the solution.
5. The reverse transcription reaction conditions are as follows:

   Incubation at 42°C for 15 min.

   Incubate for 3 min at 95°C.

6. Set up PCR reaction.

Trouble Shooting

1. For low concentration template, the amplification curve is chaotic and the fluorescence intensity becomes weak.

Possible reasons	Suggestions
Too few copies of target DNA	When the copy number of the target DNA in the reaction solution is only a few times to several tens of times, the probability of scattered copy number becomes larger and linear chaos is easy to form. Please raise the sample concentration appropriately before the reaction.
Affected by primer dimers	The amplification of the target fragment is accompanied by the amplification of the primer dimer, which weakens the amplification reaction of the target fragment due to competition. Please explore the optimized reaction conditions or redesign the primer to prevent the formation of primer dimer.
DNA is attached on the reaction centrifuge tube, and the reaction template amount is less.	When the template concentration is low or the sample is stored for a long time after dilution, DNA will be attached on the reaction centrifuge tube which will cause template lost. Please increase the sample concentration before reaction. If the sample is diluted, it is recommended to use immediately after dilution.

2. NTC has high fluorescence value

Possible reasons	Suggestions
Cross contamination occurs	Please replace with new reagent or sterilized water. If there's no improvement, please try to operate in a new experimental environment.
Instrument specification error (when performing multiplex PCR, etc.)	When using several kinds of fluorescent probes, please set up fluorescence measurement correctly to prevent the difference of detection signals caused by the spectrum crossing of different dyes.

3. 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	When the DNA solution with lower concentration is stored for a long time, the actual concentration will be lower because it is attacjed on the tube wall. It is recommended that the reaction be carried out after diluting the stock solution again. In addition, the standard sample diluted by gradient is preferably diluted directly from the stock solution each time it is used.
The quality of primer or probe 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 tends to produce poor reproducibility. Adjust by changing the concentrations of primers and probes or PCR reaction conditions. 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 re-experiment according to the reaction volume recommended by the quantitative PCR instrument.

4. Amplification efficiency is less than 90% (slope<-3.6)

Possible reasons	Suggestions
Primers are of poor quality	When the primer quality is not good, the amplification efficiency will be greatly reduced. Dilute the primer again from the primer stock solution or synthesize again.
Ct values deviating from straight lines are not excluded when calculating amplification efficiency	The calculation of amplification efficiency includes Ct value deviating from straight line, which increases the error of calculation value. Ct value deviating from straight line should be excluded before calculation.
The reaction conditions are not suitable	Please optimize primer, probe concentration and PCR reaction conditions.

5. The amplification efficiency is higher than 110% (slope>-3.1)

Possible reasons	Suggestions
Ct values deviating from straight lines are not excluded when calculating amplification efficiency	The calculation of amplification efficiency includes Ct value deviating from straight line, which increases the error of calculation value. Ct value deviating from straight line should be excluded before calculation.
Impurities in the sample obviously inhibit high-concentration templates	When the sample contains impurities, the template with high concentration is obviously inhibited, thus decreasing the amplification efficiency. Please reduce the sample concentration or further purify the template.

6. The fluorescence signal of the amplification curve is very weak or the amplification curve is serrated.

Possible reasons	Suggestions
Specification error of detection light spectrum	Due to the difference in the principle of different fluorescence quantitative PCR instruments and the spectral range of detection light provided at present, it is necessary to select the luminescent group and quenching group of the probe according to the detectable fluorescence signal range set by the instrument model. Please refer to the operation instructions of the instrument to re-determine the parameter settings.
Low purity of fluorescence probe	Please use purified Probe above HPLC grade, otherwise the residual unbound fluorescent dye will increase the fluorescent baseline, resulting in a lower fluorescence value generated by the amplification product.
The fluorescent probe is of poor quality	Due to decomposition during probe storage, the baseline increases, resulting in a lower fluorescence value generated by amplification products.In addition, some fluorescent dyes are not suitable for buffer containing EDTA for storage. Please follow the storage conditions recommended by Probe Synthesis Company.
Fluorescence acquisition time is too short	For some instruments, a longer extension time is needed to fully collect fluorescence. When the serration of the amplification curve is obvious, setting the extension time to 45-60 sec can improve this phenomenon.