TIANGEN®

FastFire qPCR PreMix (Probe)

For fast, quantitative, specific real-time PCR using sequence-specific probe

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FastFire qPCR PreMix (Probe)

Cat. no. GFP208

Kit Contents

Contents	GFP208-02 20 μl×500 rxn
2× FastFire qPCR PreMix (Probe)	4×1.25 ml
50× ROX Reference Dye	1 ml
RNase-Free ddH_2O	5×1 ml
Handbook	1

Storage

FastFire qPCR PreMix (Probe) can be stored at -30~-15°C for one year. It should be stored immediately upon receipt at -30~-15°C. 2× FastFire qPCR PreMix (Probe) and 50× ROX Reference Dye should be thawed and then mixed upside down gently to be homogenous before using. If the Reagents have been thawed but 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 decrease product performance.) The reagents could be stored for up to 3 months at 2-8°C if frequently used. Please avoid refreezing and thawing repeatedly.

Introduction

FastFire qPCR PreMix (Probe) is designed for probe based quantitative PCR assays, enables fast and specific quantitative results with total running time 60% reduced compared with regular real-time PCR.

FastFire qPCR PreMix is ready to use with premix including hot-start DNA Polymerase and unique PCR buffer. It could ensure a sensitive PCR detection on any Real-Time PCR thermal cycler.

Kit features

1. FastFire qPCR PreMix employs a specific antibody-modified hot-start DNA polymerase in conjunction with a carefully optimized Buffer system, resulting in a 60% shorter PCR reaction time than typical qPCRs and featuring accurate quantitation, high amplification efficiency, reproducibility, and a wide confidence range.
2. This product has been optimized to achieve excellent fluorescence signal release, while fully degrading the signal release from the fluorescence quenching groups of the probe, and a stronger signal will be obtained with the same amount of template.
3. FastFire qPCR PreMix is in the form of 2× pre-mix. When preparing the PCR reaction solution, users only need to add templates, primers, probe, and ddH_2O to carry out the Real-Time PCR reaction, which is easy and convenient to operate.
4. This product comes with ROX Reference Dye, which is used to eliminate the signal background as well as calibration of fluorescence signal error generated between wells, making it convenient for customers to choose the corresponding concentration for different models of fluorescence quantitative PCR instruments.

Principle of the kit

This product employs a specific antibody-modified hot-start DNA polymerase for PCR amplification. The purpose of detecting PCR product amplification is achieved by adding a fluorescent probe to the PCR reaction solution and then detecting the fluorescence intensity during the reaction process.

1. The specific antibody modified hot start DNA polymerase in this product can activate all the enzyme activities by incubating at 95°C for 1 min. Meanwhile, together with the carefully optimized Buffer system, it can greatly shorten the denaturation, annealing and extension time, so as to shorten the total running time of PCR by 60%, obtain the results of the experiment faster without affecting the effect of the PCR reaction, and it has the characteristics of accurate quantitation, high amplification efficiency, good reproducibility and wide confidence range.
2. This product is specially optimized for the differences in the structural composition of cDNA templates and gDNA templates, and the PCR reaction steps are optimized so that good PCR results can be obtained even for the more difficult-to-amplify gDNA templates.
3. This product has been optimized to facilitate the 5'-3' exonuclease activity of Taq enzyme, so that the release of fluorescent signals can achieve excellent results. In addition the FastFire qPCR PreMix system also sufficiently degrades the signal release from the fluorescence quenching moiety of the probe, and with the above optimization, a stronger signal will be obtained with the same amount of template.

Important Notes

1. The hot start DNA polymerase is recommended to be activated at the start of reaction: for cDNA template by a 30-60 sec, 95°C incubation step; for gDNA template by 98°C, 1-2 min.
2. The performance will decrease if the reagents are not mixed thoroughly. Gently mix the reagents by inverting the tubes and centrifuge briefly prior to use. DO NOT vortex and avoid producing bubble.
3. Fluorescence probe is not included in this kit.
4. 0.2 μM concentration of probe, 0.3 μM concentration of primer could achieve excellent amplification result for most systems. If need further optimization, primer concentration could be adjusted between 0.05-0.9 μM, and probe concentration between 0.1-0.5 μM.
5. In a 20 μl reaction volume, the amounts of genome DNA or cDNA template is usually less than 100 ng. The reverse transcription product, if used as template, should not comprise more than 20% of the total PCR reaction volume.

Protocol

<1> Set up the Real-Time reaction

1. Thaw FastFire qPCR PreMix (if stored at -30~-15°C), ROX Reference Dye, template, primers, probe and RNase-Free ddH_2O. Completely mix and equilibrate all the 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×FastFire qPCR 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[*1]
Reverse Primer (10 µM)	1.5 µl	0.75 µl	0.6 µl	0.3 µM[*1]
fluorescence probe (10 µM)	1.0 µl	0.5 µl	0.4 µl	0.2 µM[*2]
DNA template	-	-	-	≤200 ng
50× ROX Reference Dye[*3]	-	-	-	-
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, the primer concentration should be decreased. For further optimization, a primer titration from 0.05 µM to 0.9 µM can be performed.

[*2] The optimal concentration of probe is related with Real-time PCR instruments, probe form and fluorescence dyes according to the information provided in handbook with the instruments or fluorescence probe. A final probe concentration of 0.2 µM is optimal for most applications. For further optimization, a probe titration from 0.1 µM to 0.5 µM can be performed.

[*3] The optimal concentration of ROX Reference Dye for commonly used Real-Time PCR instruments is as below:

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

<2> Real-Time Amplification

Typically, optimal results are obtained using a two-step PCR. Three-step PCR could be adopted because low copies of template, etc. cause the low amplification efficiency.

Two-step PCR procedure

Stage	Cycle	Temperature	Time	Step	Signal Collection
Initial denaturation	1×	95°C △1	1 min	Initial denaturation	N
PCR	40×	95°C △1	5 sec	Denaturation	N
		60°C △2	15 sec △3	Annealing/ Extension	Y

Three-step PCR procedure

Stage	Cycle	Temperature	Time	Step	Signal Collection
Initial denaturation	1×	95°C △1	1 min	Initial denaturation	N
PCR	40×	95°C △1	5 sec	denaturation	N
		50-60°C △4	10 sec	Annealing	N
		72°C	15 sec △3	Extension	Y

△1 the initial denaturation temperature is 95°C for cDNA template and 98°C for gDNA.

△2 60°C, 15 sec is optimal for most application. If for further optimization, please try 56-66°C.

△3 Set time should be referred to the manual of specific instrument. The optimal annealing and extended time for commonly used Real-Time PCR instruments is as below:

Roche/ABI 7500 Fast/BioRad/Agilent: 15 sec.
ABI 7900HT/7900HT Fast/ViiA 7/StepOne/StepOnePlus: 20 sec.
ABI 7000/7300: 31 sec.
ABI 7500: 32 sec.

△ [4] Annealing temperature of primers is usually 5°C lower than Melting temperature (Tm). The annealing temperature could be increased properly if the base number is low, which could increase the specification. On the contrary, the annealing temperature could be decreased if the base number is high.

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.

Suggestions for operating when performing RT-qPCR reactions

Three cDNA first-strand synthesis kits are available for performing RT-PCR reactions: the FastKing RT Kit (with gDNase) (GKR116), FastKing gDNA Dispelling RT SuperMix (GKR118), and the TIANScript II cDNA First-Strand Synthesis Kit (GKR107).

FastKing RT Kit (with gDNase) (GKR116) removes the residue of genomic DNA in 3 min, which makes the results of gene quantification more real and credible. It is suitable for rapid reverse transcription of total RNA with template amount of 50 ng-2 μg (it takes a total of 21 min), and it is an excellent choice for reverse transcription experiments of fluorescence quantitative PCR.

1. The template RNA is thawed on ice; 5× gDNA Buffer, FQ-RT Primer Mix, 10× King RT Buffer, and RNase-Free ddH2O are thawed at room temperature and placed on ice quickly after thawing. Vortex and shake each solution before use and centrifuge briefly to collect any liquid remaining on the walls of the tubes.

   Perform the following procedure on ice. In order to ensure the accuracy of the reaction solution preparation, each reaction should be carried out by first preparing the Mix and then dispensing it into each reaction tube.
2. Prepare the mixture according to the removal system for genomic DNA in Table 1 and mix thoroughly. Centrifuge briefly and place at 42°C and incubate for 3 min. Then place it on ice.

Table 1 gDNA removal reaction system

Components	Dosage
5×gDNA Buffer	2 μl
Total RNA	-
RNase-Free ddH_2O	Make up to 10 μl

3. The mixture is prepared according to the reverse transcription reaction system in Table 2.

Table 2 Reverse transcription reaction system

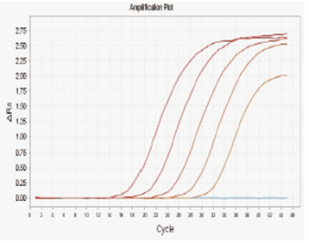
Reagent	Dosage
10×KingRT Buffer	2 μl
FastKingRT Enzyme Mix	1 μl
FQ-RT Primer Mix	2 μl
RNase-Free ddH_2O	Make up to 10 μl

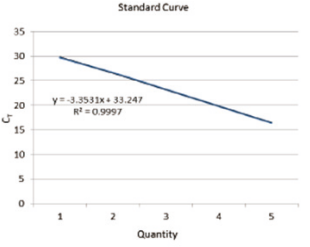
4. Mix from the reverse transcription reaction, add to the reaction solution from the gDNA removal step and mix well.
5. 42°C and incubated for 15 min for reverse transcription.
6. 95°C, and put on ice after incubation for 3 min. The cDNA obtained can be used for subsequent experiments, or cryopreserved.

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

Human GAPDH mRNA is detected by Real-Time RT-PCR.

Using FastFire qPCR PreMix, the amount of cDNA is equivalent to Total RNA 1 pg-10 ng. Negative Control uses ddH_2O as a template.





Description of primer design

The design of PCR primer is very important when performing Real-time PCR reactions. The design of primer with high PCR amplification efficiency and reaction specificity can be referred to the following requirements.

The requirements for designing primer are as follows:

Length of primer	18-30 bases
GC content	40-60%
Tm value	The primer software can all give Tm, which is related to primer length, base composition, and the ionic strength of the buffer used for the primer as well. The Tm values of the upstream and downstream primer should be as close as possible. The simple Tm formula is: Tm = 4°C (G + C) + 2°C (A + T). Generally 5°C lower than the primer Tm value is used as the PCR annealing temperature. Increasing the annealing temperature increases the specificity of the PCR reaction.
Primer and PCR amplification product sequences	The appropriate length of PCR amplification products is between 100-150 bp. Try to avoid designing primer in the secondary structure region of the template. Avoid formation of 2 or more complementary bases between the 3’ ends of upstream and downstream primer to minimize primer dimer formation. The base at the 3' end of the primer cannot have more than 3 consecutive G's or C's. There should be no complementary sequences in the primer itself, otherwise the primer itself will fold into a hairpin-like structure. Avoid primer with T at the 3' end base. A, T, G, and C should be distributed as evenly as possible in the primer sequence.

Description of probe design

Please prepare a fluorescent probe suitable for the target gene sequence. For the design of Probe sequences, please refer to the design guide for each Probe.

In addition, please use Probe purified at HPLC grade or above whenever possible, otherwise residual unbound fluorescent dyes will cause the baseline to drift upwards thus reducing the sensitivity of the assay.

Trouble shooting

1. Chaos of amplification curve and weak fluorescence signal due to low concentration of template.

Comments	Suggestions
Insufficient amount of starting template	Increase the amount of template, if possible. Ensure that sufficient copies of the target nucleic acids are present in your sample.
Competition with primer dimer	Amplification of target DNA will be weakened by amplification of primer dimer. Optimize the PCR thermal cycling or redesign the primers to prevent the formation of primer dimers.
Template loss by adsorption of DNA to the reaction tube	Long-term storage of low concentration of template or diluted template will cause template loss by adsorption to the tube. Please raise the template concentration. PCR reaction should be performed immediately after the sample is diluted.

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

Comments	Suggestions
Cross contamination	Discard all the components of the assay (e.g., master mix, primers, and probes). Repeat the assay using new components.
PCR instruments configuration error	When using several fluorescence probes together, ensure that the correct detection channel is activated or the correct filter set is chosen for the reporter dye.

3. Poor repeatability of CT value

Comments	Suggestions
Instrument malfunction	Thermo cycling is not suitable or performs in improper temperature or has poor repeatability. Operate and checkup the Real-Time PCR instrument according to the instruction manual.
Contamination of templates	Contamination of templates may lead to the poor linearity.
Long-term stored of diluted template	DNA concentration will decrease due to adsorption to the tube for long term storage. Make new serial dilutions of template from the stock solutions. Repeat the PCR using the newly diluted template immediately.
Poor quality of primers and probes	The batch difference of primers should be avoided. Good quality of primers previously synthesized could be used as the control.
Unsuitable PCR programs, primer concentration or primer sequence	Poor amplification efficiency will cause poor repeatability. Please use optimal primer and probe concentrations and modify the PCR thermal cycling according to the information provided in this handbook. Generally, lower annealing temperature, higher concentration of primers, prolongation of the extension time will help. For high GC content template, please prolong the denature time. If still no improvement, redesign the primers and probes.
Metering inaccuracies	Too small volume of reaction will decrease the detection accuracy. Use the recommended volume in manual and repeat the PCR amplification.

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

Comments	Suggestions
Poor quality of primers	Poor quality of primers will decrease the amplification efficiency largely. Make new dilution of primer from the stock solution or re-synthesize the primers.
Non-exclusion of deviation CT	The deviation CT will increase the calculation error. Please Exclude the deviating CT value when calculating the amplification efficiency
Incorrect reaction setup	Optimize the concentration of primers, probes and PCR reaction condition.

5. Amplification efficiency over 110% (slope>-3.1)

Comments	Suggestions
Non-exclusion of deviation CT	The deviation CT will increase the calculation error. Please Exclude the deviating CT value when calculating the amplification efficiency
Impurities in sample	Impurities in sample inhibit the amplification of high concentration of template largely and therefore increase the amplification efficiency. Please lower the concentration of sample or purify the template further.

6. Poor fluorescence signal or zigzag amplification curve

Comments	Suggestions
Incorrect detection filter/channel	The detection spectroscopy differs with different qPCR instruments. Please select the luminophore and quencher according to different instrument models. Please refer to the instruction manual and reset the parameters.
Low fluorescence probe purity	Please select the probe with purity above HPLC grade, otherwise the remaining unbinded fluorescence dye will cause high background signal and affect the sensitivity of detection.
Poor quality of fluorescence probe	Degradation of probe will raise the baseline and the fluorescence signals of amplificationproducts at the same time. Besides, some fluorescence dyes are not suitable for storagein the buffer contains EDTA. Please use the condition recommended by probes synthesizer.
The fluorescence signals collection time is too short	For some instruments, collection time should be prolonged to collect enough fluorescence signals. Please set 45-60 sec for extension time to improve the zigzag amplification curve.