TIANGEN®

FastFire qPCR PreMix (SYBR Green)

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

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FastFire qPCR PreMix (SYBR Green)

Cat. no. 4992217/4992218/4992249

Kit Contents

Contents	4992217 20 μl × 125 rxn	4992218 20 μl × 500 rxn	4992249 20 μl × 5000 rxn
2 × FastFire qPCR PreMix (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

FastFire qPCR PreMix (SYBR Green) 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 (SYBR Green I) 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 (SYBR Green) is designed for SYBR Green I based quantitative PCR assays, enables fast and specific quantitative results. Optimized premix could reduce the running time and is suitable for regular and fast real-time PCR thermal cycler.

FastFire qPCR PreMix adopts antibody modified Anti Taq DNA polymerase. Combined with the unique PCR buffer, it could ensure a sensitive PCR detection on any Real-Time PCR thermal cycler. Total running time could be reduced by 60% compared with regular real-time PCR program. Meantime accurate quantification, high amplification efficiency, high specificity and wide credibility range could be achieved.

Important Notes

1. FastFire qPCR PreMix (SYBR Green) contains SYBR Green I. Store the reagent in dark and avoid direct exposure to strong light during the preparation of PCR reaction mixtures.
2. Gently mix the reagents by inverting the tubes and centrifuge briefly prior to use. Do NOT vortex and avoid producing bubble.
3. The purity of primers is important for the specificity of PCR. Primers purified by PAGE or more superior methods are recommended.
4. 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 titer from 0.2 μM to 0.5 μM can be performed.
5. In a 20 μl reaction volume, the amount of cDNA template is usually less than 100 ng, and genomic DNA is less than 50 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

Note: FastFire qPCR PreMix and 50× ROX Reference Dye should be stored protected from light.

1. Thaw FastFire qPCR PreMix (if stored at -30~-15°C), ROX Reference Dye, template, primers, and RNase-Free ddH_2O. Completely mix and equilibrate all the reagents to room temperature before use.
2. Prepare 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[*]
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$_2$O	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, the primer concentration should be decreased. 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 thermal cycler:

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/Mx4000 etc.	1× (e.g. 1 μl ROX/ 50 μl volume)
Instruments of Roche, Bio-Rad and Eppendorf etc.	No need

<2> Real-Time PCR

Typically, optimal results are obtained using a two-step PCR. Three-step PCR could be considered if low amplification efficiency due to low copies of template is observed.

Two-step PCR procedure

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

Three-step PCR procedure

Stage	Cycle	Temperature	Time	Step	Signal Collection
Initial denaturation	1×	95°C	1 min	Initial denaturation	N
PCR	40×	95°C	5 sec	Denaturation	N
		50-60°C △[3]	10 sec	Annealing	N
		72°C	15 sec △[2]	Extension	Y
Melting/Dissociation Curve Stage					

△[1] 60°C for 15 sec is optimal for most application. For further optimization, please try 56-66°C.

△[2] Set time according to different instruments requirement. The optimal annealing and extension time for commonly used Real-Time PCR instruments is as below:

ABI 7700/7900HT/7500 Fast, Roche, BioRad and Agilent etc: 15 sec
ABI 7000/7300: 31 sec
ABI 7500: 32 sec

△[3] Annealing temperature of primers is usually 5°C lower than its melting temperature (Tm). The annealing temperature could be increased properly if the base number is low, which could increase the specificity. 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.

<3>Take ABI 7500 Fast Real Time PCR system for an example

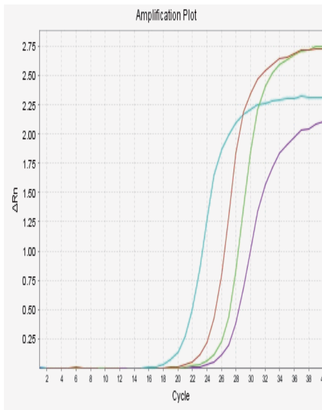

Figure1: Amplification Curve

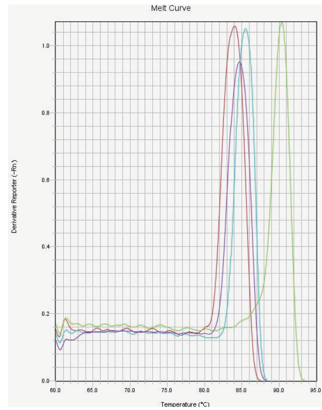

Figure2: Melting Curve

Figure 1: the result from four different PCR primers with FastFire qPCR PreMix single strand cDNA is used as template.

By the analysis of melting curve in figure 2, four separate peaks could be obtained with non-specific amplification and no primer dimer formed. cDNA used as the template is synthesized with FastQuant cDNA first strand systhesis kit.

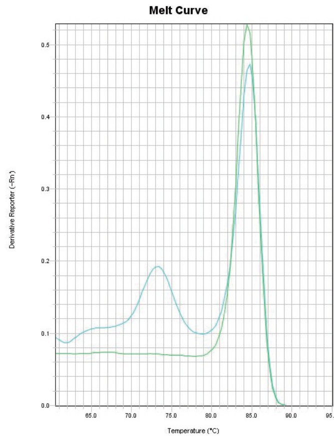

Figure 3: Analysis of Melting Curve

One single specific peak could be observed with FastFire qPCR PreMix in Figure 3 with the Tm value of 83.5°C. By comparison, primer dimer could be obtained besides specific amplification products with the reagent from company A with a Tm of about 75°C.

Trouble shooting

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 3.5 mM in FastFire qPCR PreMix. 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 3.5 mM in FastFire qPCR PreMix. 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.