2× SYBR Green qPCR Master Mix (ROX1 plus)

Catalog No.: A0001-R1

Description

The *EZ*Bioscience® 2× SYBR Green qPCR Master Mix (ROX1 plus) uses a specially modified *Taq* DNA polymerase protected by a hot-start activation technique, and optimized qPCR buffer system to perform SYBR Green I based Real-time quantitative PCR (qPCR). The Mix is prepared at 2× reaction concentration, and can be directly used on both high and low-template qPCR with high sensitivity, specificity and reliability. Besides, the relatively *high concentration of ROX (ROX1) is premixed in the mixture* to make it an excellent ready to use, one-tube qPCR reagent.

Components

Components	A0001-R1	A0001-R1-L
2× SYBR Green qPCR Master Mix*	5 ml	25 ml

*: Contain hot-start DNA Polymerase, dNTPs, Mg^{2+},buffer, SYBR Green I dye, and premixed with ROX1 passive reference dye, to rectify the error of fluorescence signals between different wells.

Storage

All components should be stored at -20°C and avoid of light. The quality of this product is guaranteed for 18 months under recommended storage conditions.

Notice

Appropriate Real-time PCR instruments for this Kit are as follows:

ABI 5700, 7000, 7300, 7700, 7900, 7900HT, 7900HT Fast; StepOne™, StepOne Plus™
Bio-Rad CFX96™, CFX384™, iCycler iQ™, iQ™5, MyiQ™, MiniOpticon™, Opticon®, Opticon 2, Chromo4™; Cepheid SmartCycler®; Roche LightCyclerTM 480; Eppendorf Mastercycler® ep realplex, realplex 2 s; Illumina Eco qPCR; Qiagen/Corbett Rotor-Gene® Q, Rotor-Gene® 3000, Rotor-Gene® 6000; Thermo Scientific PikoReal Cycler.

Protocol (Suggestions for qPCR Amplification)

1. The suggested template amount is 10 ~ 100 ng for genomic DNA or 1 ~ 10 ng for cDNA template. The following loading table is designed for reaction volume of both 50 µl and 20 µl:

Components	Amount/ reaction(µl)	Amount/ reaction(µl)	Final concentration
Total Reaction Volume	50	20	-
2× SYBR Green qPCR Master Mix	25	10	1×
Template	2.5 ~ 10	1 ~ 4	1 ~ 100 ng
Forward Primer (10 µM)	1	0.4	125 nM
Reverse Primer (10 µM)	1	0.4	125 nM
ddH_2O	Add to 50	Add to 20	-

2. Standard 2-step amplification program:

Step	1	2	
	Hot-Start DNA Polymerase Activation	PCR	
	Hold	CYCLE (40 cycles)	
		Denature	Anneal/ Extend
Temp	95°C	95°C	60°C
Time	5 min	10 sec	30 sec
Volume	20 µl/ 50 µl		

Melt Curve could be set following the recommendation of the equipment. A representative program for Melting Curve could be set up as follows:

Step	1	2	3
Heating/ Cooling rate	100%	100%	1%
Temp	95°C	60°C	95°C
Time	15 sec	1 min	30 sec
Data Collection	-	-	During temperature rising stage

Important: The 95°C for 5 mins step is required to activate the Hot-Start DNA Polymerase.

Data Analysis

Two types of quantification methods are commonly applied to quantify target gene expression when using this SYBR Green qPCR Master Mix.

1. Relative Quantitation: target gene expression is measured against an internal standard, such as GAPDH or Actin-b.

Gene expression can be measured by the quantitation of cDNA converted from a messenger RNA corresponding to this gene relative to a calibrator sample serving as a physiological reference. In a typical experiment, gene expression levels are studied as a function of either a treatment of cells in culture, of patients, or of tissue type. The calibrator sample in each case is the cDNA from either the untreated cells or patients, or a specific tissue type. All quantitations are also normalized to an endogenous control such as 18S rRNA to account for variability in the initial concentration and quality of the total RNA and in the conversion efficiency of the reverse transcription reaction.

Set Ct^{A1} as the Ct value of the target gene of sample 1, and Ct^{B1} as the Ct value of the internal control gene of sample 1; set Ct^{A2} as the Ct value of the target gene of sample 2, and Ct^{B2} as the Ct value of the internal control gene of sample 2. The expression difference (in folder) of the target gene in sample 1 and in sample 2 can be calculated this way($2^{-\Delta\Delta Ct}$ approach):

$$\Delta\Delta Ct=(Ct^{A2}-Ct^{B2})-(Ct^{A1}-Ct^{B1})$$

The expression level of the target gene in sample 2 is $2^{-\Delta\Delta Ct}$ times that of sample 1.

Note: This calculation method is based on the assumption that the amplification efficiency is 100%(the amount of products after each cycle is doubled). If the amplification efficiency is not 100%, the calculation formula needs to be amended according to actual reaction efficiency. For example: if the amplification efficiency of the target gene and internal control genes is 1.90, then the formula should be amended into $1.90^{-\Delta\Delta Ct}$.

2. Absolute quantitation: compares the Ct of an unknown sample against a standard curve with known copy numbers.

Absolute quantitation is applicable only if isolation procedure and sample contents do not effect PCR amplification. The quantitation of genomic DNA may lend itself for absolute quantitation against a standard curve.

Determination of baseline and threshold: please refer to the real-time PCR system software used to calculate baseline and threshold values for a detector or manually set up according to amplification curves.

Passive reference ROX: is a dye molecule included in the SYBR Green PCR Master Mix that does not participate in the PCR amplification. On applied Biosystems real-time PCR system, the passive reference provides an internal reference to which the SYBR Green/ dsDNA complex signal can be normalized during data analysis. Normalization is necessary to correct for well-to-well fluorescent fluctuation.

Representative Results

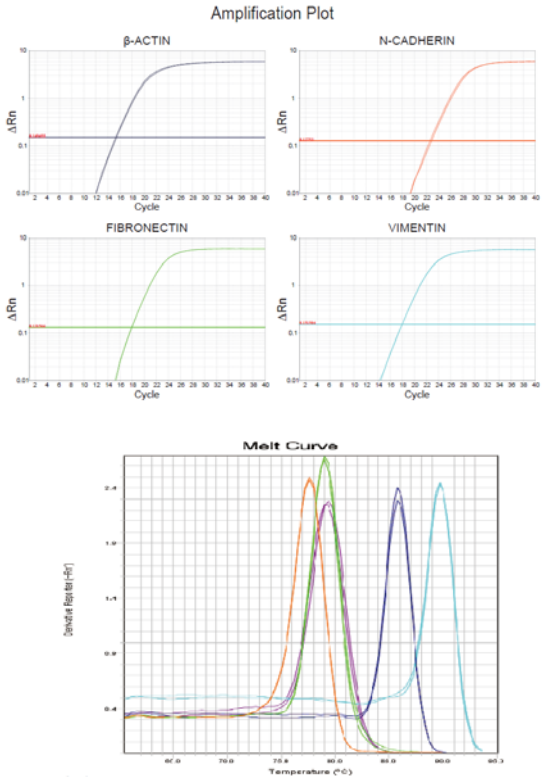

Figure 1. β-Actin, N-cadherin, Fibronectin and Vimentin mRNA expression level in cDNA from A549 cells were detected. The amplification plots and dissociation curve above indicate that, all of the four genes could be amplified efficiently and specifically. (Notes: Quantitation is fine when Ct value is within 15 ~ 32; if the Ct value is larger than 35, the amplification is invalid; If the Ct value is too small (<15), please dilute the template and repeat the quantitation; if the Ct value is within 32 ~ 35, 3 replicates are required to validate the results. A typical valid dissociation curve should be unimodal due to highly specific amplification and no primer dimer formation (Figure 1. below). If the dissociation curve is apparently multimodal, it often suggests primer dimer formation, non-specific amplification, or other contamination. *Alternatively, confirmation of non-specific amplification can be analyzed by agarose gel electrophoresis*).

Suggestions for Primer Design

Forward and reverse primers for target gene quantification should be unique or as specific as possible to avoid non-specific amplification. Primer sequence design and specificity check can be conducted using NCBI's "BLAST" (an open source tool and free), or other primer-design softwares, such as "Primer Bank" and "Primer 3". Additionally, the following general principals may be considered when designing primers:

1) The amplicon length is recommended to be within the range of 100 bp ~ 200 bp.

2) The optimal length of primers is 17 bp ~ 25 bp.

3) The 3' end of primers should avoid GC-rich or AT-rich areas. The last base at 3' end of the primers should be G or C and avoid T if possible.

4) GC content of primers should be within 40% ~ 60%.

5) The holistic distribution of A, G, C and T within primers should be as even as possible. Avoid using GC- or TA-rich areas. Avoid continuous structures of T/ C or A/ G.

Trouble Shooting

Problem	Potential Cause(s)	Suggestion(s)
Apparent amplification can be observed in negative control.	The reagents or water used is contaminated.	Change new reagents or water and retry. The reaction should be set up in a super clean bench to minimize contamination from the air.
	Appearance of primer dimer.	It is normal that amplification occurs in negative control after 35 cycles. Analysis can be performed combining with the melt curve.
Ct value appears too late(large).	Low amplification efficiency.	Optimize the reaction. Try three-step program or re-design the primers.
	The concentration of templates is too low.	Increase the amount of the template, meanwhile avoid destroying the amplification.
	Degradation of templates.	Prepare new templates and retry.
	The amplicon is too long.	The length of the amplicon is recommended to be within 100 bp ~ 200 bp.
	There are PCR inhibitors in the reaction.	Inhibitors are usually brought in when adding templates. Increase the dilution folds or prepare new templates and retry.
Abnormal shape of amplification plot.	Rough amplification plot.	It is caused by system rectification due to weak signal. Elevate the template concentration and repeat the reaction.
	Broken or downward amplification plot.	The concentration of templates is too high. The end value of the baseline is bigger than Ct value. Decrease the end of the baseline (Ct value - 4), and re-analyze the data.
	Amplification plot goes downward suddenly.	There are bubbles left in the reaction tube, which break up when the temperature rises, thus the instrument detects the sudden decrease of the fluorescence value. Spin briefly and check closely if there are bubbles left before reaction.
No amplification plot.	Cycling number is insufficient.	Generally the cycling number is set to be 40. But notice that too many cycles will result in excessive background, thus reducing the data reliability.
	Check if there is signal collection procedure during cycling.	In two-step program, signal collection is usually positioned at annealing and extension stage; for three-step program, signal collection should be positioned at 72°C extension stage.
	Check if the primers are degraded.	Test the integrity of primers after long-term storage through PAGE electrophoresis to confirm if the primers are degraded already.
	The concentration of templates is too low.	Reduce the dilution fold and retry. For target gene with unknown expression level, begin without template dilution.
	Degradation of templates.	Prepare new templates and retry.