2× Color SYBR Green qPCR Master Mix

Catalog No.: A0012

Description

The *EZ*Bioscience® 2× Color SYBR Green qPCR Master Mix 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). This Mix is supplemented with an inert red dye. And the *EZ*Bioscience® Color Reverse Transcription Kit (*Cat. No.: A0010C, A0010CG or A0010CGQ*) contains an inert blue dye to avoid of pipetting errors. Mixing cDNAs and other components together in a qPCR reaction turns the solution into purple. This provides a visual aid when pipetting and decreases the risk of pipetting errors during reaction setup, especially when using white reaction vessels. The dyes do not affect the specificity or sensitivity of qPCR reactions. 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.

Components

Components	A0012 (500 Rxns)
2× Color SYBR Green qPCR Master Mix*1	5 ml (1 ml × 5 tubes)
50× ROX Reference Dye 1*2	220 µl
50× ROX Reference Dye 2*2	220 µl

*1: Contains hot-start DNA Polymerase, dNTPs, Mg^{2+}, and SYBR Green I dye and red dye.

*2: Used 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

Please select an appropriate ROX according to the Real-time PCR instrument used:

Do Not Use ROX	Bio-Rad CFX96™, CFX384™, iCycler iQ™, iQ™5, MyiQ™, MiniOpticon™, Opticon®, Opticon 2, Chromo4™; Cepheid SmartCycler®; Eppendorf Mastercycler® ep realplex, realplex 2 s; Illumina Eco qPCR; Qiagen/Corbett Rotor-Gene® Q, Rotor-Gene® 3000, Rotor-Gene® 6000; Roche LightCycler™ 480; Thermo Scientific PikoReal Cycler.
Use ROX 1 (1×)	ABI 5700, 7000, 7300, 7700, 7900, 7900HT, 7900HT Fast; StepOne™, StepOne Plus™
Use ROX 2 (1×)	ABI 7500, 7500 Fast, Quant-Studio 3, 5, 6, 7, ViiA™7; Stratagene MX4000™, MX3000P™, MX3005P™

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 table is designed for reaction volume of both 10 µl and 20 µl. First, dilute the cDNA templates derived from the *EZ*Bioscience® Color Reverse Transcription Kit (*Cat. No.: A0010C, A0010CG or A0010CGQ*) for 5 times by ddH_2O and mix thoroughly, and then set up reaction systems as the following table:

Components	Amount/ reaction (µl)	Amount/ reaction (µl)	Final concentration
Total Reaction Volume	10	20	-
2× Color SYBR Green qPCR Master Mix	5	10	1×
Template (diluted by 5 times)	1	2	1 ~ 100 ng
Forward Primer (10 µM)	0.2	0.4	125 nM
Reverse Primer (10 µM)	0.2	0.4	125 nM
ROX Reference Dye	0.2	0.4	1×
ddH_2O	Add to 10	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 mins	10 secs	30 secs
Volume	50 µl/ 20 µl		

Melting Curve could be set following the recommendation of the equipment. A representative program for Melt 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 min 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 Color 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/patients, or a specific type of tissue. 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 Color 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

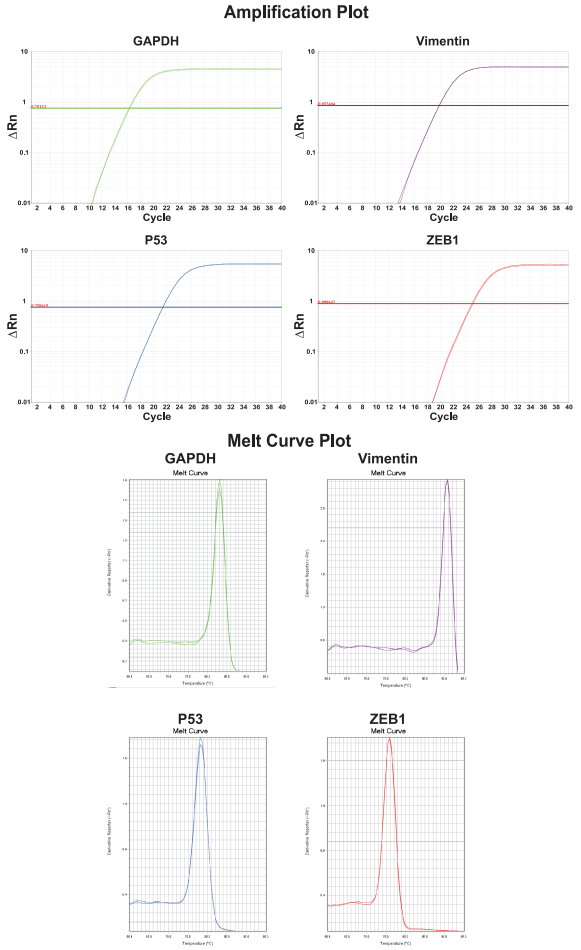

Figure 1. GAPDH, P53, Vimentin and ZEB1 mRNA expression level in cDNA from HEK293T cells were detected. The amplification plots and dissociation curve above indicate that, all of the four genes could be amplified efficiently and specifically. (Notes: Quantitation is fine when Ct value is within 15 ~ 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 nt ~ 25 nt.

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.