EZ-Probe qPCR Master Mix-UDG (No ROX)

Catalog No.: EZB-Probe-U0

Description

The *EZ*Bioscience® **EZ-Probe qPCR Master Mix-UDG (No ROX)** uses a specially modified *Taq* DNA polymerase protected by Hot Start technique, and optimized qPCR buffer system to perform probe based real-time quantitative PCR (qPCR). The Mix is supplied at 2× concentration, which allows accurate target quantification and detection over a broad dynamic range and makes it possible to conduct highly reproducible and reliable qPCR analyses. In addition, the Mix contains Uracil-DNA N-Glycosylase (UDG) which can prevent carryover contamination in PCR and ensure the accuracy of the results.

Components

Components	EZB-Probe-U0 (500 Rxns)	EZB-Probe-U0-L (2,500 Rxns)
2× EZ-Probe qPCR Master Mix-UDG (No ROX)*1	5 ml	25 ml

***1:** primers/probe is not supplied in this product; it contains UDG, dNTP blend containing dUTP/dTTP, Hot Start DNA Polymerase, optimized buffer.

Storage

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

Notice

Appropriate real-time PCR instruments for this kit are as follows:

Bio-Rad CFX96™, CFX384™, iCycler iQ™, iQ™5, MyiQ™, MiniOpticon™, Opticon®, Opticon 2, Chromo4™; **Roche** LightCycler™ 96, Roche LightCycler™ 480; **Eppendorf** Mastercycler® ep realplex, realplex 2s; **Illumina** Eco qPCR; **Qiagen/Corbett** Rotor-Gene® Q, Rotor-Gene® 3000, Rotor-Gene® 6000; **Thermo Scientific** PikoReal Cycler; **Analytikjena** qTOWER 3G; **Cepheid** SmartCycler®.

Protocol

1. Thaw the 2× EZ-Probe qPCR Master Mix-UDG (No ROX), DNA templates, probe and primers on ice, mix by inverting the tube up and down 10 times, centrifuge briefly, then store on ice.

2. Determine the number of reactions to be set up, and include 10% overage. Prepare the appropriate number of reactions by mixing 2× EZ-Probe qPCR Master Mix-UDG (No ROX), probe, forward primer and reverse primer into a reaction mix, and mixing cDNA or genomic DNA template and Nuclease free ddH_2O into a reaction mix. When cDNAs are used as templates, it is suggested to be diluted by 5 ~ 10 times. Assume that the cDNA is diluted by 5 times, the table below is designed of 10 µl and 20 µl reaction systems:

Components	Volume (10 µl /well)	Volume (20 µl /well)	Final concentration
2× EZ-Probe qPCR Master Mix-UDG (No ROX)	5 µl	10 µl	1×
Probe*1 (10 µM)	0.1 µl	0.2 µl	100 nM
Forward Primer (10 µM)	0.2 µl	0.4 µl	200 nM
Reverse Primer (10 µM)	0.2 µl	0.4 µl	200 nM
cDNA*2	1 µl (0.5 ~ 2 µl)	2 µl (1 ~ 4 µl)	1 ~ 10 ng
Nuclease free ddH_2O	Add to 10 µl	Add to 20 µl	-

*1: The probe (dual labeled probe, et. al.) should be protected from light, and the final concentration of the probe should be between 50 ~ 250 nM.

*2: When genomic DNA is used as template, it is suggested that the final concentration is 10 ~ 100 ng.

3. Mix each reaction mix thoroughly, then centrifuge briefly to spin down the contents.

4. Transfer the appropriate volume of each reaction mix to each PCR tube or well of an optical-grade PCR plate.

5. Seal the tubes or optical plate, and centrifuge at 1000 rpm for 1 minute to collect the contents of the wells at the bottom. The samples are ready for thermal cycling.

6. Set the thermal cycling conditions using the default PCR thermal cycling conditions specified in the following tables:

Step	1	2	3	
	UDG activation*1	Hot Start*2	PCR	
	Hold	Hold	Cycle (40 cycles)	
			Denature	Anneal/ Extend*3
Temp.	50°C	95°C	95°C	60°C
Time	2 min	5 min	10 s	30 s
Volume	10 µl/ 20 µl			

Note: Melting Curve step is not included in the probe detection program.

Important: *1: The purpose of this step is to prevent carryover contamination in PCR. ***2:** The Hot Start DNA Polymerase requires activation at 95°C for 5 minutes. ***3:** Collect fluorescence signal during the Annealing & Extension step.

Data Analysis

Two types of quantification methods are commonly applied to quantify target gene expression.

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 ($2^{-\triangle\triangle Ct}$ approach):

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

The expression level of the target gene in sample 2 is $2^{-\triangle\triangle Ct}$ 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 $1.90^{-\triangle\triangle Ct}$.

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 affect 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.*

Representative Results

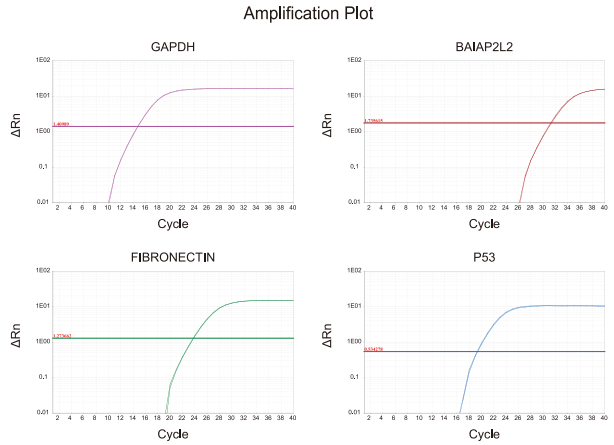

Figure1. GAPDH, BAIAP2L2, FIBRONECTIN and P53 mRNA expression level in cDNA from A549 cells were detected. The amplification plots above indicate that, all of the four genes could be amplified efficiently and specifically (**Notes:** Quantitation is fine when Ct value is within 13 ~ 32; if the Ct value is larger than 35, the amplification is invalid; If the Ct value is too small (＜13), please dilute the template and repeat the quantitation; if the Ct value is within 33 ~ 35, 3 replicates are required to validate the results).

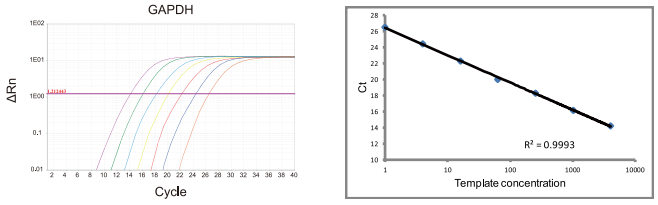

Figure2. GAPDH mRNA expression level in serially diluted cDNAs from A549 cells were detected. The amplification plots above indicate that, the target gene could be amplified efficiently and specifically. And the linear relationship within the detection range is very good.

Suggestions for Primer and Probe Design

For Probe **(it is recommended to design the probe first, and design the primer according to the probe)*:***

1) The probe length is generally 18 ~ 35 bp (18 ~ 30 bp is the best).

2) Probe position: the probe cannot be located near the 5' or 3' end of the gene (because the primer needs to be located on the outside of the probe).

3) Select the strand in which the probe contains more C bases than G bases.

4) The base G should be avoided at the 5' end of the probe.

5) Continuous identical bases should be avoided, especially GGGG or more poly G.

6) Keep the GC content in the 20 ~ 80% range.

7) For singleplex assays, keep the Tm between 65°C to 70°C.

8) If there is SNP site in the probe region, it should be located in the middle of the probe sequence.

For Primer:

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 tools, such as "Primer Bank" and "Primer 3". Additionally, the following general principals may be considered when designing primers:

1) Choose the primers after the probe.

2) The forward or reverse primer sequence should be as close as possible to probe, but it should not have a coincidence region with them.

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

4) Keep the GC content in the 20 ~ 80% range.

5) Continuous identical bases should be avoided, especially GGGG or more poly G.

6) Keep the Tm between 58°C to 60°C.

7) Be sure the last 5 nucleotides at the 3' end contain no more than two G and/or C bases.

Trouble Shooting

Problem	Potential Cause	Suggestion
Apparent amplification can be observed in negative control.	The reagents, primers or water used is contaminated.	Change new reagents, primers, or water and retry. The reaction should be set up in a super clean bench to minimize contamination from the air.
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.
	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 whether 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.