EZ-Probe qPCR Master Mix for microRNA (ROX2 plus)

Catalog No.: EZB-miProbe-R2

Description

The ***EZ*Bioscience**® **EZ-Probe qPCR Master Mix for microRNA（ROX2 plus）** uses a specially modified *Taq* DNA polymerase protected by Hot start technique, and specifically optimized qPCR buffer system to perform probe based real-time quantitative PCR (qPCR) for microRNAs detection. The mix is supplied at 2× concentration. Meanwhile, it provides a tube of appropriative dedicated probe, Universal 3' qPCR Primer, and U6 Primer, which is well matched with the cDNA obtained by the ***EZ*Bioscience**® *microRNA Reverse Transcription Kits (Cat. No.: EZB-miRT4, or EZB-miRT1)*. So, it allows accurate target quantification and detection over a broad dynamic range and makes it possible to conduct highly reproducible and reliable qPCR analyses for microRNAs.

Components

Components	EZB-miProbe-R2-S (200 Rxns)	EZB-miProbe-R2 (500 Rxns)
2× EZ-Probe qPCR Master Mix for microRNA (ROX2 plus)*1	2 ml	5 ml
Probe	90 µl	220 µl
Universal 3' qPCR Primer	200 µl	500 µl
U6 Primer	100 µl	250 µl

***1:** The ROX2 premixed is used to rectify the error of fluorescence signals between different wells.

Storage

All components should be stored at -20°C and protected from light. and repeated thawing. The Probe is suggested to divide into 3 ~ 4 parts, and If not used for a long time, store at -80°C. The quality of this product can be guaranteed for 12 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®.
ABI 7500, 7500 Fast, Quant-Studio 3, 5, 6, 7, 12K Flex, Dx, ViiA™7; **Stratagene** MX3000P™, MX3005P™, MX4000™.

Protocol

1. Thaw the 2× EZ-Probe qPCR Master Mix for microRNA (ROX2 plus), Universal 3' qPCR Primer, U6 Primer, Probe and cDNA on ice, mix by inverting the tube several times, then centrifuge briefly and put 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 for microRNA (ROX2 plus), Probe, Specific forward qPCR Primer and Universal 3' qPCR Primer into a reaction mix, and mixing cDNA and Nuclease free ddH_2O into a reaction mix. cDNAs derived from the ***EZ*Bioscience**® *microRNA Reverse Transcription Kit (Cat. No.: EZB-miRT4, or EZB-miRT1)* are used as templates, it is suggested to be diluted by 5 ~ 10 times. Assume that the cDNA is diluted by 5 times, the following table is designed of 10 µl and 20 µl reaction systems:

Components	Volume/ 10 µl	Volume/ 20 µl	Final concentration
2× EZ-Probe qPCR Master Mix for microRNA (ROX2 plus)	5 µl	10 µl	1×
Probe*1	0.2 µl	0.4 µl	200 nM
Specific Forward Primer (10 µM)	0.4 µl	0.8 µl	400 nM
Universal 3' qPCR Primer	0.4 µl	0.8 µl	400 nM
cDNA	1 µl (0.5 ~ 2 µl)	2 µl (1 ~ 4 µl)	1 ~ 10 ng
ddH_2O	Add to 10 µl	Add to 20 µl	-

*1: The probe (labeled with FAM and MGB) should be protected from light and avoid repeated thawing.

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. Place the reaction plate in the real-time PCR instrument.

7. Set the thermal cycling conditions using the default PCR thermal cycling conditions specified in the following table:

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

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

Important: *1: The Hot Start DNA Polymerase requires activation at 95°C for 5 minutes. ***2:** Collect fluorescence signal during the Annealing & Extension step.

Data Analysis

Two types of quantification methods are commonly applied to quantify target microRNA expression when using this EZ-Probe qPCR Master Mix for microRNA (ROX2 plus).

1. Relative Quantitation: target microRNA expression is measured against an internal standard, such as U6.

Gene expression can be measured by the quantitation of cDNA converted from a microRNA 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 U6 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.*

Passive reference ROX: *is a dye molecule included in the 2× EZ-Probe qPCR Master Mix for microRNA (ROX2 plus) 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 free fluorophore signal can be normalized during data analysis. Normalization is necessary to correct for well-to-well fluorescent fluctuation.*

Representative Results

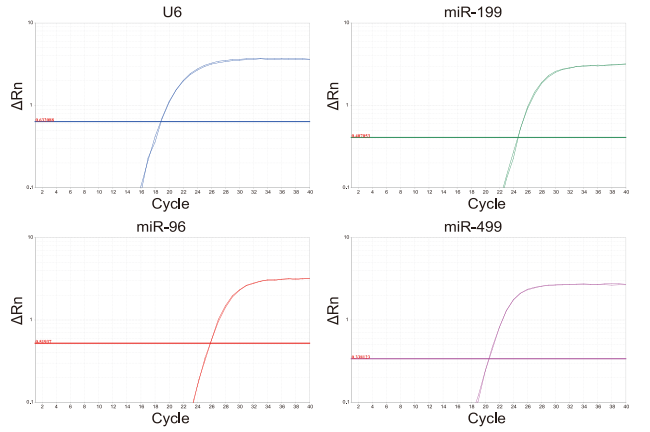

Figure 1. U6, miR-199, miR-96 and miR-499 expression level in cDNA produced by microRNA Reverse Transcription Kit (Cat. No.: EZB-miRT4) from A549 cells were detected. The amplification plots above indicate that, all of the four miRNAs could be amplified efficiently (**Notes:** Quantitation is fine when 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).

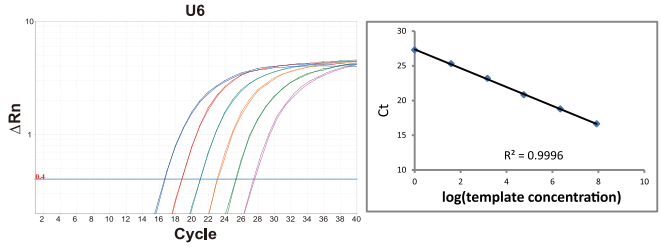

Figure 2. U6 expression level in equivalently diluted cDNAs from A549 cells were detected. The amplification plots above indicate that, the target gene could be amplified efficiently and specifically. And the linear relationship within the detection range is very good.

Suggestions for Primer Design

The reverse primer for microRNA quantification (a universal qPCR primer) is already provided in this kit, which is corresponding with the ***EZBioscience®*** *microRNA Reverse Transcription Kit (Cat. No.: EZB-miRT4, or EZB-miRT1).* The following general principals may be considered when designing primers for target microRNAs:

1) First, get the target microRNA sequence from microRNA data base (such as miRBase: http://www.mirbase.org/).

2) Copy the target microRNA sequence, change the U in the sequence to T, then delete the last 6 nucleotides at the 3' end.

3) Add 3 ~ 6 nucleotides to the 5' end of the primer, to justify the Tm value of the primer to 55 ~ 60°C (the added sequence mainly contains G&C, such as CGGGC, GCGGGC, or A/TGCCCG).

4) Synthesize the primer and evaluate the quality of the primer by qPCR. If the Ct value derived is between 13 ~ 32, the amplification curve displays representative S form curve, the primer could be approved.

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.