2× qPCR Mix for microRNA (ROX2 plus)

Catalog No.: EZB-miQP2

Description

The ***EZ*Bioscience**® **2× qPCR Mix for microRNA (ROX2 plus)** uses a specially modified *Taq* DNA polymerase protected by a Hot Start technique, and optimized qPCR buffer system to perform SYBR Green I based real-time quantitative PCR (qPCR) for microRNA expression detection. The mix is supplied at 2× concentration, and is specially optimized for microRNA, which strikingly improves the microRNA amplification and reduces the non-specific amplification. Meanwhile, the ROX2 passive reference dye (low ROX) is premixed in the mixture to make it an excellent ready to use qPCR reagent for microRNA expression detection. Besides, *a universal 3' qPCR primer* is also supplied in this kit. It is recommended that the kit is used *together with the* ***EZ*Bioscience**® *microRNA Reverse Transcription Kit (Cat. No.: EZB-miRT2).*

Component

Component	EZB-miQP2-S (200 Rxns)	EZB-miQP2-L (500 Rxns)
2× qPCR Mix for microRNA*	2 ml (1 ml × 2 tubes)	5 ml (1 ml × 5 tubes)
Universal 3' qPCR Primer (10 μM)	100 μl	250 μl
U6 Primer (10 μM)	50 μl	125 μl

*: Contain Hot start DNA Polymerase, dNTPs, Mg^{2+}, SYBR Green I dye, and premixed with and ROX2, to rectify the error of fluorescence signals between different wells.

Storage

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

Notice

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

ABI 7500, 7500 Fast, Quant-Studio 3, 5, 6, 7, 12K Flex, Dx, ViiA™7; **Stratagene** MX4000™, MX3000P™, MX3005P™.
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 (Suggestions for qPCR Amplification)

1. The suggested amount of template cDNA is 2 μl (the cDNA derived from the ***EZ*Bioscience**® microRNA Reverse Transcription Kit, Cat. No.: EZB-miRT2, then diluted for 5 times, which is 100 μl).The following table is designed for reaction volume of both 10 μl and 20 μl:

Component	Volume/ 10 μl	Volume/ 20 μl	Final concentration
2× qPCR Mix for microRNA	5	10	1×
Specific forword qPCR Primer (10 μM)	0.2	0.4	200 nM
Universal 3' qPCR Primer (10 μM)	0.2	0.4	200 nM
Template cDNA	1 μl (0.5 ~ 2 μl)	2 μl (1 ~ 4 μl)	1 ~ 100 ng
ddH_2O	Add to 10 μl	Add to 20 μl	-

2. Standard 2-step amplification program:

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		

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

A melt curve could be set up according to the default program of the equipment. A representative program for a 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

Data Analysis

Two types of quantification methods are commonly applied to quantify target microRNA expression when using this 2× qPCR Mix for microRNA.

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

microRNA expression can be measured by the quantitation of cDNA converted from a microRNA corresponding to this microRNA relative to a calibrator sample serving as a physiological reference. In a typical experiment, microRNA expression levels are studied as a function of either a treatment of cells in culture, of patients, or of tissue type, or of exosomes. The calibrator sample in each case is the cDNA from either the untreated cells or patients, or a specific tissue type, or of exosomes. 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 microRNA 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 microRNA 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 microRNA 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 microRNA 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 microRNA 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 affect PCR amplification.

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× qPCR Mix for microRNA 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.

Representative Results

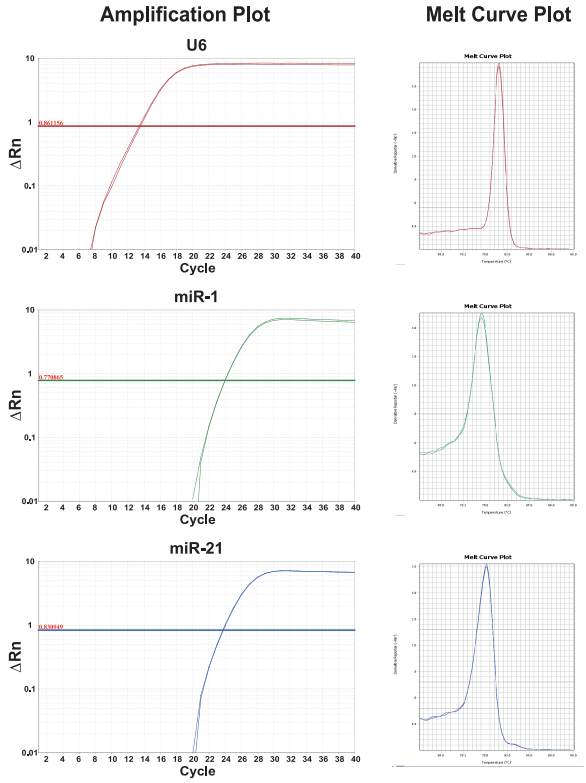

Figure1. miR-21, miR-1 and U6 expression level in cDNA from A549 cells were detected. The amplification plots and melt curves above indicate that, all the above microRNAs could be amplified efficiently and specifically (**Notes:** Quantitation is fine when Ct value is within 13 ~ 32; if the Ct value 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. A typical valid melt curve should be unimodal due to highly specific amplification and no primer dimer formation. If the melt 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

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.EZB-miRT2)*. 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 exam the quality of the primer by qPCR. If the Ct value derived is between 15 ~ 32, the amplification curve displays representative S form curve, and the melt curve has a typical single peak, the primer could be approved. If there are apparently double peaks in the melt curve, optimize the primer by add or delete the specific nucleotides to the 5' or 3' end of the primer.

Trouble Shooting

Problem	Potential Cause	Suggestion
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.
	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.
	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.