EZ-press One Step qRT-PCR Kit

Catalog No.: EZB-qRT

Description

The ***EZ*Bioscience**® **EZ-press One Step qRT-PCR Kit** is a one-step kit to quantify RNA using SYBR Green I. With the exception of primers and template, this kit contains in four vials all the components required to perform rapid, sensitive and reproducible qRT-PCR.

20× One Step qRT Enzyme Mix contains M-MLV (RNase H-) reverse transcriptase, RNase Inhibitor, Hot Start DNA Polymerase; 2× One Step qRT Buffer contains SYBR Green I, dNTPs, Oligo dT18, Random Hexamer and optimized buffer; 50× ROX Reference Dyes are used to rectify the error of fluorescence signals between different wells.

The M-MLV Reverse Transcriptase, which is an RNA-dependent DNA polymerase with a significantly attenuated RNase H activity, can synthesize cDNA at a temperature range of 42°C to 60°C and is inactivated during the activation step of the Hot Start DNA Polymerase.

RNase inhibitor can protect RNA templates from degradation.

This buffer has been optimized to allow both reverse transcription and PCR amplification to occur in the same reaction across a wide range of templates.

Hot Start DNA Polymerase requires an activation step at 95°C for 5 minutes, which prevents non-specific amplification during cDNA synthesis.

Components

Components	EZB-qRT-S (100 Rxns)	EZB-qRT (500 Rxns)
20× One Step qRT Enzyme Mix*1	100 µl	500 µl
2× One Step qRT Buffer*2	1 ml	5 ml
50× ROX Reference Dye 1*3	44 µl	220 µl
50× ROX Reference Dye 2*3	44 µl	220 µl

*1: Contain reverse transcriptase, RNase Inhibitor, Hot Start DNA Polymerase.

*2: Contain SYBR Green I, dNTPs, Oligo dT18, Random Hexamer and optimized buffer.

*3: Used to rectify the error of fluorescence signals between different wells.

Storage

The kit should be stored at -20°C and protected from light. Avoid repeated freeze thawing. The quality of this product can be 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™; **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®.
Use ROX1 (1×)	**ABI** 5700, 7000, 7300, 7700, 7900, 7900HT, 7900HT Fast; StepOne™, StepOne Plus™.
Use ROX2 (1×)	**ABI** 7500, 7500 Fast, Quant-Studio 3, 5, 6, 7, 12K Flex, Dx, ViiA™7; Stratagene MX3000P™, MX3005P™, MX4000™.

Protocol

1. Thaw the 20× One Step qRT Enzyme Mix, 2× One Step qRT Buffer, ROX Reference Dye, total RNA and primers 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 20× One Step qRT Enzyme Mix, 2× One Step qRT Buffer, ROX Reference Dye, forward primer and reverse primer into a reaction mix, and mixing total RNA and Nuclease free ddH_2O into a reaction mix. **The recommended amount of total RNA** added as a template used is **between 0.1 pg and 100 ng.** The following table is designed for reaction volume of both 10 µl and 20 µl:

Components	Volume/ 10 µl	Volume/ 20 µl	Final concentration
Total RNA	40 ng (0.5 pg ~ 50 ng)	80 ng (1 pg ~ 100 ng)	0.1 pg ~ 100 ng
20× One Step qRT Enzyme Mix	0.5 µl	1 µl	1×
2× One Step qRT Buffer	5 µl	10 µl	1×
Forward Primer (10 µM)*1	0.2 µl	0.4 µl	200 nM
Reverse Primer (10 µM)*1	0.2 µl	0.4 µl	200 nM
ROX Reference Dye (50×)*2	0.2 µl	0.4 µl	1×
ddH_2O	Add to 10 µl	Add to 20 µl	-

*1: Usually for optimization, the final concentration of the primer is 0.2 µM, and it can be adjusted within the range of 0.1 to 1.0 µM according to the situation.

*2: Please select an appropriate ROX according to the real-time PCR instrument used.

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. The standard reaction procedure for One Step qRT-PCR is as follows:

Step	1	2	3	
	cDNA Synthesis	Hot Start DNA Polymerase Activation*1	PCR	
		Hold	Cycle (40 cycles)	
			Denature	Anneal/ Extend*2
Temp.	42°C	95°C	95°C	60°C
Time	5 min	5 min	10 sec	30 sec
Volume	10 µl/ 20 µl			

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

8. 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 gene expression when using this EZ-press One Step qRT-PCR Kit.

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.*

Passive reference ROX: *is a dye molecule 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.*

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.

Representative Results

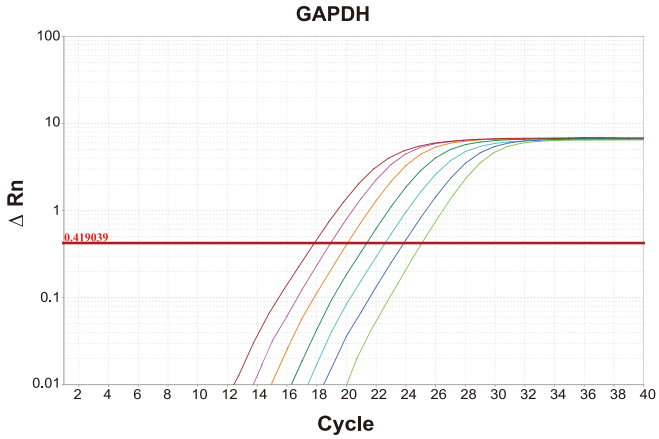

Figure1. GAPDH mRNA expression level in serially diluted RNA from Lentivirus were detected. The amplification plots above indicate that the target gene could be amplified efficiently and specifically.

Trouble Shooting

Problem	Potential Cause	Suggestion
No amplification product; Relative fluorescent signal ≤ background or no-template control.	cDNA synthesis temperature too high, low priming efficiency.	Lower incubation temperature.
	RT or cDNA primer blocked by Secondary structure.	Raise incubation temperature. Redesign primer(s).
	RNA has been damaged/degraded.	Replace RNA if necessary.
	RNase contamination.	Maintain aseptic conditions; add RNase inhibitor.
Product detected at higher than expected cycle number.	RNA has been damaged/degraded.	Replace RNA if necessary.
	RNase contamination.	Maintain aseptic conditions; add RNase inhibitor.
	RT inhibitors are present in RNA.	Remove inhibitors in the RNA preparation by an additional 70% ethanol wash. Inhibitors of RT include SDS, EDTA, guanidium salts, formamide, sodium phosphate and spermidine.
	Inefficient cDNA synthesis.	Adjust cDNA synthesis temperature and/or primer design. Double the amount of reverse primer.
	Inefficient PCR amplification.	Adjust annealing temperature as necessary.
		Redesign primers.
Higher than expected signal.	Too much sample added to reactions.	Decrease the concentration of template RNA.
Unexpected bands after electrophoresis.	Genomic DNA contamination.	Pre-treat RNA with DNase I.
	Low specificity in PCR.	Optimize PCR conditions as described above.