BioChain®
www.biochain.com
Tel: 1-888-762-2568 Fax: 1-510-783-5386 Email: info@biochain.com

User's Manual and Instructions

QCell-Eva One-Step qRT-PCR SuperMix Kit

Catalog Number: K5054200, K5054400

Introduction

qRT-PCR is a highly sensitive technique that is widely used for detection and quantification of RNA in tissues and cultured cells. Traditionally, quantitative PCR is performed in two steps: a first-strand cDNA synthesis step using reverse transcriptase, followed by a PCR step using a thermostable DNA polymerase. This Kit combines Reverse Transcriptase (MMLV-RTase) and RNase Inhibitor in a single mixture, with Eva fluorescent dye and hotstart Taq DNA polymerase in a separate 2x reaction mix optimized for qRT-PCR. Both cDNA synthesis and PCR are performed in a single tube using gene-specific primers and either cell lysate or RNA. A cell lysis buffer is provided in the kit to make cell lysates in less than 5 minutes at room temperature. The cell lysate can be used directly for qRT-PCR, bypassing RNA isolation procedure. The passive reference dye ROX is included in a separate tube to make the QCell-Eva One-Step qRT-PCR SuperMix adaptable for many real-time QPCR platforms. Human GAPDH primer set for RT-PCR is also included in the kit as a control. This primer was designed to span an exon-exon boundary in the human GAPDH cDNA, which can eliminate the undesired amplification of genomic DNA in the RNA or cell lysate.

BioChain's QRT-PCR SuperMix contains BioChain's Taq polymerase with hot start capability. BioChain's hot-start Taq polymerase improves PCR amplification reactions by decreasing non-specific amplification and preventing primer-dimer formation. This enzyme is activated after an initial 10 minutes heating at 95°C. And the real-time RT-PCR buffer is specially formulated to provide superior specificity and increase reverse transcription and amplification efficiency.

Eva Dye

Eva Dye binds double–stranded DNA. Detection is monitored by measuring the increase in fluorescence intensity throughout the cycle. Eva Dye has higher affinity to double-stranded DNA than SYBR Green dye and shows stronger fluorescence intensity than SYBR Green upon binding to DNA. Eva Dye is more stable than SYBR Green and the absorption and emission spectra of Eva Dye are very similar to SYBR Green Dye or FAM, so the same optical setting for SYBR Green Dye or FAM can also be used for Eva Dye.

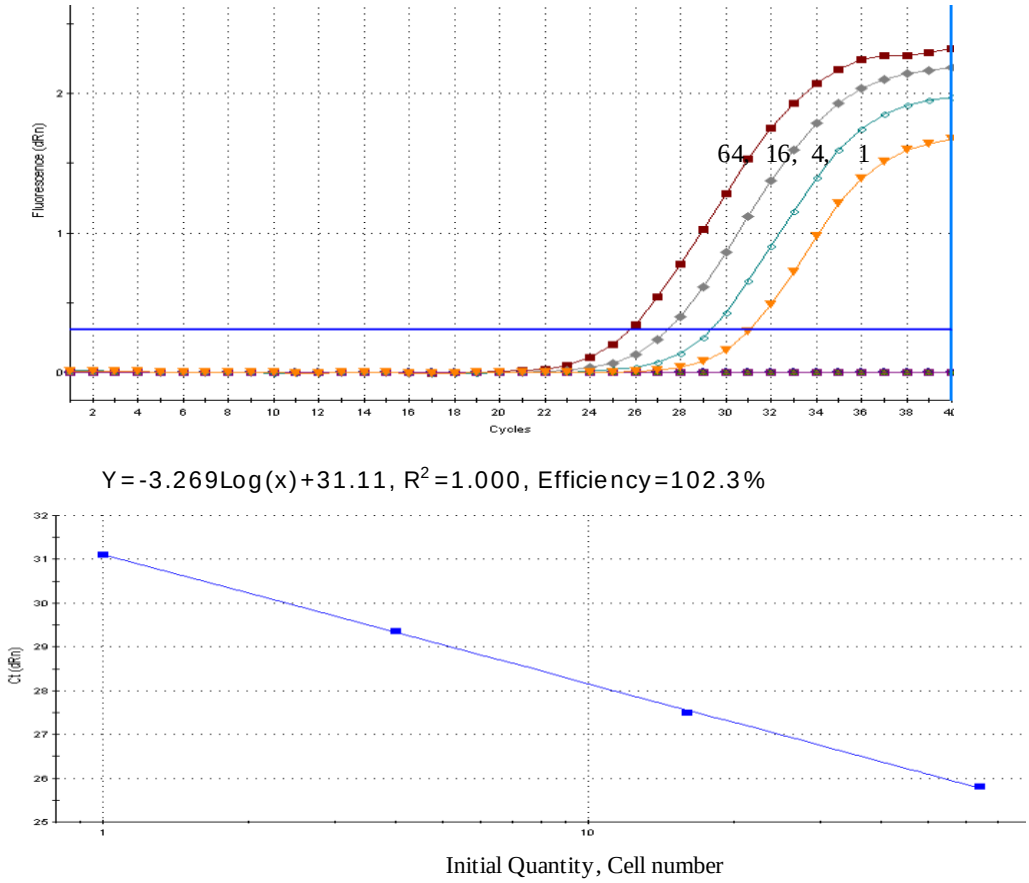

Figure 1. BioChain's QCell-Eva One-Step QRT-PCR SuperMix provides sensitive detection down to a single cell. K562 cells were lysed according to the cell lysis protocol. 4-fold serial dilution of cell lsyate were prepared from 64 cells to 1 cell. GAPDH gene expression was detected using BioChain's QCell-Eva qRT-PCR kit on Stratagene's Mx3005P instrument. Efficiency as measured from standard curve was 102.3%, with a R^2 value of 1.000.

Features

- Flexible and convenient – quantitating gene expression in cells (without isolating RNA) or RNA in one-step format
- Save time – quick cell lysis procedure, and ready-to-use supermix reducing setup time and liquid handling steps
- High Sensitivity – qRT-PCR from as low as 1 cell or 1 pg total RNA.
- Versatile – compatible with a wide variety of cell lines

Applications

- Real-Time RT-PCR
- Gene expression profiling
- Gene knockdown verification
- Array Validation

Tel: 1-888-762-2568 Fax: 1-510-783-5386 Email: info@biochain.com

Description

Components in this kit are prepared with pure chemicals according to our proprietary technology. QCell-Eva One-Step qRT-PCR SuperMix Kit provides a one-step, simple, robust, inexpensive assay for detection and quantitative analysis of gene expression directly from cells or RNA with intercalator format.

Quality Control

1 kit of this lot has been tested for quantitating human GAPDH gene expression in a serial dilution of cell lysate from 64 cells to 1 cell using Stratagene's Mx3005P as a real time PCR instrument. Good linearity and great PCR efficiency is observed and consistent with the previous lot.

Components

Catalog Number: K5054200: Reagents are sufficient for 200 assays

Item	Amount	Part No.
1. Cell Lysis Buffer	20 ml	K5054200-1
2. Eva qRT-PCR Reaction Mixture, 2x (containing Eva Dye and Hotstart Taq DNA polymerase)	1.25 ml x 2	K5054200-2
3. Reverse Transcriptase / RNase Inhibitor Mixture	100 µl	K5054200-3
4. ROX Reference Dye	50 µl x 2	K5054200-4
5. Human GAPDH control F/R primer pair (25x)	200 µl	K5054200-5
6. Nuclease-Free PCR Grade Water	3 ml	K5054200-6

Catalog Number: K5054400: Reagents are sufficient for 400 assays

Item	Amount	Part No.
1. Cell Lysis Buffer	40 ml	K5054400-1
2. Eva qRT-PCR Reaction Mixture, 2x (containing Eva Dye and Hotstart Taq DNA Polymerase)	1.25 ml x 4	K5054400-2
3. Reverse Transcriptase / RNase Inhibitor Mixture	100 µl x 2	K5054400-3
4. ROX Reference Dye	50 µl x 4	K5054400-4
5. Human GAPDH control F/R primer pair (25x)	200 µl	K5054400-5
6. Nuclease-Free PCR Grade Water	3 ml x 2	K5054400-6

Reagents and Equipments Required but not Supplied in this Kit:

1. PBS (Ca^{2+}, Mg^{2+} free)
2. Spectrofluorometric thermal cycler

Storage and Stability

Upon receipt, store all components at -20 ºC in a constant temperature freezer. Avoid repeated freeze/thaw cycles. When stored under these conditions the supermix is stable for one year after ship date. The Eva Dye and the ROX reference dye are light sensitive and should be kept away from light whenever possible.

Tel: 1-888-762-2568 Fax: 1-510-783-5386 Email: info@biochain.com

Protocol

Primer Design

Design QPCR primers to generate amplicons of 250 bp. Since the cell lysate contains genomic DNA, the primers should be designed to amplify cDNA but minimize amplification of genomic DNA. It is useful to choose primers that span an exon-exon boundary in the target mRNA, or choose primers that flank a large intron. If possible, design primers to avoid regions of secondary structure in the mRNA. Since reverse transcription and PCR are performed in one-step, we recommend to use the reverse PCR primer as the gene specific primer for reverse transcription.

Recommended Control Reactions

No Template Control (NTC): no-template control reactions are recommended in each experiment to screen for contamination of reagents or false amplification.
No-RT Control: no-RT control reactions are recommended for each experimental sample by omitting reverse transcriptase from the reaction. The no-RT control should generate no signal if the primers are specific for the cDNA and does not amplify genomic DNA.

Use of the ROX Reference Dye

ROX reference dye is included in this kit and may be added to compensate for non-PCR related variations in fluorescence. Addition of the reference dye is optional. Optimizing the ROX dye concentration within the qPCR reaction is an important aspect of setup. Too much ROX in the qPCR reaction will reduce background but also makes a low target signal difficult to distinguish from background. Conversely, too little ROX can increase background, meaning that low or weak target signals can be lost. For instruments that allow excitation at ~584 nm (such as Stratagene's Mx instrument and ABI 7500), firstly 1:10 dilute the ROX reference dye provided in the kit, then begin optimization using 0.5 μl **diluted** ROX reference dye in 25 μl qRT-PCR reaction. For instruments that do not allow excitation near 584 nm (such as ABI PRISM®/GENEAmp® 5700 instruments), begin optimization using 0.5 μl **undiluted** ROX reference dye in 25 μl qRT-PCR reaction.

Reagent Preparation and Storage

Thaw the tube containing 2x qRT-PCR Reaction Mixture on ice and store it on ice while setting up the reactions. Avoid direct light in preparation of the PCR reaction mixture because Eva Dye is light sensitive.

1. If the ROX reference dye will be included in the reaction, keep all solutions containing the ROX protected from light.
2. Due to the sensitivity of quantitative PCR, results can be easily affected by pipetting errors. Always prepare a master mix of qRT-PCR supermix containing the primers and the reference dye (if reference dye is used). Individual pipetting of replicate samples is not recommended.

Cell Lysis Procedure

The lysis buffer can be used to prepare lysates from a variety of mammalian culture cells. Lysates may be prepared with the maximum cell density (10^4 cells /μl). When used for qRT-PCR, the lysate may be diluted in the cell lysis buffer prior to adding to the qRT-PCR reaction. High concentration of either cellular materials or lysis buffer may inhibit qRT-PCR reaction, so the total amount of cell lysate added to the qRT-PCR reaction should not exceed 1/10 volume of the reaction. And the number of cells added to the 25 μl qRT-PCR reaction should be <= 500. This is a general guideline. For some cells lines, 500 cells may inhibit the qRT-PCR reaction. Prior to the experiment, perform a pilot standard curve to determine the maximum number of the cells that may be added to the qRT-PCR reaction, and determine the cell number range that give linear amplification of the specific target under your specific reaction conditions.

1. Harvest cells using the method appropriate to the properties of the cell line. For adherent cells, trypsinize the cells using standard techniques. Count the cell.
2. Pelleting the cells by centrifuging at 200 – 300x g for 5 min. Carefully remove the supernatant by aspiration.
3. Wash the pellet once with ice-cold PBS. Pelleting the cells by centrifuging at 200 – 300x g for 5 min. Carefully remove the supernatant by aspiration. Keep the pellet on ice.
4. Add appropriate volume of Cell Lysis Buffer to the cell pellet. Vortexing for 1 minute to lyse the cells.
5. Analyze the lysate by qRT-PCR. RNAs in the lysate are stable at 4°C for up to 4 hr.

QRT-PCR setup and cycling

1. Prepare the following RT-PCR reaction mixture. (First make the master mix without the template. After making the master mix, gently mix the reaction without creating bubbles, aliquot and then add 1 – 2.5 μl of template to each experimental reaction)

per reaction: 25 μl

Regents	Volume	Final Concentration
Eva QRT-PCR Reaction Mixture (2x)	12.5 μl	1x
Reverse Transcriptase / RNase Inhibitor Mixture	0.5 μl	
PCR forward primer	X μl	150 – 200 nM
PCR reverse primer	X μl	150 – 200 nM
ROX Reference Dye [a]	0.5 μl	
Template (cell lysate or RNA)[b]	1 – 2.5 μl	
Nuclease-free PCR grade water	Add up to 25 μl	

[a] See page 5: Use of the ROX Reference Dye
[b] If cell lysate is used as the template, the volume of cell lysate should not exceed 1/10 volume of the qRT-PCR reaction. If RNA is used as the template, it is recommended to use RNA template in less than 250 ng.

2. Gently mix the reactions without creating bubbles since bubbles interfere with fluorescence detection. Then centrifuge the reactions briefly.

3. Place the reactions in the instrument and run the appropriate RT-PCR program. Try the following protocol first, and optimize the reaction conditions if needed.
PCR program for RT-PCR:

Cycles	Temperature	Time	Detection	Remark
1	42°C	15 min	OFF	
1	95°C	10 min.	OFF	This step inactivates the reverse transcriptase and activates the hotstart Taq DNA polymerase. 10 minutes incubation is required to fully activate hotstart Taq DNA polymerase.
40	95°C	15 sec	OFF	
	50-60°C [a]	15 sec	ON	
	72°C	30 sec	OFF	

   a. Set an appropriate annealing temperature for the primer set used.

4. Dissociation Program for all PCR products

Follow manufacturer's guidelines for setting up dissociation depending on the instrument's software version.

QRT-PCR Setup and Cycling Program for human GAPDH control primer set (amplicon size = 226 bp)

1. Prepare the following RT-PCR reaction mixture. (First make the master mix without the template. After making the master mix, gently mix the reaction without creating bubbles, aliquot and then add 1 – 2.5 μl of template to each experimental reaction).

per reaction: 25 μl

Regents	Volume
Eva QRT-PCR Reaction Mixture (2x)	12.5 μl
Reverse Transcriptase / RNase Inhibitor Mixture	0.5 μl
Human GAPDH primer set (25x)	1 μl
Reference Dye ROX [a]	0.5 μl
Template	2.5 μl
Nuclease-free PCR grade water	Add up to 25 μl

[a] See page 5: Use of the ROX Reference Dye

2. PCR program for amplification of human GAPDH amplicon.

Cycles	Temperature	Time	Detection
1	42°C	15 min	OFF
1	95°C	10 min.	OFF
40	95°C	30 sec	OFF
	55°C	15 sec	ON
	72C	30 sec	OFF

3. Dissociation Program: Follow manufacturer's guidelines for setting up dissociation depending on the instrument's software version.

Related Products

QCell-Pro One-Step qRT-PCR SuperMix Kit (Cat# K5055200, K5055400), Eva QPCR SuperMix (Cat# K5052200, K5052400), Pro QPCR SuperMix (Cat# K5053200, K5053400), dNTP set for PCR (Cat# K6011100), PCR mix (Cat# 5051100), PCR Optimization Kit (K5051100), Taq Polymerase (Cat#7051200), RNA, PCR ready cDNA, and PCR ready genomic DNA.

References

1. Biotium, Inc. at http://www.biotium.com/product/product_info/allcolor.pdf
2. Higuchi R, Dollinger G, Walsh P S and Griffith R (1992): Simultaneous amplification and detection of specific DNA sequences. *BioTechnology* 10:413-417.
3. Higuchi R, Fockler C, Dollinger G and Watson R (1993): Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *BioTechnology* 11:1026-1030