BioChain®
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User's Manual and Instructions

Mouse Mesenchymal Stem Cells

Catalog Number: KS080xxx

Introduction

MicroRNAs (miRNAs) are endogenous regulators of gene expression that are encoded in the genomes of animals, plants and viruses. miRNAs function as a critical regulatory layer in development, differentiation, and the maintenance of cell fate. Mature miRNAs are 18-24 nt, single-stranded molecules that become incorporated into the RNA-induced silencing complex (RISC). RISC mediates down-regulation of gene expression through translational inhibition, transcript cleavage, or both (Figure 1, ref.1. FFL: combined transcriptional/post-transcriptional Feed-Forward Loops)

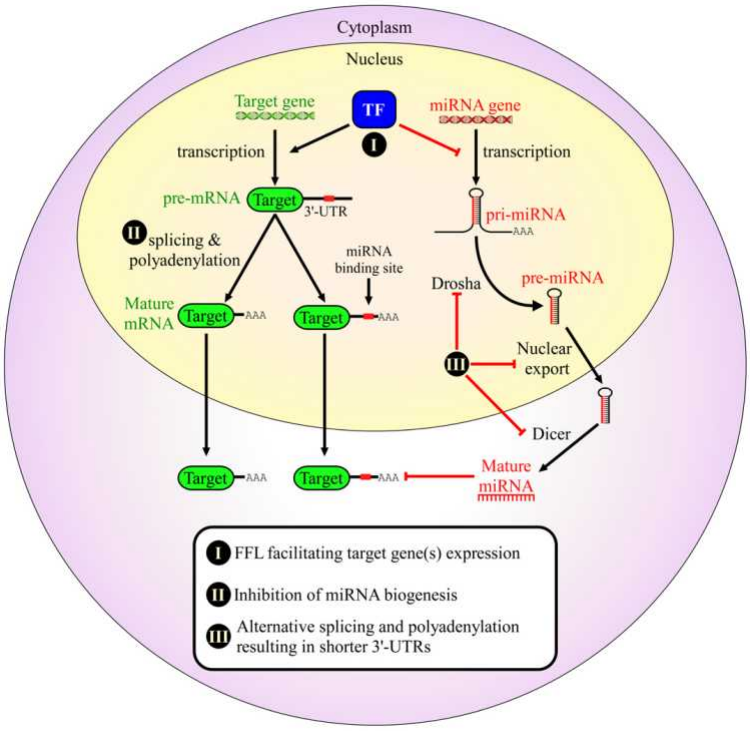


Figure 1. Mechanisms of regulation by miRNAs in cancer cells

The microRNA Biomarker Real-time RT-qPCR Detection Kit provides a simple, robust and specific assay for quantitative analysis of miRNA expression from total RNA samples (containing miRNAs) or small RNAs enriched samples. This kit contains all the reagents needed for running real-time RT-qPCR assay, including reverse transcription reagents and primer, qPCR reaction reagents and specific primer/probe mixture. The synthesized RNA oligo could be used as positive control and facilitate quantitative analysis. The passive reference dye ROX is included in a separate tube to make this kit adaptable for particular qPCR instruments. The components are suitable for different PCR formats as well, such as individual reaction tube, strip and 96-well plate.

The proprietary design of "End Guide" primer is facilitate the high quality of assay. The PCR primers/probe mixture includes a forward primer, a reverse primer and a TaqMan probe dual-labeled with 5'-FAM and BHQ-1-3', which is used for real-time detection and quantification of target cDNA. The specific primers and probe were uniquely designed and optimized for detecting mature miRNA with high specificity and sensitivity (Figure 2 and 3).

BioChain also provides custom service to design specific RT primer and qPCR primers/probe set for particular miRNA detection. For researchers interested in detecting other miRNAs, please contact BioChain Technical Support.

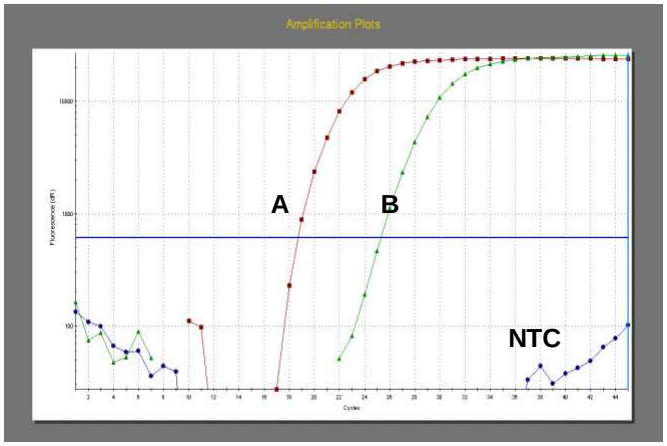
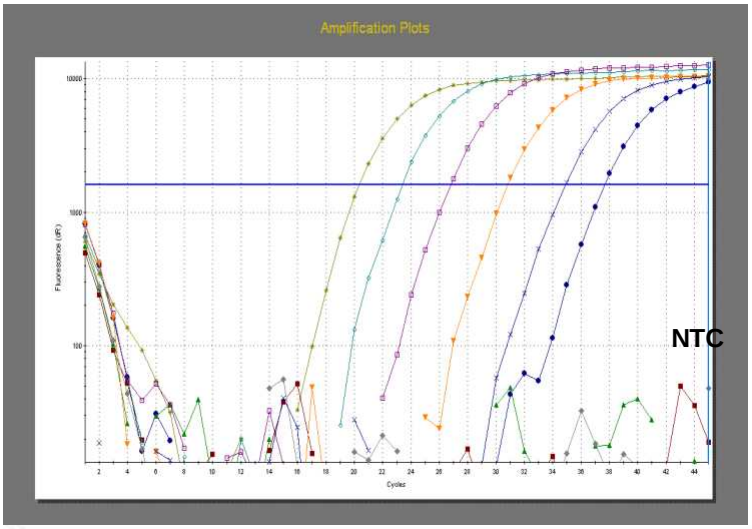


Figure 2. Twenty nanogram small RNA (<200 nt) (A) and 80 ng total RNA (B) isolated from human placenta were analyzed for expression of miR-191 using BioChain's miR191 Biomarker Real-time RT-qPCR Detection Kit. NTC: non-template control.



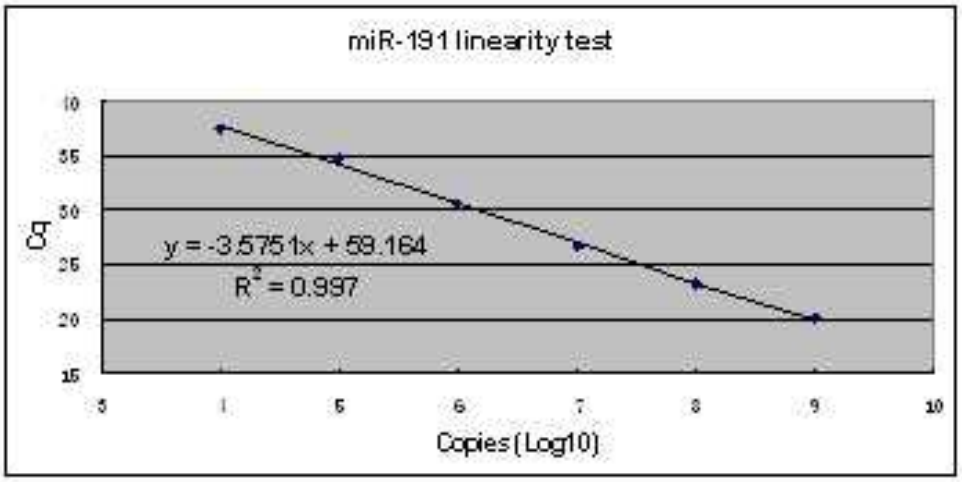


Figure 3. Linear amplification of miR-191 with a serial dilutions of synthesized RNA using BioChain's miR191 Biomarker Real-time RT-qPCR Detection Kit. Results demonstrated good linearity and excellent PCR efficiency over at least 6 logs of dynamic range. NTC: non-template control.

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Features

- Convenient - All reaction components are supplied and ready to use mixtures reduce setup time and liquid handling steps for quick and easy set up
- High sensitivity - Detect miRNA expression with both total RNA (containing miRNAs) or small RNA enriched samples
- Wide dynamic range - Good linearity and excellent PCR efficiency over 6 logs of dynamic range
- Flexible - Compatible with various real-time qPCR instruments and different qPCR formats

Applications

- MicroRNA biomarker detection and quantification
- MicroRNA expression profiling
- MicroRNA array data validation

Description

Components in this kit are prepared with pure chemicals according to our proprietary technology. The microRNA Biomarker Real-time RT-qPCR Detection Kit provides a simple and robust assay for detection and quantitative analysis of miRNA expression from total RNA samples (containing miRNAs) or small RNAs enriched samples.

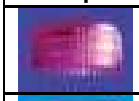
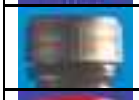
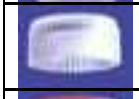
Quality Control

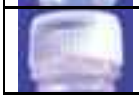
At least one kit of each lot has been tested for amplifying miRNA from synthesized target RNA over 6 logs of dynamic range using Stratagene's Mx3005P as qPCR instrument. Good linearity and great PCR efficiency is observed and consistent with the previous lot.

Components

Reagents are sufficient for 200 assays.

microRNA Biomarker Real-time RT-qPCR Detection Kit (KS08xxx2)

Cap	Color	Item	Amount	Part No.
	Violet	RT Primer (10x)	80 µl	KS080xxx-1
	Amber	Primer/probe Mix (25x)	200 µl	KS080xxx-2
	Red	Synthesized RNA control (1.5x10^9 copies/µl)	10 µl	KS080xxx-3
	Yellow	RT Reaction Buffer (5x)	200 µl	K4201100-2
	Natural	dNTP (10 mM each)	100 µl	K6011105-100
	Orange	MMLV reverse transcriptase/RNasein (100/10U/µl)	50 µl	K5055200-3
	Blue	Pro qPCR SuperMix (2x)	1.25 ml x 2	K5053200-1

	Green	ROX Reference Dye	50 µl x 2	K5053200-2
	Natural	Nuclease-Free PCR Grade Water	1.5 ml x 2	K5055200-5

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 this kit is stable for one year after ship date. The miRNA probe mix and the ROX reference dye are light sensitive and should be kept away from light whenever possible.

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Protocol

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 RT-qPCR reaction. For instruments that do not allow excitation near 584 nm (such as ABI PRISM®/GENEAmp® 5700 instrument), begin optimization using 0.5 µl **undiluted** ROX reference dye in 25 µl RT-qPCR reaction.

Reagent Preparation

Thaw the tubes containing reaction reagents on ice. Store them on ice while setting up the reactions. Avoid direct light in preparation of the PCR reaction mixture because probe and ROX reference dye are light sensitive.

1. Include no RT Enzyme and/or no RNA controls for genomic DNA contamination and RT reaction control for each new sample.
2. Set up a no-template control (NTC) to test for potential cross contamination for each round test.
3. Due to the sensitivity of qPCR, results can be easily affected by pipetting errors. Always prepare a master mix containing enzyme, primers and probe (and the reference dye if it is used), and the mix volume should be more than test needs to prevent pipetting inaccuracy during reagent preparation. Individual pipetting of replicate samples is not recommended.

Reverse transcription

Prepare the RT reaction mixture on ice. First make the master mix (5 x RT Reaction Mix + RT Primer + Water), gently mix without creating the bubbles, aliquot, and then add RNA sample to final volume per reaction at 15 µl.

Set no MMLV RT Enzyme and/or no RNA sample as RT controls are strongly recommended for facilitating to interpret results.

Final volume per reaction: **15 µl**

Reagents	Volume (µl)
BCI RT Reaction Buffer (5x)	3
dNTP (10 mM each)	2
MMLV reverse transcriptase/RNasein (100/10U/µl)	1
RT Primer	1.5
RNA sample or synthesized RNA control	X (2) [a]
Nuclease-free PCR grade water	Add up to 15 µl

[a] 2 µl RNA control was validated. Frozen-thaw cycle impacts stable of synthesized RNA and induce lower control sensitivity.

Standard Program for RT:

Step 1: 22°C for 15 minutes (this step facilitates RT primer annealing to the RNA template. If the 22°C incubation can not be done at the real-time PCR instrument, this step can be done separately in regular thermal cycler).
Step 2: 37°C for 15 minutes (this step is for the reverse transcription process)
Step 3: 85°C for 5 minutes (this step to stop reaction)
Step 4: 4°C hold (ready for PCR or storage)

qPCR

Prepare the PCR reaction mixture on ice. Firstly make the master mix (Pro qPCR SuperMix + primers/probe (+ Rox) + Water), gently mix without creating the bubbles, aliquot, and then add cDNA template or RT controls to each experimental reaction well or tube. Also set non-template control(s) (NTC) facilitating to interpret results.

Final volume per reaction: **25 µl**

Reagents	Volume
Pro qPCR SuperMix (2x)	12.5 µl
primers/probe Mix (25x)	1 µl
Reference Dye ROX [a]	0.5 µl
cDNA [b]	2 µl
Nuclease-free PCR grade water	Add up to 25 µl

[a] Optional. See: Use of the ROX Reference Dye
[b] Two micro liter cDNA from 2ul synthesized RNA control was validated. Optimal amount should be determined by preparing the dilution series.

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

Quick Guide: For each master mix and **n** reactions:
Set (**n + 1**) **× 23** µl Master Mix
Vortex gently to mix
Aliquot **23** µl of master mix into each reaction well
Add **2** µl of appropriate Template to each well
Total reaction volume = **25** µl

Place the reactions in the instrument and run the appropriate PCR program. It is highly recommended to use the following protocol.

Cycles	Temp	Time	Detection	Remark
1	95°C	10 min	OFF	This step will inactivate the Reverse Transcriptase and activate the Hotstart Taq DNA Polymerase
40	95°C	15 sec	OFF	Set the instrument to detect FAM (and Rox if applicable) and report fluorescence at the annealing step of each cycle
	55°C	15 sec	ON (FAM)	

As the samples are run on the machine, reaction are automatically detected, analyzed. Data are stored for review or comparison with other test results. Runs are around 90 minutes in duration.

Quality Control

Different controls are furnished with the kit and should be run at least in singlicate each time the assay is performed to ensure proper performance of the assay. All assay controls must be examined prior to interpretation of sample results.
The actual quantification cycle (Cq) is depending on the platform of detection and the version of the analysis software used. The Cq range for positive control has been determined using all supplied BCI reagents and Stratagene Mx3005P v.4.00. Once identified, the Cq range as determined on your specific platform will be consistent from run to run.

1. If the controls do not yield the correct results, the assay is not valid and the tests should not be interpreted.
2. If run in duplicate (recommended), test should be repeated on sample with inconsistent results between duplicates (Cqs > 1).

The following describes the analysis of each control, and the decisions necessary based upon the results (Table).

Table Expected results of controls

Type of Control	Expected Result	Aberrant Result	Potential Trouble
RT control: No Reverse Transcriptase	No amplification curve present and no Cq value generated	Show amplification curve with Cq value	(RNA sample) DNA contamination
RT control: No RNA Input	No amplification curve present and no Cq value generated	Show amplification curve with Cq value	(RT or PCR buffer) DNA contamination
PCR Negative Control: Non-Template (NTC)	No amplification curve present and no Cq value generated	Show amplification curve with Cq value	Contamination of the master mix or cross-contamination of PCR reactions due to improper procedure
PCR positive Control (2ul 1:5 diluted cDNA from 2ul RNA control)	Show amplification curve and with Cq value **less than 25**	No amplification curve present and no Cq value generated or Cq value greater than **25**	Wrong practice during reaction preparation or PCR inhibited

Warnings and Precautions

1. This product is for ***Research Use Only***.
2. The assay kit should be used as a system. Do not substitute other manufacturer's reagents. Dilution, reducing amplification reaction volumes, or other deviation in this protocol may affect the performance of this test and/or nullify any limited sublicense that comes with the purchase of this testing kit.
3. Close adherence to the protocol will assure optimal performance and reproducibility. Care should be taken to ensure use of correct thermocycler program, as other programs may provide inaccurate/faulty data, such as false positive and false negative results.
4. Do not mix or combine reagents from kits with different lot numbers.
5. Materials are stable until the labeled expiration date when stored and handled as directed. Do not use kits beyond their expiration date.
6. Due to the analytical sensitivity of this test, extreme care should be taken to avoid the contamination of reagents or amplification mixtures with samples, controls or amplified materials. To minimize contamination:

a. Wear clean gloves when handling samples and reagents and routinely clean work areas and pipettes prior to doing PCR;
b. Work flow in the PCR laboratory should always be in a one way direction between separate work areas; beginning in Master Mix Preparation, moving to the Specimen Preparation, then to the

Amplification, and finally to Detection. Do not bring amplified DNA into the areas designated for master mix or specimen preparation;
c. Laboratory personnel are reminded to wear appropriate personal protective equipment and universal precautions when working with specimens. It is recommended that glass distilled de-ionized molecular biology grade water be used with the preparation of specimen RNA;
d. All pipettes, pipette tips, and any equipment used in a particular area should be dedicated to and kept to that area of the laboratory;
e. Sterile, disposable plastic ware should be used whenever possible to avoid RNase, DNase, or cross-contamination;
f. All reagents should be closely monitored for signs of contamination (e.g., negative controls giving positive signals). Discard reagents suspected of contamination.

End-point PCR

This kit can also be used for end-point PCR.

For end-point PCR, amplify for an appropriate number of cycles (usually ~30 cycles) so that the reaction remains in the exponential phase of amplification, while the PCR amplification product is readily visible on an agarose gel.

Analyze the PCR product in 3.5% agarose gel in 1xTAE and stained with Ethidium Bromide or other DNA binding dyes. Specific microRNA amplicon is around 70 bp (Figure 4).

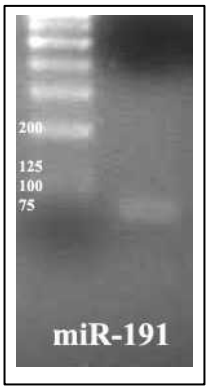

Figure 4. miR191 Biomarker Real-time RT-PCR product analysis by gel electrophoresis

Related Products

MicroRNA Isolation Kit (Cat# KS 341025)
Broad Range Total RNA Isolation Kit (Cat# K1341050)
BioChain qRT-PCR ready RNA (containing miRNAs)

References

1. Shalgi R, *et al. Aging* 2009. 1:762-770.
2. Ach, R., *et al. BMC Biotechnology* 2008. 8:69.
3. Tang, G., *et al. Trends Biochem. Sci.* 2005. 30:106-114.
4. Liu, J., *et al. Science* 2004. 305:1437-1441.