hsa-mir-373 Real-time RT-PCR Detection Kit User Manual

Catalog # CPK1190

For the detection and quantification of miRNA hsa-mir-373 using Real-Time RT-PCR detection instruments.

For research use only. Not for diagnostic or therapeutic procedures.

Cohesion BIOSCIENCES

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I. INTRODUCTION

The miRNAs RT-PCR Quantitation Kit is a sensitive and specific method using real-time PCR for the detection and quantification of miRNAs (miRNA) from total RNA samples. miRNAs are small, single-stranded, ~19 - 23 nt RNA molecules encoded in the genomes of plants, animals, and viruses. Mature miRNAs enter the RNA-induced silencing complex (RISC) and guide the RISC to induce translational repression or endonucleolytic cleavage of specific target mRNAs. Unlike commonly used methods for detection of miRNAs, the miRNAs qPCR Quantitation Kit is more rapid and sensitive.

This kit contains miRNA high specific RT and PCR primer set, with SYBR Green dye included. The stem-loop like miRNAs RT primer and the miRNAs high specific primer set ensure the RT and PCR reaction would not be interfered by the miRNAs precursors. The reaction template could be total RNA or cell lysates.

The system enables highly sensitive detection from as few as 10 copies of a target miRNAs, with a broad dynamic range that supports accurate quantification of high-copy mRNA from up to 1 µg of total RNA.

High specificity ensurenced by stemloop RT primer and high specific miRNAs primer set; even highly homogized miRNAs can be accurately dicriminated.

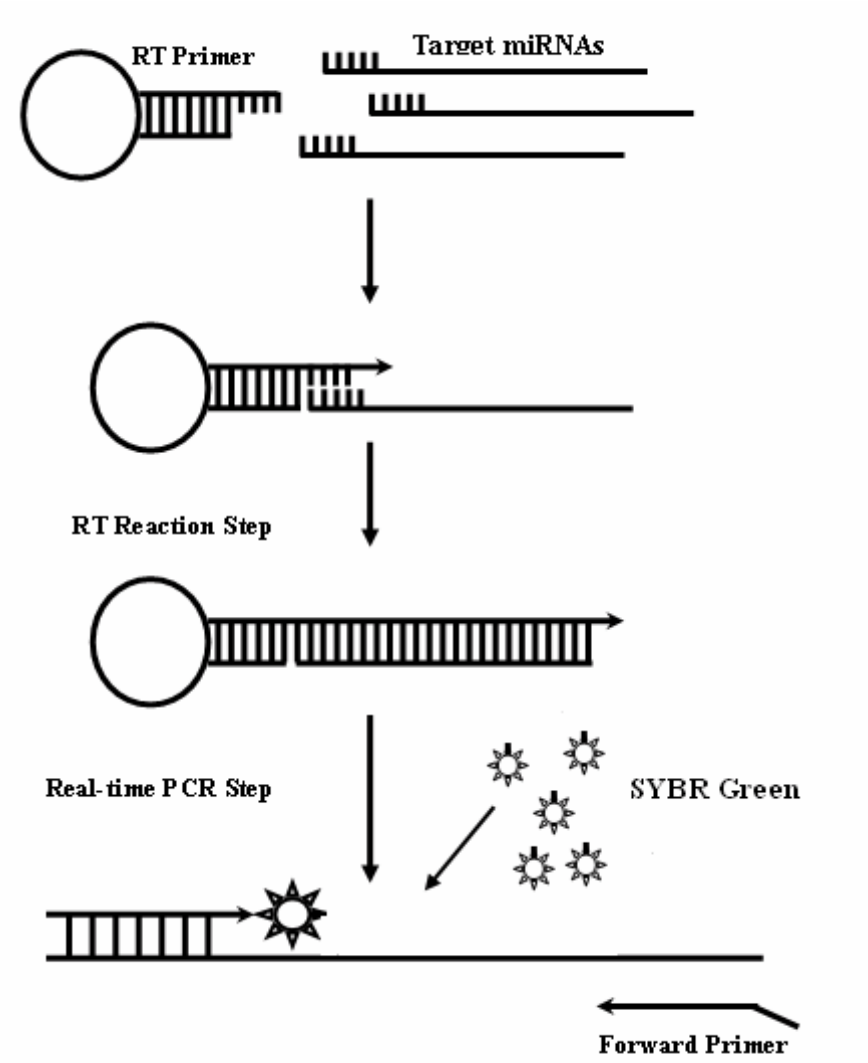
Higher dynamic range and sensitivity of miRNAs quantification than conventional method (such as Northern blot & micro-array) with broad dynamic range of at least seven orders of magnitude, and in this ready to use formulation can quantify as few as 7 copies of a miRNA target in as little as 0.1 pg of total RNA.

Less sample requirement Total RNA, cell lysate or purified samll RNA can work as the qPCR Quantitation Kit's template, even the genomic DNA contamination would not interfere in miRNAs quantification.

miRNA synthetic standard can work as a standard curve when you need to know the absolute number of a miRNA in one cell or work as positive control when you need to know the relative expression ratio between miRNA and U6 snRNA or 5S rRNA.

II. PRINCIPLES

miRNAs real-time Quantitation assay of miRNAs includes two steps, stem-loop RT and real-time PCR. Stem-loop RT primers bind to at the 3' portion of miRNA molecules and are reverse transcribed with reverse transcriptase. Then, the RT product is quantified using real-time PCR that includes miRNA-specific forward primer, reverse primer and SYBR Green dye.



III. KIT COMPONENTS

Component	50rxns	100rxns	200rxns	Storage
RT Buffer (5X)	200 µl	400 µl	800 µl	4 °C
dNTP (10 mM)	40 µl	80 µl	160 µl	-20 °C
M-MLV Reverse Transcriptase (200 U/µl)	10 µl	20 µl	40 µl	-20 °C
Real-time PCR Master Mix (2X)	0.5 ml	1 ml	1 ml x2	4 °C dark
hsa-mir-373 RT Primer (10µM)	10 µl	20 µl	40 µl	4 °C
hsa-mir-373 Specific Primer Set (10 µM)	20 µl	40 µl	80 µl	4 °C
ROX Reference Dye (50X)	100 µl	200 µl	400 µl	4 °C dark
rTaq DNA Polymerase (5 U/µl)	10 µl	20 µl	40 µl	-20 °C
PCR enhancer	100 µl	200 µl	400 µl	4 °C dark
Synthetic hsa-mir-373 Standard	1 pmol	1 pmol	1 pmol	-20 °C
RNase Free H_2O	1.5 ml	1.5 ml	1.5 ml x2	4 °C
Sterilized H_2O	1.0 ml	1.0 ml	1.0 ml	4 °C
RNA Dilution Buffer	1.0 ml	1.0 ml	1.0 ml	4 °C
Manual	1	1	1	

Note: ROX dye provides an internal passive reference to which the SYBR Green signal can be normalized during data analysis which is necessary to correct for well-to-well fluorescent fluctuations. Applied Biosystems 7000/7300/7500/7500 Fast/ 7900 Real-Time PCR System need low concentration of ROX dye, Applied Biosystems Step OnePlus Real-Time PCR System need high concentration. Other brands of real time PCR machine don't need ROX dye.

IV. MATERIALS REQUIRED BUT NOT PROVIDED

1. RNase Inhibitor

To keep the integrality of RNA template and good quality of RT reaction, adding RNase Inhibitor in your RT system was recommended. The final concentration usually was 0.25 U/µl.

2. Optical PCR plates or tubes

As to eliminate the impact on fluorescence detection caused by PCR tubes or plates, using optical PCR plates and tubes was recommended to corporate the requirements of the Real-Time PCR instruments such as ABI PRISM 7000/7300/7500/7900, MX3000p/4000p.

V. ASSAY PROCEDURE

1. Handling the synthetic miRNA standard

The synthetic miRNA standard is supplied as 1 pmol dry powder Oligo stock. Following the guideline below when handling the synthetic miRNA standard stock.

Dilution: Centrifuge the tube at 10000 rpm/min for 1 minute. Carefully open the tube, add 1 ml RNA dilution buffer (supplied by the kit) to dilute the stock to 1 nM and get another DEPC treated, steriled tubes to dilute the 1nM stock to 0.1 nM for working concentration. Then prepare some other DEPC treated, steriled 1.5 ml tubes to dilute the 1 nM miRNA to serial orders (5 - 7 orders) to make the standard curve required for experiment.

Storage: It is important to store the 1 nM miRNA standard solution and the 0.1 nM work concentration solution at -20 °C. And it is preferred to store the solution at -70 °C for longer time.

RNase-free conditions: Take precautions to ensure that the stock solution does not become contaminated with RNase.

a. Use RNase-free steriled pipette tips and supplies for all manipulations.

b. Wear gloves when handling reagents and solutions.

2. Handling the miRNA RT primer

The miRNA RT primer is supplied as 10 µM and please dillute it to 1 µM working concentration according to the amount that you need in an experiment. Then store the 1 µM miRNA RT primer solution at -20 °C. For a standard miRNA RT reaction, the final concerntration of RT primer is 60 nM.

3. Preparing the RT reaction mix

The template for the miRNAs real-time Quantitation assay can be total RNA, cell lysate or purified miRNA. The RNA template's input range can vary from 1 µg to 3 µg

or more as far as the requirement of the experiment. We recommended a standard 20 µl RT reaction size.

The following table provides RT reaction Mix volumes for a standard 20 µl reaction size.

Component	Final Con.	Vol /1 rxns
5X RT Master Mix	1X	4 µl
dNTP (10 mM)	0.375 mM	0.75 µl
MiR-RT Primers (1 µM)	60 nM	1.20 µl
MMLV Reverse Transcriptase (200 U/µl)	40 U	0.2 µl
RNA Sample	1-3 µg	X µl
RNase Free H_2O		To 20 µl

Note: Mix the RT reaction reagents by flipping the reagent tubes and pipette Mix for several times before RT reaction. Remember not to vortex.

4. Performing miRNAs RT reaction

Standard RT Reaction Program

30 minutes at 25 °C, 30 minutes at 42 °C, 5 minutes at 85 °C, store at 4 °C.

Note: Keep all components, reaction mixes and samples on ice. After assembly, transfer the reaction mixes to a thermal cycler preheated to the cDNA synthesis temperature and begin RT reaction. Please take out the RT product quickly and put them on ice as soon as possible. The following procedures must be carried out at this low temperature all the time.

5. Handling the miRNA RT product

For the miRNAs real-time Quantitation assay, mix cDNA and pipette 2 µl (20 µl size) or 4 µl (40 µl size) miRNA RT reaction product as the template for real-time PCR step subsequently. Store the surplus miRNA RT reaction product at -20 °C.

6. Preparing Real-Time PCR reaction mix

The following table provides Real-time PCR reaction Mix volumes for a 20 µl and 40 µl reaction size. Note that preparation of a master mix is crucial in quantitative applications to reduce pipetting errors in 40 µl reaction size.

Component	Final Con.	Vol /1 rxns
2X Real-time PCR Master Mix	1X	10 µl
miR Specific Primer Set (10 µM)	0.2 µM	0.4 µl
ROX Reference Dye[1]	1X or 5X	0.4 µl or 2 µl
PCR enhancer[2]	0 / 2.5% / 5%	0 / 1.5 / 1 µl
Taq DNA Polymerase (5 U/µl)	1 U	0.2 µl
miRNA RT Product		2 µl
dd H_2O		To 20 µl

[1] ROX dye provides an internal passive reference to which the SYBR Green signal can be normalized during data analysis which is necessary to correct for well-to-well fluorescent fluctuations. Applied Biosystems 7000/7300/7500/7500 Fast/ 7900 Real-Time PCR System need low concentration of ROX dye, Applied Biosystems Step OnePlus Real-Time PCR System need high concentration. Other brands of real time PCR machine don't need ROX dye.

[2] Appropriate concentration (0 / 1.5 / 1 ul) of PCR enhancer is suggested when dissociation curve show some unspecific curve such as primer dimer curve.

7. Performing miRNAs Real-Time PCR reaction

Program the real-time PCR instrument to perform PCR amplification as shown below.

95 °C for 3 minutes hold,

40 cycles of:

95 °C, 12 seconds

62 °C, 40 seconds

Note: You should select FAM or SYBR for detection and ROX for reference dye and detection step is at 62 °C.

VI. CONSTRUCTION A STANDARD CURVE

The synthetic miRNAs standard provided by this kit can work as a miRNA control when spiked into the total RNA extracted from tissue or cells. If the exact copy number of the miRNA in a certain amount RNA sample was wanted, you can construct a standard curve follow the recommendation step below:

1. Add 1 ml RNA dilution buffer to dilute the synthetic miRNAs standard to 1 nM stock. Prepare another 1.5 ml tube to dilute the 1 nM miRNA standard solution to 0.1 nM as work concentration to a probably volume. And stored 1 nM stock solution separated from primers and Master Mix at -70 °C.
2. Dilute the 0.1 nM synthetic miRNAs to 5 - 7 order of magnitude with RNase free H_2O.
3. Add 2 µl RNA sample dilution to 20 µl RT reaction system. Note that each order should be prepared for 2 or 3 repeats in RT reaction.
4. Add 4 µl RT reaction product to 40 µl real-time PCR system. Note that each order should also be prepared for 2 or 3 repeats in PCR reaction.
5. For the standard curve, the 2 µl 0. 1 nM synthetic miRNAs sample added into 20 µl RT reaction system represents 6 $\times 10^7$ copies per reaction.

Note:

1. For multiple reactions, prepare a master mix of common components, add the appropriate volume to each tube or plate well on ice, and then add the unique reaction components (e.g., template). Preparation of a master mix is crucial in qRT-PCR to reduce pipetting errors.
2. Make sure that all components are at the bottom of the tube/plate; centrifuge briefly if needed.

VII. QUALITY CONTROL

The product is tested functionally by qRT-PCR using four kinds of template: 10nM and 100pM synthetic miRNAs standard and total RNA from two cells. Kinetic analysis must demonstrate a linear dose response with decreasing target concentration.

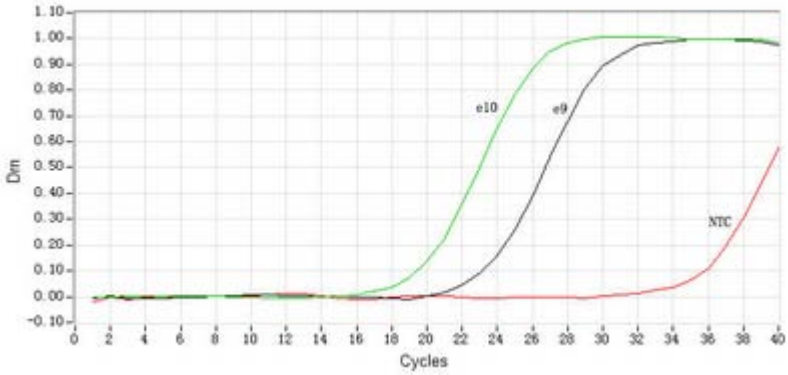


Fig. 1 hsa-mir-373 amplification curve in 10nM and 100pM synthetic miRNA standard

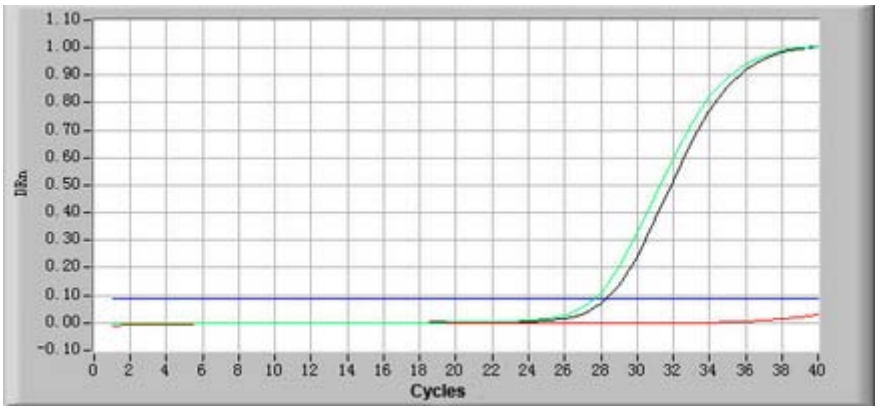


Fig. 2 Amplifiction curve in total RNA from cells

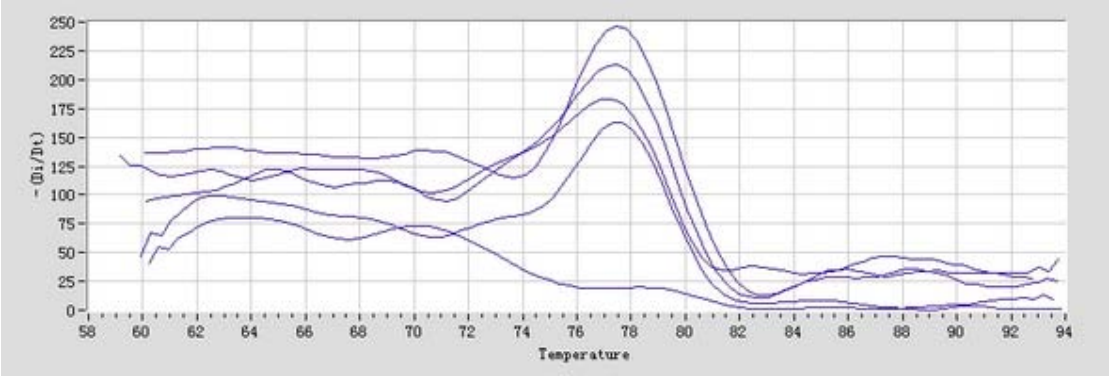


Fig. 3 Dissociation curve (Note: the minor peak on the left is the negative control)

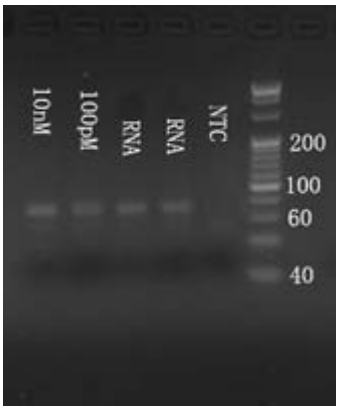


Fig. 4 Electrophoresis Image (20bp marker)

VIII. TECHNICAL SUPPORT

For troubleshooting, information or assistance, please go online to www.cohesionbio.com or contact us at techsupport@cohesionbio.com

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IX. NOTES