All-in-One™ miRNA qRT-PCR Detection System 2.0 User Manual

For quantitative detection of mature miRNA

All-in-One™ miRNA qRT-PCR Detection Kit 2.0
Cat. No. QP115 (20 RT and 200 qPCR reactions)
Cat. No. QP116 (60 RT and 600 qPCR reactions)

All-in-One™ miRNA First-Strand cDNA Synthesis Kit 2.0
Cat. No. QP113 (20 miRNA reverse transcription reactions)
Cat. No. QP114 (60 miRNA reverse transcription reactions)

User Manual

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User Manual

All-in-One™ miRNA qRT-PCR Detection System 2.0

I. Introduction and Principle

Small, non-coding miRNAs are widely present in eukaryotes. They consist of about 22 nucleotides that control many important physiological processes in cell development and differentiation. Different miRNAs express differently at different developmental stages and in different tissues. Therefore, the quantitative assaying of miRNAs is important both basic and applied research.

The **All-in-One ™ miRNA qRT-PCR Detection System 2.0** uses real-time PCR technology to quantitatively measure miRNAs.The experimental procedure includes two major steps (Figure 1).

1. **Single-step cDNA Synthesis** -- Poly A polymerase is used to add poly-A tails to the 3' end of miRNAs, and at the same time M-MLV RTase, with a unique Oligo-dT adaptor primer, to reverse transcribe the miRNA tailed poly-A.
2. **qPCR Detection** -- The All-in-One™ qPCR Mix containing SYBR® Green specifically detects the reverse transcribed miRNA (the miRNA-specific forward primer is used with the Universal Adaptor PCR Primer).

Compared to traditional hybridization-based miRNA detection methods such as Northern blot analysis, the method provided by the All-in-One™ qRT-PCR Kit is faster, more specific and sensitive, and uses less sample material.

Advantages of the All-in-One™ miRNA qRT-PCR DetectionSystem 2.0

1. Provides efficient reverse transcription of miRNAs into cDNA in a single step

2. Delivers a precise quantitative and accurate measurement of miRNA expression profiles

3. Differentiates between mature and precursor miRNA with specific, mature miRNA qPCR primer

4. Co-developed with validated primers, miRNA clones and other tools used for functional studies of miRNA

5. All - in - One™ miRNA qRT-PCR Detection System 2.0 can be used to simultaneously detect multiple miRNAs from cDNA of a synthetic reaction,which is a qPCR detection system based on SYBR Green Dye. It not only reduces errors and saves samples, but also realizes high-throughput detection.

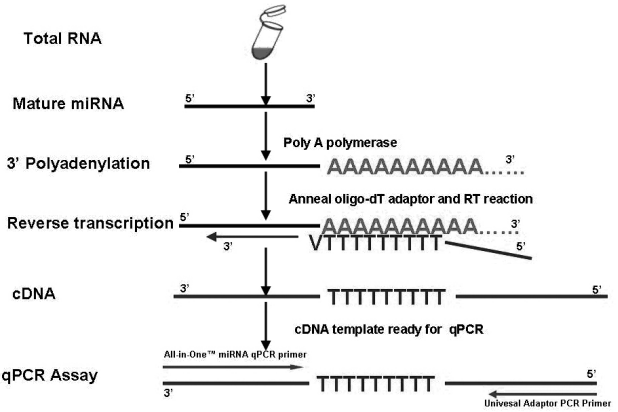

Figure 1. A graphic representation of the steps involved in the All-in-One™ qRT-PCR miRNA Detection Kit 2.0.

II. Related Products

GeneCopoeia offers comprehensive solutions for studying miRNAs. A careful process of co-development ensures that they work well together and provide robust and reproducible results.

Product	Description
miExpress™ Precursor miRNA Expression Clones	750 human, 450 mouse and 270 rat precursor miRNA expression-ready clones for studying the regulatory effect of miRNA on genes and proteins
miTarget™ miRNA Target Expression Clones	20,000 human and 15,000 mouse expression-ready miRNA target clones for validating miRNA function
miArrest™ miRNA Inhibitor Expression Clones	750 human, 450 mouse and 270 rat miRNA inhibitor expression-ready clones for analyzing miRNA function
OmicsLink™ Expression-Ready ORF cDNA Clones	20,000 human and 15,000 mouse expression-ready clones for gain-of-function studies
All-in-One™ miRNA qPCR Primers	1700 human, 800 mouse and 400 rat validated miRNA qPCR primers for robust, reproducible and reliable quantitation of miRNA
Endofectin™ Transfection Reagents	Allows efficient transfection with low toxicity using reagents optimized for specific cell types

III. Contents and Storage

The kits are stable for at least 12 months when stored at –20°C, the inside reagent can also be stored at –80°C in aliquots. Avoid repeated freezing-thawing.

1. All-in-One™ miRNA qRT-PCR Detection Kit 2.0

Contents of the kits (Cat. Nos. QP115 and QP116) are provided in the following table.

Cat. No	Contents	Quantity	Description
QP113-01	2 U/μl Poly A Polymerase	1×20 μl 3×20 μl	Adds Poly-A tails to the 3' end of miRNA
QP056-01	SureScript™ RTase Mix (20x)	1×20 μl 3×20 μl	Contains M-MLV RTase and RNase Inhibitor for reverse transcription reactions
QP113-03	5×PAP/RT Buffer II	1×80 μl 3×80 μl	Contains the rATP, dNTP, oligo-dT adaptor RT primer etc.
QP006-07	dd H_2O(RNase/DNase free)	1×1 ml 3×1 ml	Diethyl pyrocarbonate- (DEPC) treated ddH_2O
QP001-01	2×All-in-One™ qPCR Mix	2×1 ml 3×(2×1 ml)	2×Master mix contains hot-start Taq, dNTP and reaction buffer used in qPCR reactions
QP001-02	50×ROX Reference Dye	1×80 μl 3×80 μl	Used in qPCR instruments requiring ROX for calibration
QP010-03	50 μM Universal Adaptor PCR Primer(Tm = 64.5 GC content = 50%)	1×20 μl 3×20 μl	The reverse qPCR primer which matches the oligo-dT adaptor RT primer combines with All-in-One™ specific-miRNA qPCR primer to detect miRNA

2. All-in-One™ miRNA First-Strand cDNA Synthesis Kit 2.0

Contents of the kits (Cat. Nos. QP113 and QP114) are provided in the following table.

Cat. No	Contents	Quantity	Description
QP113-01	2 U/μl Poly A Polymerase	1×20 μl 3×20 μl	Adds Poly-A tails to the 3' end of miRNA
QP056-01	SureScript™ RTase Mix (20x)	1×20 μl 3×20 μl	Contains M-MLV RTase and RNase Inhibitor for reverse transcription reactions
QP113-03	5×PAP/RT Buffer II	1×80 μl 3×80 μl	Contains the rATP, dNTP, oligo-dT adaptor RT primer etc.
QP006-07	dd H_2O(RNase/DNase free)	1×1 ml 3×1 ml	Diethyl pyrocarbonate- (DEPC) treated ddH_2O

IV. Preparation

Wear a lab coat, disposable gloves and protective goggles when handling chemicals.

RNA Sample Preparation

When working with RNA it is important to avoid RNases in your solutions, consumables and labware. When preparing your RNA samples, always wear a mask and disposable gloves in all procedures. Follow the described procedures you are using for RNA extraction carefully. Ready-to-use solutions that are RNase-free can be purchased. Alternatively treat solutions with diethyl pyrocarbonate (DEPC) and then autoclave. RNases on labware can also be inactivated by DEPC treatment or by baking at 250°C for 3 hours. Use DEPC to treat all microcentrifuge tubes, pipettes and pipette tips (if no RNase free) and then autoclave to deactivate RNases. RNase-free consumables are available for purchase from many commercial sources.

Primer Design

The reverse primer called "Universal Adaptor PCR Primer" (T_m = 64.5, GC% = 50%) has been provided in the All-in-One™ miRNA qPCR Kit.

You may wish to design and make specific forward primers for your miRNA of interest or order from GeneCopoeia. Please contact us for further information.

Since the length of miRNA is generally between 18 ~ 24 nucleotides for some "easy" miRNA, a forward primer may be designed directly according to the sequence. However, for some potentially "difficult" miRNA (e.g. very high or very low T_m or highly homologous miRNA) or miRNA from specific tissues (e.g. tissues with high pre-miRNA/ pri-miRNA) special primers may need to be designed to optimize the primer sequence in order to obtain specific amplification and avoid interference from pre- miRNA/pri-miRNA.

IMPORTANT NOTES:

1. Store kit at –20°C. Avoid storage or leaving reagents at 4°C or room temperature.

2. Mix reagents thoroughly by gently inverting tubes several times avoiding bubbles, and then briefly centrifuge before use.

3. Following the procedure carefully to avoid contamination with RNases which can rapidly degrade RNA and lead to inconclusive results.

4. Set up all reactions on ice to reduce risk of RNA degradation.

V. Procedure

1. Reverse transcription of miRNA

a. Thaw template RNA on ice. Thaw 5×PAP/RT Buffer II and ddH_2O (Rnase and DNase free) at room temperature (15°C ~25°C).

b. Gently mix miRNA reverse transcription reagents by flicking to dissolve all reagents thoroughly. Briefly centrifuge to collect residual liquid from the sides of the tubes and then place on ice.

c. Prepare miRNA reverse transcriptase reaction solution.

Place RNase-free reaction tubes on ice and then add the following reagents to a final volume of 20 µl.

Reagent	**Volume**	**Quantity**
Total RNA* or small-molecule RNA		1 µg† 100 ng
2 U/µl Poly A Polymerase	1 µl	
SureScript™ RTase Mix (20x)	1 µl	
5×PAP/RT Buffer	4 µl	1×
dd H_2O (RNase/Dnase free)	To final **20 µl**	

* Total RNA must contain small-molecule RNA.
† The amount of total RNA can be between 1 ng ~ 5 µg. If using purified small-molecule RNA, the amount can be between 0.1 ng ~ 1 µg.

d. Prepare reverse transcription reaction.
Mix the prepared reaction mix gently, but thoroughly. Incubate at 37°C for 60 minutes after a brief centrifugation.

Incubate at 85°C for 5 minutes to inactivate the enzyme.

The resulting reverse transcription reaction product should be diluted **5 ~ 50 times** with sterile H_2O before using for the next qPCR experiment or it can be directly stored at –20°C.

2. Detection of miRNA with qPCR.

a. Mix 2×All-in-One™ qPCR Mix by gently inverting. Briefly centrifuge and place on ice. If required, Mix 50×ROX Reference Dye.

b. Dilute the **50 μM** Universal Adaptor PCR Primer to **2 μM** with sterile ddH_2O before using for the next qPCR experiment.

c. Prepare qPCR solution on ice. See the following example.

Reagent	**Volume**	**Final concentration**
2×All-in-One qPCR Mix[i]	10 μl	1×
All-in-One™ miRNA qPCR Primer (2 μM) [ii]	2 μl	0.2 μM
Universal Adaptor PCR Primer (2 μM)	2 μl	0.2 μM
First-strand cDNA (diluted 1:5) [iii]	2 μl	
ROX Reference Dye[iv] (30μM) if needed	0.4~0.1 μl	600nM-150nM
Water (double distilled)		
▪ Not using ROX Reference Dye	4 μl	
▪ Using ROX Reference Dye	3.9~3.6 μl	
Final volume	**20 μl**	

Notes

i. Use the 2×All-in-One™ qPCR Mix as half of the total reaction volume and adjust other reagents accordingly. If the total reaction volume is changed, maintain each component in proper proportion.

ii. Primer concentration should be in the range of 0.2 to 0.4 μM. In general, a PCR reaction using 0.2 μM primers produces good results.

iii. The first-strand cDNA should be diluted before using for the PCR reaction in order to avoid interference to the qPCR from the reverse transcription system.

iv. ROX Reference Dye is added only for qPCR instruments that require ROX for calibration. ROX Reference Dye provides an internal reference to which the reporter-dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescence fluctuations due to changes in concentration or volume. Adjust the ROX Reference Dye to optimal concentration according to different qPCR instruments.

Instrument	ROX per 20μl PCR Reaction	Final Concentration
BioRad iCycler, MyiQ, iQ5, CFX-96, CFX-384, Eppendorf Mastercycler realplex, Roche LightCycler 480, LightCycler 2.0	None	No ROX
ABI PRISM 7000/7300/7700/7900HT and 7900HTFast, ABI Step One, ABI Step One Plus	0.4 μl（0.2-0.4μl）	600 nM（300-600nM）
ABI 7500，7500 Fast, ABI Viia7, Stratagene Mx3000P , Mx3005P, Mx4000,	0.1 μl（0.02-0.1μl）	150 nM（30-150nM）

For other instruments which need calibration of ROX but have not been listed out in the table, please optimize the concentration of ROX according to the guide line of specific instrument.

d. Thoroughly mix the qPCR reaction solution, add to PCR tubes, and briefly centrifuge to make sure that all the reagents are in the bottom of the tubes.

e. The following standard 3-step method for the qPCR reaction is recommended (example adapted from the iQ5 real-time PCR detection system from Bio-Rad)

Cycles	Steps	Temperature	Time	Detection
1	Initial denaturation	95°C	10 min	No
40	Denaturation	95°C	10 sec	No
	Annealing	T_m – 2°C	20 sec	No
	Extension	72°C	At least 10 sec	Yes

Notes

i. When using SYBR Green dye to monitor the qPCR reaction, a melting curve analysis should be performed immediately after qPCR cycling. For instructions, consult the documentation for your qPCR instrument. The following is an example adapted from the iQ5 real-time detection system from Bio-Rad Laboratories. The conditions for your instrument may differ.

Temperature Range	Heating Rate	Constant Temperature	Detection
65°C ~ 95°C	0.5°C/ time	6 sec/ time	Yes
30°C		30 sec	No

ii. The DNA polymerase used in the 2×All-in-One™ qPCR Mix is a chemically especially modified hot-start enzyme. Incubation for 10 minutes at 95°C will sufficiently activate the enzyme.

iii. Specific properties of a miRNA lead to special properties of the designed primer. Therefore the annealing temperature needs to be strictly controlled in order to avoid non-specific amplifications. For validated miRNA primers purchased from GeneCopoeia, please refer to the optimal conditions for the experiment.

iv. The Oligo-dT Adaptor primer for reverse transcription is 53 nucleotides, therefore the resulting PCR amplification fragment is about 75bp (assuming the sequence of miRNA is about 22 nucleotides), which requires at least about 10 seconds extension time. From the melting temperature of the products, the T_m value is generally determined to be between 75°C ~ 83°C. If the melting temperature exceeds this range, other assaying methods such as electrophoresis are suggested for the specific properties of the product.

v. The main conditions for the above reactions are for use with the iQ5 qPCR instrument from Bio-Rad. If a qPCR instrument from another commercial source is used, please reference the instrument manual and adjust the extension time and melting curve conditions accordingly.

VI. Trouble Shooting Guide

miRNA sequence homology problems	• Because the sequence of miRNA is short and some have a high degree of homology, the primer design can sometimes be tricky. Thus one needs to fully consider the specificity problems when designing the miRNA forward primers. Specifically for miRNA that have a single nucleotide difference only, the demand for specificity is higher for designing and synthesizing primers, in addition to designing reaction conditions.
Confusion of amplification curves	• The fluorescence detection temperature may not be appropriate. Adjust accordingly. • The set up position for samples may not be right. Adjust accordingly. • Try to use 3.0% agarose gel electrophoresis to check the PCR products. Check the purity of the primers using electrophoresis or use PAGE-purified primers if the bands are diffused. One may also use phenol/chloroform extraction and ethanol precipitation methods to treat the primers before experiment.

Abnormal melting curves	• **Signals in blank (No Template Control) sample** • There may be contamination or positive samples in the PCR reaction system if the T_m of the melting curves of the blank control is the same as the positive control. Eliminate sample application error first. If the situation still persists, change PCR grade water, primers or use new 2×All-in-One™ q-PCR Mix. • If the T_m of melting curves of blank control is lower than the positive control, the PCR reaction may have produced nonspecific amplification such as primer-dimers. Please prepare PCR reaction mix on ice and increase the temperature of fluorescence detection. If the C_t value of the negative control is >35 and the difference with the positive samples is more than 5 cycles, the PCR reaction system is up to the standard. On the other hand, if the Ct value cannot reach the aforementioned value, then redesign the primer or optimize the reaction conditions.
	• **Double peaks and multiple peaks in melting curves of positive control** • In the absence of other primers present in the reaction, double or multiple peaks in the positive control means that the PCR reaction produces nonspecific amplification fragments. Prepare the PCR reaction mix on ice; optimize the PCR reaction conditions such as by increasing the annealing temperature, decreasing the primer concentration or increasing the fluorescence detection temperature (no more than the T_m value of the expected product). If this does not work, optimize and redesign the forward primer.
No signal (Ct) or Ct value is too high	• Check if there are PCR products to exclude the possibility of instrument detector malfunction. • Not enough PCR cycles. For good sensitivity, one should generally set up more than 35 PCR cycles, but more than 45 cycles may result in too much background signals. • The amount of template may not be enough or the template may be degraded. Use the highest concentration of diluted template samples possible to set up PCR. At the same time, avoid freezing and thawing samples repeatedly. • Amplification efficiency is low and PCR reaction conditions are not optimal. Redesign primers and optimize reaction conditions.

VII. Limited Use License and Warranty

Limited Use License

Following terms and conditions apply to use of all All-in-One™ miRNA qRT-PCR Detection Kit 2.0 (the Product). If the terms and conditions are not acceptable, the Product in its entirety must be returned to GeneCopoeia within 5 calendar days. A limited End-User license is granted to the purchaser of the Product. The Product shall be used by the purchaser for internal research purposes only. The Product is expressly not designed, intended, or warranted for use in humans or for therapeutic or diagnostic use. The Product must not be resold, repackaged or modified for resale, or used to manufacture commercial products without prior written consent from GeneCopoeia. This Product should be used in accordance with the NIH guidelines developed for recombinant DNA and genetic research. Use of any part of the Product constitutes acceptance of the above terms.

Limited Warranty

GeneCopoeia warrants that the Product meets the specifications described in the accompanying Product Datasheet. If it is proven to the satisfaction of GeneCopoeia that the Product fails to meet these specifications, GeneCopoeia will replace the Product. In the event a replacement cannot be provided, GeneCopoeia will provide the purchaser with a refund. This limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to GeneCopoeia within 30 days of receipt of the Product. GeneCopoeia's liability is expressly limited to replacement of Product or a refund limited to the actual purchase price. GeneCopoeia's liability does not extend to any damages arising from use or improper use of the Product, or losses associated with the use of additional materials or reagents. This limited warranty is the sole and exclusive warranty. GeneCopoeia does not provide any other warranties of any kind, expressed or implied, including the merchantability or fitness of the Product for a particular purpose.

GeneCopoeia is committed to providing our customers with high-quality products. If you should have any questions or
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