



All-in-One™ miRNA qRT-PCR Detection Kit

For quantitative detection of mature miRNA

Cat. No. AOMD-Q020 (20 RT and 200 qPCR reactions)

Cat. No. AOMD-Q050 (50 RT and 500 qPCR reactions)

Used in combination with the All-In-One™ miRNA qPCR Primers

User Manual II

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USER MANUAL II

All-in-One™ miRNA qRT-PCR Detection Kit

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I. Introduction and Principle

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

The **All-in-One™ miRNA qRT-PCR Detection Kit** uses real-time PCR technology to quantitatively measure miRNAs. The experimental procedure includes three 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
- 2) **cDNA Synthesis** - At the same time M-MLV RTase and a unique Oligo-dT Adaptor primer reverse transcribes the poly A miRNAs (The Universal Adaptor PCR primer in combination with a miRNA-specific primer allows detection of specific miRNA)
- 3) **qPCR** - 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 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 Detection Kit

- Provides efficient reverse transcription of miRNAs into cDNA in a single step
- Delivers a precise quantitative and accurate measurement of miRNA expression profiles
- Differentiates between mature and precursor miRNA
- Co-developed with validated primers, miRNA clones and other tools used for functional studies of miRNA

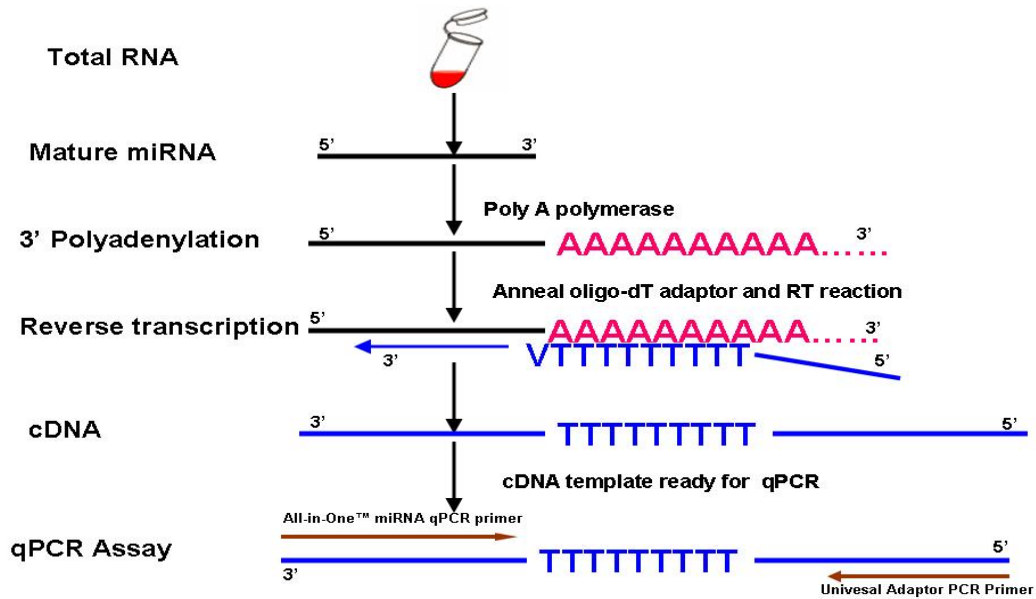


Figure 1. A graphic representation of the steps involved in the All-in-One qRT-PCR miRNA Detection Kit.

II. Related Products

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

GeneCopoeia		Description
miExpress™ Precursor miRNA Expression Clones	750 human 450 mouse 270 rat	Study miRNA regulation on target genes and proteins
miTarget™ miRNA Target Validation Expression Clones	25,000 human 25,000 mouse	Cross validate data using luciferase reporter genes
OmicsLink™ Expression-Ready ORF cDNA Clones	20,000 human 15,000 mouse	Perform gain-of-function studies with expression-ready clones
All-in-One™ miRNA qRT-PCR Detection Kits		Accurately quantify miRNA expression
All-in-One™ miRNA qPCR Primers		Validated for robust, reproducible and reliable quantitation of miRNA activity
Endofectin™ Transfection Reagents	Optimized for specific cell types	Transfect efficiently and with low toxicity for reliable and reproducible results

III. Contents and Storage

Contents and storage recommendations for the All-in-One miRNA qRT-PCR Detection Kits (Cat. Nos. AOMD-Q020 and AOMD-Q050) are provided in the following table.

Contents	Quantity	Storage temperature/ conditions
2.5 U/μl Poly A Polymerase	20 μl 50 μl	-20°C (Stable for at least 12 months) Alternatively, the solution can also be stored at -80°C in aliquots. Avoid repeated freezing/ thawing.
RTase Mix	20 μl 50 μl	-20°C (Stable for at least 12 months) Alternatively, the solution can also be stored at -80°C in aliquots. Avoid repeated freezing/ thawing.
5X Reaction Buffer	100 μl 250 μl	-20°C (Stable for at least 12 months) Alternatively, the solution can also be stored at -80°C in aliquots. Avoid repeated freezing/ thawing.
dd H ₂ O (RNase and DNase free)	1 ml 1 ml	-20°C (Stable for at least 12 months) Alternatively, the solution can also be stored at -80 °C in aliquots. Avoid repeated freezing/ thawing.
2X All-in-One qPCR Mix	1 ml x 2 1 ml x 5	-20°C (Stable for at least 12 months) Alternatively, the solution can also be stored at -80 °C in aliquots. Avoid repeated freezing/ thawing.
50X ROX Reference Dye	80 μl 200 μl	-20°C (Stable for at least 12 months) Alternatively, the solution can also be stored at -80 °C in aliquots. Avoid repeated freezing/ thawing.
100 μM Universal Adaptor PCR Primer T _m = 64.5 GC content = 50%	20 μl 50 μl	-20°C (Stable for at least 12 months) Alternatively, the solution can also be stored at -80 °C in aliquots. Avoid repeated freezing/ thawing.

IV. Preparation

Wearing a lab coat, disposable gloves and protective goggles are recommended 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 qRT-PCR Detection Kit.

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

Since the length of miRNAs is generally between 18 ~ 24 nucleotides for some “easy” miRNAs, a forward primer may be designed directly according to the sequence of the miRNA. However, for some potentially “difficult” miRNAs (e.g. very high or very low T_m or highly homologous miRNAs) or miRNAs 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 5X RT Buffer and ddH₂O (RNase/ DNase free) at room temperature (15–25°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 25 μl .

Reagent	Volume	Quantity
Total RNA or small-molecule RNA		2 µg [†] 100 ng
2.5 U/ µl Poly A Polymerase	1 µl	
RTase Mix	1 µl	
5X Reaction Buffer	5 µl	1X
dd H ₂ O (RNase-/DNase- free)	To final 25 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 H₂O before using for the next qPCR experiment or it can be directly stored at –20°C.

2. Detection of miRNA with qPCR.

- a. Dissolve 2X All-in-One qPCR Mix by gently inverting. Briefly centrifuge and place on ice. If required, dissolve 50X ROX Reference Dye.
- b. Dilute the 100µM Universal Adaptor PCR Primer to 2µM with sterile ddH₂O before using for the next qPCR experiment.
- c. Prepare qRT-PCR solution on ice. See example.

Reagent	Volume	Final concentration
2X All-in-One qPCR Mix ⁱ	10 µl	1X
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	
50X ROX Reference Dye ^{iv}	0.4 µl	1X
Water (double distilled)		
■ Not using ROX Reference Dye	4 µl	
■ Using ROX Reference Dye	3.6 µl	
Final volume	20 µl	

Notes

- i. Use the 2X 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 used in Real-Time PCR instruments that require ROX for calibration, such as the ABI qPCR 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 2X 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 GeneCopeia, 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 $75^{\circ}\text{C} \sim 83^{\circ}\text{C}$. 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. Examples

- a) Example 1: Specificity assay using the All-in-One miRNA qRT-PCR Detection Kit

With 200 ng total RNA mixture from human brain and heart as template, the miRNA qRT-PCR Detection Kit and the All-in-One miRNA qPCR Primers were used to detect 30 miRNA and an internal reference snRNA U6. Results from qRT-PCR and electrophoresis showed neither non-specific amplification products nor primer-dimer formation.

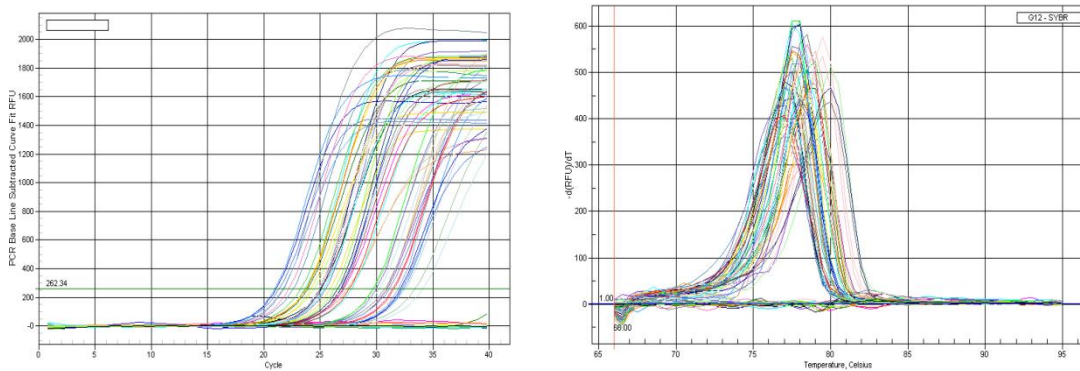


Figure 1. Amplification and melting curves of 31 miRNA and the internal reference snRNA U6, in which double channel detection was used for the positive control, and single channel detection was used for the NTC (No Template Control).



Figure 2. Agarose gel electrophoresis (3% agarose gel) of the amplification products of 31 miRNA and the internal reference snRNA U6, in which double channel detection was used for the positive control, and single channel detection was used for the NTC (No Template Control).

b) Example 2: Sensitivity assay using the All-in-One miRNA qRT-PCR Detection Kit

Starting with different amounts (5µg, 1µg, 200ng, 20ng, 2ng, 100pg) of human brain total RNA, the All-in-One miRNA qRT-PCR Detection Kit was used to detect the expression level of hsa-miR-124. The results showed that linear amplification can be detected between 5µg ~ 100pg of total RNA.

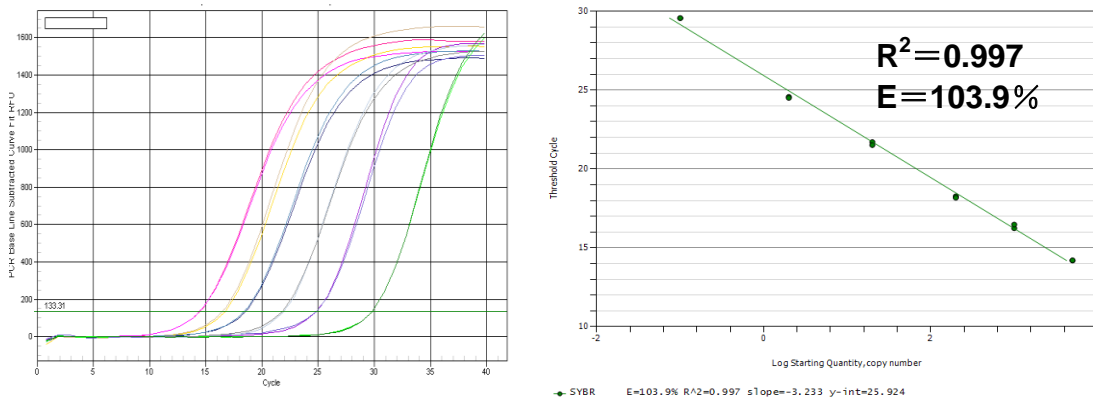


Figure 3. The amplification curve and standard curve generated from different amount of human brain total RNA as template, and using All-in-One miRNA qRT-PCR Detection Kit to detect hsa-miR-124 expression level.

VII. Trouble Shooting Guide

<p>miRNA sequence homology problems</p>	<ul style="list-style-type: none"> 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 miRNAs that have a single nucleotide difference only, the demand for specificity is higher for designing and synthesizing primers, in addition to designing reaction conditions.
<p>Confusion of amplification curves</p>	<ul style="list-style-type: none"> 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 3.5% 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.
<p>Abnormal melting curves</p>	<ul style="list-style-type: none"> 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 2X 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 C_t 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	<ul style="list-style-type: none"> • 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.

VIII. 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 (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 Manual. 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.

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