GeneCopoeia™
Expressway to Discovery

All-in-One™ miRNA qPCR Primer

Catalog number: HmiRQP0032

User Manual and Primer Validation Report

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Primer Manual and Validation Report

I. Introduction

miRNAs are single-stranded non-coding RNA molecules that are on average about 22 nucleotides in length that regulate gene expression and thereby regulate different physiological activity in the cell. However, they are difficult to detect due to their short lengths. All-in-One miRNA qPCR primers are specific miRNA upstream detection primers for qPCR. When combined with the GeneCopoeia All-in-One miRNA qRT-PCR Detection Kit, the primers can be used for miRNA quantitation. GeneCopoeia All-in-One miRNA qPCR primers have been validated in qPCR reactions using their specific cDNAs as templates.

II. Product Information

Catalog#	Primer ID	Mature_Acc	Mature_ID	PCR size	Conc.	Package size
HmiRQP0032	hsmq-0632	MIMAT0000253	hsa-miR-10a	72bp	100 μM	500 rxn
HmiRQT0001	Positive Control cDNA Mix				10× Mix	3 rxn

III. Additional Materials Required or Recommended

GeneCopoeia All-in-One™ miRNA qRT-PCR Detection Kit (Cat Nos. AOMD-Q020 or AOMD-Q050)

IV. Procedure

1. Upon receiving centrifuge the tubes at 12,000 rpm for 30 seconds so that the liquid stays at the bottom of the tubes.
2. The primers are dissolved in TE (10mM Tris-Cl, 1mM EDTA). The concentration of each primer is 100 μM. Dilute the primers to the concentration of 2 μM with sterilized ddH_2O before use.
3. For each reaction, take one of the primers and add it to a PCR primer reaction tube (2 μl primer for 20 μl reaction). The final working concentration of the primer should be 0.2 μM. The products are used together with the All-in-One miRNA qRT-PCR Detection Kit. For details, refer to the appendix of primer-specific All-in-One miRNA qPCR Validation Report, Part B, Procedure, Section "qPCR to detect miRNA".
4. The primers are provided with a positive control cDNA mix for use as templates for validation. The quality of the primers may be validated with the method described in the appendix of primer-specific All-in-One miRNA qPCR Validation Report, Part B, Procedure, Section "qPCR to detect miRNA".
5. These primers should be used with a miRNA Poly (A) tailing kit. It is not suitable for use with a TaqMan® miRNA detection kit.
6. The positive control cDNA mix is synthesized with the All-in-One miRNA qRT-PCR Detection kit which uses a uniquely designed oligo-dT adaptor as the reverse transcription primer and a universal reverse qPCR primer that specifically matches to the oligo-dT adaptor. The mix cannot be used in conjunction with other miRNA Poly (A) tailing kits to validate the All-in-One miRNA qPCR primer.

V. Storage

Store in -20℃ and avoid repeated freeze-thaw cycles .

VI. Applications

All-in-One miRNA qPCR Primers are used for detection of miRNA expression. When combined with the All-in-One miRNA qRT-PCR Detection Kit the primers can be used to detect miRNAs both qualitatively and quantitatively.

VII. All-in-One miRNA qPCR Primer Validation Report

A. Materials and Methods

1. Instrument
   iQ5 Real Time PCR Detection System: Bio-Rad

2. Reagents
   All-in-One™ miRNA qRT-PCR Detection Kit (Catalog Nos. AOMD-Q020 or AOMD-Q050)
   Validation templates: cDNAs extracted from 10 different human tissues

B. Procedure

RNA Extraction

1. Sample preparation

Place a small amount of material from a human tissue sample into a pre-cooled mortar. Add a small amount of liquid nitrogen and grind the tissue to a fine powder. Transfer the powder to a centrifuge tube containing 1 ml of TRIzol (Invitrogen). Vortex for 5 minutes. (If the samples are cultured cells, use about 10^6~10^7 cells with 1 ml of TRIzol). Pipette up and down until the cells are completely lysed).

2. Phase separation

Leave the cell or tissue samples at room temperature for about 10 minutes. Add 200 µl of chloroform per 1 ml of TRIzol. Close the cap and vortex vigorously for 1 minute. Let the samples settle down at room temperature for 2-5 minutes. Centrifuge at 12,000 *g* for 15 minutes at 6℃. Remove tubes from the centrifuge being careful not to disturb the liquid. The samples should be separated into 3 layers with RNA in the top layer.

3. RNA Precipitation

Carefully transfer about 450 µl (per 1 ml TRIzol) of the supernatant to a new centrifuge tube containing 600 µl of cold 2-propanol. Mix well and keep at –20℃ for 10 minutes. Then centrifuge at 12,000 *g* for 10 minutes (at 6℃).

4. Washing

Remove the supernatant. Add 500 µl of cold 75% ethanol. Vortex until a pellet forms at the bottom of the tube. Centrifuge at 12,000 *g* for 5 minutes (6℃). Remove the supernatant. Centrifuge briefly again and remove the remaining supernatant.

5. Dissolve RNA

Air-dry the samples for 5 to 10 minutes. (Do not over dry the samples. The samples are dry when they turn white). Dissolve the samples in 30 µl of DEPC water. Label the samples properly and store them at –80℃.

6. Determine RNA Concentration

Take 1 µl of RNA sample. Dilute the sample 1:10 in DEPC water. RNA concentration can be measured with a NanoDrop (Thermo Scientific). DEPC water should be used as a blank. Record both the RNA concentration and the ratio of A_{260}/A_{280}.

7. RNA electrophoresis

7.1. Prepare denaturing gel

Add 1 g of agarose into 75 ml of de-ionized water. Boil the agarose for 1–2 minutes and cool it down to about 70℃. Add 10 ml of 10X MOPS, 15 ml of formaldehyde and 5 µl of ethidium bromide (EB). Pour the gel into a tray with big combs. Cover the tray.

7.2. Prepare electrophoresis buffer (1X MOPS)

Take 50 ml of 10X MOPS and dilute 1:10 to the final volume of 500 ml with de-ionized water. Pour the buffer into a gel box, and add some EB to it.

7.3. Preparation of RNA sample

Take 3 µl of the RNA sample and add DEPC to a total volume of 18 µl. Heat the sample at 65℃ for 10 minutes. Cool it down immediately. Add 2 µl of 1X RNA loading buffer into the sample.

7.4. RNA electrophoresis

Place the RNA gel in a gel box with 1X MOPS electrophoresis buffer. Pre-run the gel at 100 V for 5 minutes. Load the treated RNA samples into the gel and run at 100 V until the bromophenol blue runs to about one third the length of the gel. Take a picture using a UV scanner.

8. RNA detection results

Electrophoresis results of RNAs from 10 different tissues（3 µl RNA each well). See table 8.2 for the specific lane information.

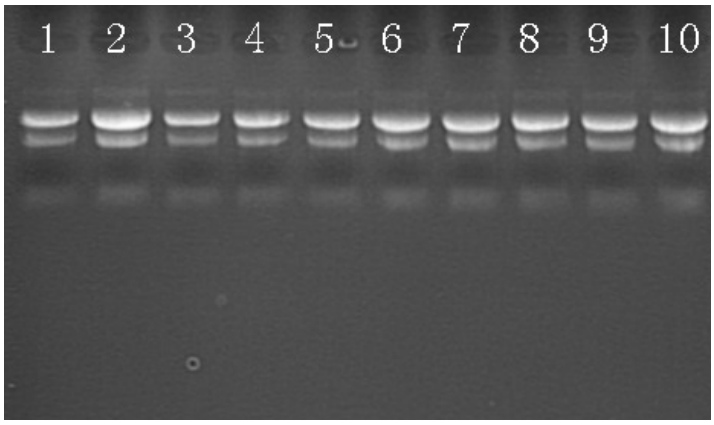


Table 8.2. RNA sample source, concentration and the ratio of OD260/OD280.

Lane	Tissue	Concentration (ng/μl)	OD A_{260}/A_{280}
1	Brain	2385	1.9
2	Lung	2430	1.94
3	Liver	2521	1.91
4	Kidney	3444	1.94
5	Breast	2785	1.87
6	Testis	2972	1.9
7	Placenta	3515	1.91
8	Spleen	3344	1.91
9	Heart	3394	1.91
10	Pancreas	3101	1.91

Note: In order to detect miRNAs, the extracted RNA must contain small molecular weight RNAs. The kit used for RNA extraction must be applicable for isolation of total RNA or small RNA. The quality of RNA is critical to the success of downstream experiments. Follow the instructions of the RNA extraction kit exactly. Examine RNA samples by electrophoresis to ensure high quality.

miRNA reverse transcription

1. Thaw all the reagents needed for miRNA reverse transcription. Mix reagents well by gently inverting the tubes. Spin down briefly and keep on ice.

2. Prepare miRNA reverse transcription reaction: Add the following reagents into an RNase-free reaction tube which is pre-cooled on ice. The final volume should be 25 μl.

Reagents	Volume	Final concentration
Total RNA		2 μg
2.5 U/μl Poly A Polymerase	1 μl	2.5 U
RTase Mix	1 μl	
5X Reaction Buffer	5 μl	1X
ddH_2O (RNase/DNase free)	to 25 μl	

Note: The total RNA in the reaction must contain small molecular RNA. If total RNA is used, the amount of RNA should be between 1 ng ~ 5 μg. If the purified small molecular RNA is used, the amount of RNA should be between 0.1 ng ~ 1 μg

3. Reverse Transcription Reaction: Mix reaction solution well. Spin down briefly. Incubate the reaction solution at 37℃ for 60 minutes. Terminate the reac tion by heating at 85℃ for 5 minutes. The products of reverse transcription can be diluted 1:5 with sterile water for the downstream qPCR reaction.

qPCR to detect miRNA

1. Thaw 2X All-in-One qPCR mix from the All-in-One miRNA qRT-PCR Detection Kit. Mix well by inverting the tube several times. Spin down briefly and keep on ice.

2. Prepare qPCR reaction solution on ice. All miRNAs are tested in duplicate. NTC (No template control) is tested singly.

Reagents	Volume	Final Concentration
2×All-in-One qPCR Mix	10 μL	1X
All-in-One miRNA qPCR Primer	(2 μM) 2 μL	0.2 μM
Universal Adaptor PCR Primer	(2 μM) 2 μL	0.2 μM
First strand cDNA (diluted 1:5)	2 μL	
ddH2O	4 μL	
Final volume	20 μL	

3. Mix the qPCR reaction solution well. Transfer the solution to a PCR tube. Spin briefly to make sure that the reaction solution is at the bottom of the tube.

4. The qPCR reaction, using the standard 3-step method to detect the DNA amount (these experiments were designed based on Bio-Rad iQ5 instrument). Melting analysis can be done immediately after the amplification reactions.

Number of Cycles	Step	Temperature	Time	Detection
1	Pre-denature	95 ℃	10min	No
40	Denature	95 ℃	10sec	No
	Annealing	See reference below	20sec	No
	Extension	72 ℃	10sec	Yes

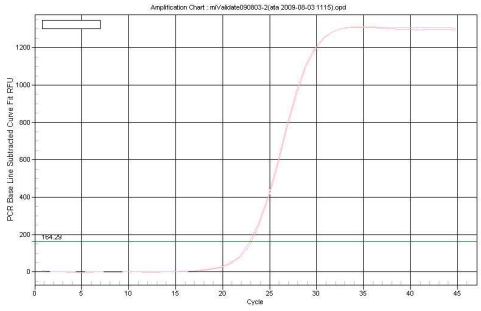
Temperature range	Rate of temperature change	Duration	Detection
66 ℃ ~ 95 ℃	0.5 ℃/ step	6 sec/ step	Yes
30 ℃		30 sec	No

Note: The above conditions are designed for use with the Bio-Rad iQ5Q-PCR instrument. If other instruments are used, adjust the extension time and conditions for analysis as recommended by the manufacturers. The annealing conditions for each miRNA assay may be different. Please refer to the details of the conditions in the following section.

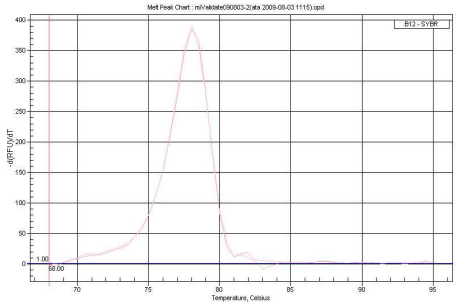
C. Test Results

hsa-miR-10a（hsmq-0632 primer）

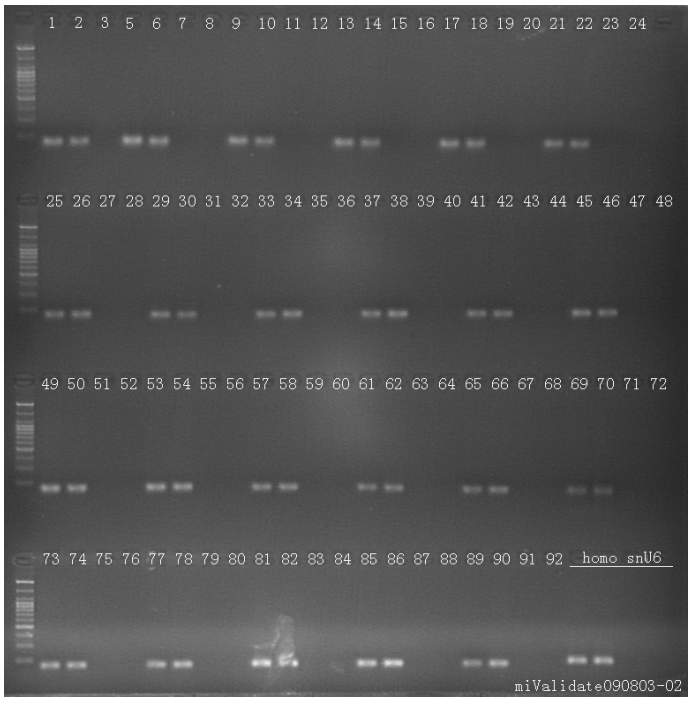
1.1 Validation.

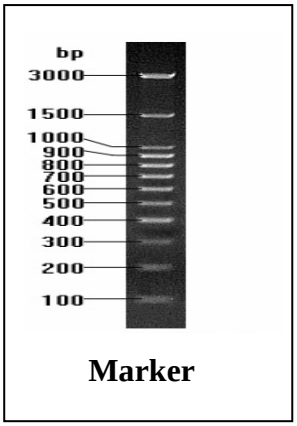


hsa-miR-10a Amplification Plot



hsa-miR-10a Melting Analysis Curve





Electrophoresis Result in lane **85-86-87-88** (contains two positive controls and two NTC) (5 µl of the PCR of products, run on 3% agarose gel)

1.2 Discrimination assay of primer with single-base differences in their sequences

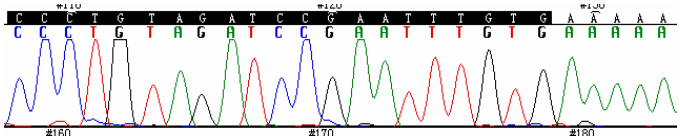
1.2.1 hsa-miR-10a BLAST

hsa-miR-10a	UACCCUGUAGAUCCGAAUUUGUG
hsa-miR-10b	UACCCUGUAGAACCGAAUUUGUG

The BLAST alignment shows that a single-base difference exists between the two miRNAs: hsa-miR-10a and hsa-miR-10b. To discriminate and validate the primer of interest (hsa-miR-10a) . Two plasmids were constructed for each of the two miRNAs.

1.2.2 quence result of the miRNA qPCR plasmids

hsa-miR-10a sequence result：



hsa-miR-10b sequence result：

1.2.3 qPCR validation

Using the All-in-One miRNA qRT-PCR Detection Kit, the hsa-miR-10a -specific primer was validated using miRNA expression plasmids for hsa-miR-10a and hsa-miR-10b as templates (about 10^7 molecules/reaction).

The percent relative discrimination was calculated using the differences between the Ct values of the mismatched template and the matching template with respect to the hsa-miR-10a-specific primer (Relative discrimination =$2^{-\Delta Ct}$ ×100%). The results are shown in the table below:

Annealing Temp.(℃)	Ct value for miRNA expression plasmid amplification		Relative Discrimination
	hsa-miR-10a	hsa-miR-10b	hsa-miR-10b
64.5	22.20	29.05	0.867%
63.3	20.24	27.02	0.908%
61.4	18.04	24.80	0.924%

1.2.4 Conclusion

Based on these results, a qPCR annealing temperature at 61.4℃ is recommended. This range gives the highest amplification efficiency as well as the best relative discrimination between the two single-base-different miRNAs.

VIII. Limited Use License and Warranty

Limited Use License

Following terms and conditions apply to use of all OmicsLink™ ORF Expression Clones in all lentiviral vectors and Packaging 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.

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