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miR-ID®: miRNA quantification assays

Experimental Protocol

20171009_miR-ID®

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Table of Contents

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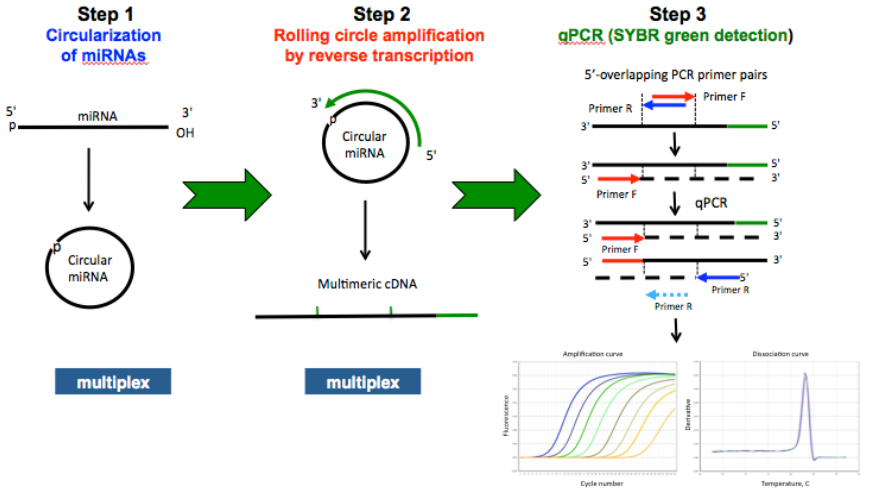
miR-ID®: miRNA Detection Assays

I. Overview

miR-ID® is a novel platform for detecting miRNA using a circularization-based RT-qPCR method. miR-ID® is highly sensitive, uses single-dye detection, and can discriminate miRNA isoforms with single nucleotide differences at any position along the molecule. The miR-ID® assay is designed to detect specific miRNAs in biological samples and works well with all sample sources, including total RNA. miR-ID® is capable of detecting 2'-OMe modifications on the 3' ends of piRNAs in animals and all small RNAs in plants.

The assay consists of a 3-step protocol in which Steps 1 and 2 (see flowchart below) can be performed in multiplex. If working with synthetic miRNAs, these RNAs must have 5'-p and 3'-OH groups at their termini.



Schematic of miR-ID®. Step 1: Circularization of linear miRNAs; **Step 2:** Reverse transcription mediated by rolling circle amplification to obtain multimeric cDNA; **Step3:** Quantification of the amplicon by RT-qPCR using highly sequence-specific 5'-overlapping primers and SYBR Green detection. Kumar et al (2011) *RNA* 17:365.

II. miR-ID® Kit Contents

Store all components at -20°C

1. Circularization Reaction Mix
2. RT Primer/s
3. RT Reaction Mix
4. RT Dilution Buffer
5. Nuclease-free Water
6. PCR Primers

III. User-supplied Reagents, Consumables, and Laboratory Equipment (not included)

Reagents

- CircLigase II (Epicentre, cat# CL9021K)
- RNAseOUT (Thermo Fisher Scientific, cat# 10777019)
- Superscript IV Reverse Transcriptase (Thermo Fisher Scientific, cat# 18090010)

  SYBR green reagents such as
  - 2X iQ SYBR Green Supermix (BioRad, cat# 170-8880)
  - Rox reference dye (Thermo Fisher Scientific, cat# 12223012)

Consumables

- Pipettes and nuclease-free filtered pipette tips for volumes 2 to 100 mL.
- RNAse-free microfuge tubes (1.5mL).
- PCR tubes or PCR strip tubes.
- Micro-Amp Fast Optical 96-Well Reaction Plate, for example Thermo Fisher Scientific cat# 4346906
- Micro-Amp Clear Adhesive Film, for example Thermo Fisher Scientific cat# 4360954

Equipment

- Ice bucket/freezer plate for reagents and enzymes
- Centrifuge for 96-well plates
- Thermal Cycler
- Real-time qPCR instrument, for example 7500-Real Time PCR System (Thermo Fisher Scientific)

IV. Experimental Protocol

Subsection A: singleplex (yields 20 µL cDNA)

1. miRNA circularization

1.1. Allow Circularization buffer and RNA samples to thaw on ice.

1.2. For each biological sample combine components listed below. When assaying multiple samples, make a combined mastermix and allow 10% overage for pipetting loss. Gently mix components and pulse-spin to collect liquid on bottom. Keep on ice. Aliquot 5 µL into each PCR tube (single or strip tubes).

Reagent	Volume
Circularization Reaction Mix	2.5 µL
RNA (1 to 20 ng total)	2 µL
RNAseOUT	0.3 µL
Circ Ligase II	0.2 µL
Total volume	**5 µL**

1.3. Incubate the circularization mixture at 60°C for 15 minutes and transfer to ice.

2. Reverse transcription

2.1. Allow RT buffer and RT primer(s) to thaw on ice.

2.2. In an RNAse-free tube, combine components listed in the table below. When assaying multiple samples, make a combined mastermix and allow 10% overage for pipetting loss. Gently mix components and pulse-spin to collect liquid on bottom. Keep on ice.

Reagent	Volume
RT Reaction Mix	6.4 µL
RT Primer/s	0.2 µL*
SuperScript IV Enzyme	0.4 µL
Nuclease-free Water	8.0 µL*
Total volume	**15 µL**

* (If only one sample is prepared, the RT primer should be diluted 10 times with RT dilution buffer. 2 µL of the diluted RT primer will be added and the volume of the nuclease-free water will be reduced to 6.2 µL)

2.3. Aliquot 15 µL of the mix into each sample, yielding a final volume of 20 µL. Gently mix components and pulse-spin to collect liquid on bottom.

2.4. Place tubes into thermal cycler and run the following parameter values:

Temperature	Time
42°C	60 min
75°C	15 min
4°C	∞

2.5. Mix gently and centrifuge briefly.

Stopping point: RT reactions maybe be used directly in qPCR or stored at -20°C.

Subsection B: multiplex (yields 80 µL cDNA)

1. miRNA circularization

1.1. Allow Circularization buffer and RNA samples to thaw on ice.

1.2. For each biological sample combine components listed below. When assaying multiple samples, make a combined mastermix and allow 10% overage for pipetting loss. Gently mix components and pulse-spin to collect liquid on bottom. Keep on ice. Aliquot 20 µL into each PCR tube (single or strip tubes).

Reagent	Volume
Circularization Reaction Mix	10 µL
RNA (1 to 20 ng total)	8 µL
RNAseOUT	1.2 µL
Circ Ligase II	0.8 µL
Total volume	**20 µL**

1.3. Incubate the circularization mixture at 60°C for 15 minutes and transfer to ice.

2. Reverse transcription (multiplex)

2.1. Allow the RT buffer and RT primers to thaw on ice.

2.2. Prepare the RT primer mix using the supplied RT primers: for each biological sample that will be analyzed, pipet 1 µl of each primer into an RNAse-free tube and add nuclease-free water to a final volume of 30 µl (or multiples thereof if multiple biological samples are being analyzed, see Appendix A for sample calculations).

2.2. In an RNAse-free tube, combine the components listed in the table below. When assaying multiple samples, make a combined mastermix and allow 10% overage for pipetting loss. Gently mix components and pulse-spin to collect liquid on bottom. Keep on ice.

Reagent	Volume
RT Reaction Mix	25.6 µL
RT Primer Mix	24.0 µL
SuperScript IV Enzyme	1.6 µL
Nuclease-free Water	8.8 µL
Total volume	**60 µL**

2.3. Aliquot 60 µL of the mix into each sample, yielding a final volume of 80 µL. Gently mix components and pulse-spin to collect liquid on bottom.

2.4. Place tubes into thermal cycler and run the following parameter values:

Temperature	Time
42°C	60 min
75°C	15 min
4°C	∞

2.5. Mix gently and centrifuge briefly.

Stopping point: RT reactions maybe be used directly in qPCR or stored at -20°C.

3. Quantitative RT-PCR

The qPCR step is always performed in singleplex (each miRNA is assayed in a single reaction), even if circularization and RT are performed in multiplex. Forward and reverse primers for each miRNA are included in the miR-ID kit. A typical 20 µL reaction for a single miRNA is described below. Note that each RT reaction of the multiplex setup serves as template for qPCR for each miRNA assay.

NOTE: Refer to the vendor's user guide for instructions on setting up the qPCR master mix. The following master mix preparation is specific for BioRad's 2x iQ SYBR Green Supermix.

3.1. Allow the 2x qPCR master mix (light sensitive), Rox reference dye, qPCR primers, and RT reactions to thaw on ice.

3.2. A separate master mix needs to be prepared for each assayed miRNA. Calculate master mix components for each miRNA by mulitplying the reagent volume by the number of samples and by the number of technical repeats (triplicate analysis is recommended). Allow 10% overage for pipetting loss. Mix gently and spin down the qPCR master mix.

Reagent	Volume
2x iQ SYBR Green Supermix	10 µL
Nuclease-free Water	7.1 µL
qPCR Primers (miRNA specific)	0.6 µL
Rox Reference Dye	0.3 µL
Total volume	**18 µL**

3.3. Pipet 18 µL of the appropriate master mix into wells of a qPCR plate.

3.4. Pipet 2 µL cDNA from the RT reaction into the appropriate individual wells.

3.5. Seal qPCR plate with optical seal and centrifuge to bring the solution to the bottom of the plate.

3.6. Set up the following program for the real-time thermal cycler and start the run.

Assay: Standard Curve (Absolute Quantification)
Run mode: Standard 7500

Step Type	Temperature	Time
HOLD	50°C	2 min
HOLD	95°C	3 min
CYCLE (**40 cycles**)	95°C	15 sec
	56°C	45 sec
	66°C + 0.1 per cycle	30 sec
	67°C + 0.2 per cycle*	30 sec
Include standard melting curve profile at the end of 40 cycles		

* Set up data collection at this step.

Data Analysis

For instructions on how to analyze and export raw Ct values, refer to the appropriate instrument user guide. The general process typically involves the following procedures:

- View the amplification plots.
- Set the baseline and threshold values and obtain Ct values.
- Analyze melting curves for PCR products.

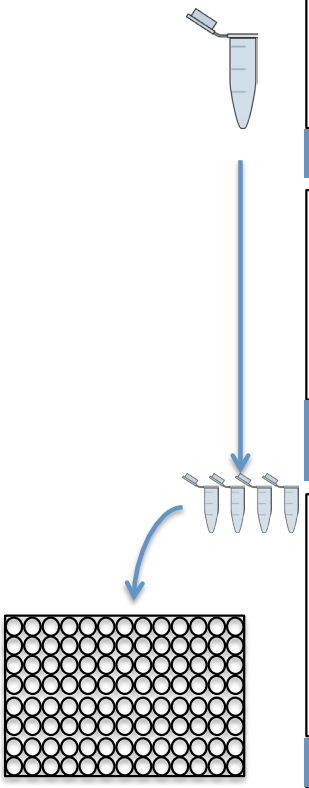
V. **Appendix A:** Table for RT Primer Mix

1 biological sample	
# of RT primers (1 µL each)	µL RT dilution buffer
1	29
2	28
3	27
4	26
5	25
6	24
7	23
8	22
9	21
10	20
11	19
12	18
13	17
14	16
15	15
16	14
17	13
18	12
19	11
20	10
21	9
22	8
23	7
24	6
25	5
26	4
27	3
28	2
29	1
30	0

2 biological samples	
# of RT primers (2 µL each)	µL RT dilution buffer
1	58
2	56
3	54
4	52
5	50
6	48
7	46
8	44
9	42
10	40
11	38
12	36
13	34
14	32
15	30
16	28
17	26
18	24
19	22
20	20
21	18
22	16
23	14
24	12
25	10
26	8
27	6
28	4
29	2
30	0

3 biological samples	
# of RT primers (3 µL each)	µL RT dilution buffer
1	87
2	84
3	81
4	78
5	75
6	72
7	69
8	66
9	63
10	60
11	57
12	54
13	51
14	48
15	45
16	42
17	39
18	36
19	33
20	30
21	27
22	24
23	21
24	18
25	15
26	12
27	9
28	6
29	3
30	0

VI. **Appendix B:** Overview of Working Steps for Singleplex Reactions



Step 1) Circularization
Combine for each sample:

- 2.5 µL CIRC Reaction Mix
- 2 µL total RNA
- 0.3 µL RNAse out (Life Technologies)
- 0.2 µL CircLigase II (Illumina)

Incubate at 60°C for 15 min, then cool to 4°C

Step 2) Reverse Transcription
Combine and add to each sample:

- 6.4 µL RT Reaction Mix
- 0.2 µL RT primer or 2 µl diluted RT primer
- 0.4 µL Superscript II (Life Technologies)
- water for a total of 15 µL

Incubate at 42°C for 1 hour, at 75°C for 15 min, then cool to 4°C

Step 3) qPCR (for BioRad iQ Supermix)
Combine for each miRNA:

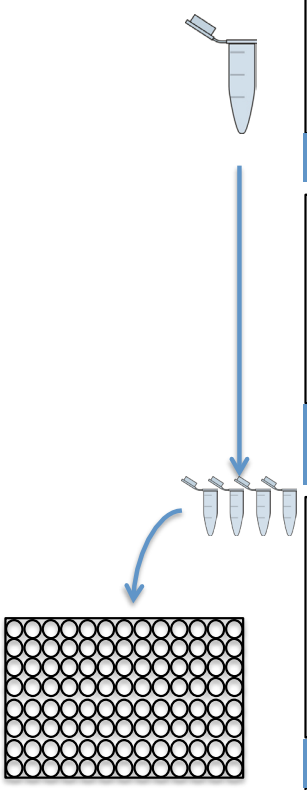
- 10 µL iQ Supermix (BioRad)
- 7.1 µL H_2O
- 0.6 µL PCR primer mix
- 0.3 µL Rox Dye (Life Technologies)
- 2 µL cDNA per sample from RT reaction

Cycle parameters

Temperature	Time
50°C	2 min
95°C	3 min
CYCLE (40 cycles):	
95°C	15 sec
56°C	45 sec
66°C (+0.1°C per cycle)	30 sec
67°C (+0.2°C per cycle)	30 sec (data collection)

Include standard melting curve profile at the end of 40 cycles

VII. **Appendix C:** Overview of Working Steps for Multiplex Reactions



Step 1) Circularization
Combine for each sample:
- 10 µL CIRC Reaction Mix
- 8 µL total RNA
- 1.2 µL RNAse out (Life Technologies)
- 0.8 µL CircLigase II (Illumina)

Incubate at 60°C for 15 min, then cool to 4°C

Step 2) Reverse Transcription
Combine and add to each sample:
- 25.6 µL RT Reaction Mix
- 24 µL RT primer or 2 µl diluted RT primer
- 1.6 µL Superscript II (Life Technologies)
- 8.8 µL water

Incubate at 42°C for 1 hour, at 75°C for 15 min, then cool to 4°C

Step 3) qPCR (for BioRad iQ Supermix)
Combine for each miRNA:
- 10 µL iQ Supermix (BioRad)
- 7.1 µL H_2O
- 0.6 µL PCR primer mix
- 0.3 µL Rox Dye (Life Technologies)
- 2 µL cDNA per sample from RT reaction

Cycle parameters

Temperature	Time
50°C	2 min
95°C	3 min
CYCLE (40 cycles):	
95°C	15 sec
56°C	45 sec
66°C (+0.1°C per cycle)	30 sec
67°C (+0.2°C per cycle)	30 sec (data collection)

Include standard melting curve profile at the end of 40 cycles

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