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miR-Direct®: assay for miRNA quantification from human plasma

Experimental Protocol

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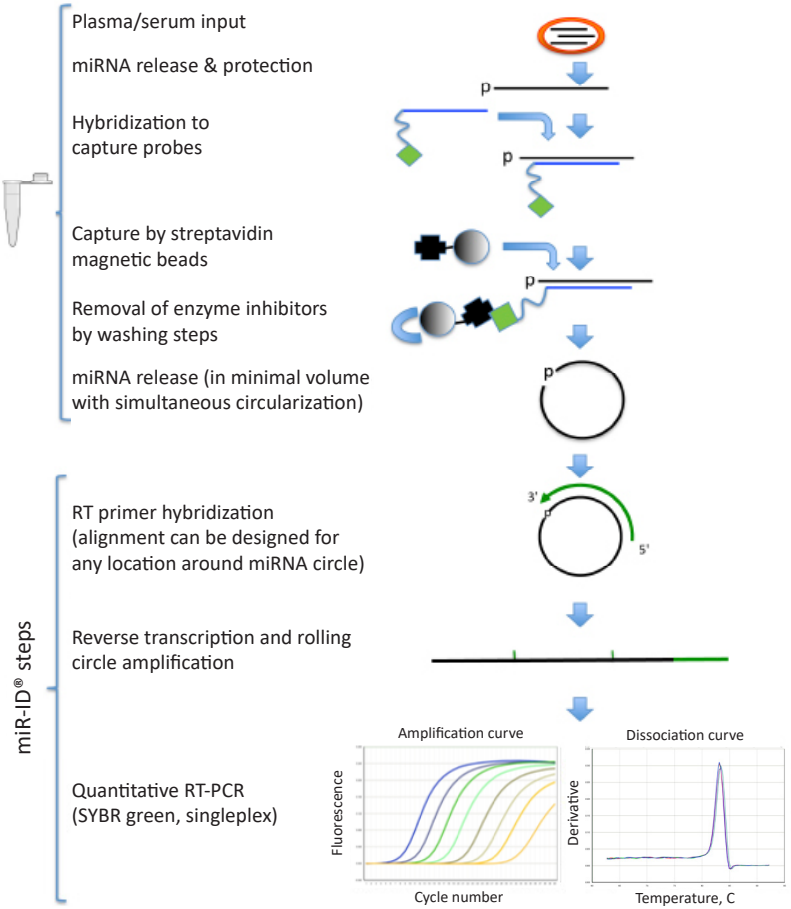
miR-Direct®: miRNA Detection from Human Plasma

I. Overview

The miR-Direct® assay is designed to detect miRNAs present in plasma (50 to 400 µl). The first part of the assay is performed in one tube and entails miRNA release from plasma, miRNA capture, washing steps, and miRNA release with simultaneous circularization. The second part is miRNA quantification by SomaGenics' miR-ID® technology, consisting of reverse transcription by rolling circle amplification and real-time qPCR using SYBR green. All steps up to quantitative qPCR can be performed in multiplex for all miRNAs of interest.



II. miR-Direct® Kit Contents

miR-Direct core kit components

Store at ambient temperature

1. Buffer 1*

Store at 4°C

1. Wash Buffer
2. Buffer 3
3. Magnetic Beads

Store at -20°C

1. miR-cel-39 synthetic spike-in miRNA control
2. Spike-in Dilution Mix
3. Protease Solution**
4. Buffer 2
5. cel-39 probe (use in probe master mix and freeze individually or as mix afterwards)
6. Circularization Reaction Mix
7. RT Reaction Mix
8. cel-39 RT Primer (use in RT master mix and freeze individually or as mix afterwards)
9. cel-39 qPCR Primer Pair
10. Nuclease-free Water
11. RT-dilution buffer

* CAUTION: Irritant. Contains guanidine hydrochloride. Use proper personal protective equipment. Do not use bleach.

** **Keep at -20°C upon receipt. Store at 4°C after thawing for first use.**

miR-Direct miRNA-specific assay components

Store at -20°C

1. miRNA-specific probe (use in probe master mix and freeze individually or as mix afterwards)
2. miRNA-specific RT Primer (use in RT master mix and freeze individually or as mix afterwards)
3. miRNA-specific qPCR Primer Pair

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

Reagents

- Streptavidin Magnetic beads (New England BioLabs, cat# S1420S)
- SuperScript II Reverse Transcriptase (ThermoFisher Scientific, cat#18064022)
- CircLigase II (Lucigen / LGC) cat# CL9021K)
- RNAseOUT (ThermoFisher Scientific, cat# 10777-019)
- 2X iQ SYBR Green Supermix (BioRad, cat#170-8880)

Consumables

- Pipettes and nuclease-free filter pipette tips for volumes 2 to 100 µl.
- RNAse-free microfuge tubes (1.5 ml).
- PCR tubes or PCR strip tubes.
- Micro-Amp Fast Optical 96-Well Reaction Plate, for example ThermoFisherScientific # 4346907
- Micro-Amp Clear Adhesive Film, for example ThermoFisherScientific # 4311971

Equipment

- 12-tube Magnetic Separation Rack, for example NEB cat# S1509S
- Centrifuge for 1.5 mL and PCR tubes
- Centrifuge for 96-well PCR plates
- End-over-end rotator (at 4°C)
- Water bath/heat block (25°C)
- Water bath/heat block (37°C)
- Water bath/heat block (60°C)
- Thermal Cycler
- RT-PCR Thermal Cycler, for example ABI 7500 Fast System

IV. Experimental Protocol

1. Component preparation

Resuspend magnetic beads by vortexing them at high speed and then placing them on a tube rotator at 4°C for at least 120 minutes .

NOTE: Magnetic beads tend to aggregate while in storage, so it is imperative that they are fully dispersed in solution before they are used. *If beads have not been used for more than 7 days, they should be rotated at 4°C overnight.*

1.1. Place at room temperature to thaw:

- miR-cel-39 spike-in miRNA
- Spike-in Dilution Mix
- Protease Solution* (first use only -- **do not vortex**)
- Buffer 2
- All Probes
- Circularization Reaction Mix
- RT Reaction Mix
- All RT Primers
- Nuclease-free Water
- Wash Buffer (although not frozen, wash buffer should be at room temperature prior to use)

**Protease solution is shipped frozen. Store at -20°C until first use. After thawing for the first time, store at 4°C (DO NOT REFREEZE).*

All previously frozen reagents listed above must be vortexed (except for protease) and centrifuged before use.

Return all reagents and enzymes to proper storage conditions after use.

1.2. Store all enzymes at -20°C (except protease solution) until they are needed in the protocol.

2. Make probe and RT primer mixes

For both of the mixes, include the probe or the RT primer of the cel-39 control assay if used.

Probe mix:

For 45 samples make a 320 µl probe mix: pipet 8 µl of each probe that is used for analysis (max. 40 different targets) into an RNAse-free tube. Add RT dilution buffer to a final volume of 320 µl.

Vortex and add to samples as directed. Any unused probe mix can be stored at -20°C.

RT primer mix:

For each biological sample that will be analyzed pipet 1 µl of each RT primer into an RNAse-free tube. Add RT-dilution buffer to a final volume of 40 µl per sample. When assaying multiple samples, allow a 10% overage for pipetting errors. Vortex and use as directed. Any unused RT primer mix can be stored at -20°C.

3. Thaw plasma samples and prepare Spike-in Mix

3.1. Use plasma IMMEDIATELY after it is thawed at 25°C.

3.2. Preparation of Spike-in Mix (use even if no Spike-in is used)

While plasma is thawing, prepare the Spike-in Mix according to the table below for the volume of plasma input into the assay. To prepare a master mix for multiple samples, scale the volumes to the desired number of plasma samples to be. Vortex the Spike-in Mix for 3 seconds after the addition of the components.

	Plasma Volume		
	50 µl	**200 µl**	**400 µl**
miR-cel-39*	4 µl	4 µl	4 µl
Spike-in Dilution Mix	0.3 µl	1.2 µl	2.4 µl

*Use RT dilution buffer if no Spike-in is used

4. Plasma lysis

NOTE: Work ***one tube at a time.***

4.1. Depending on the amount of plasma used, add the indicated reagent volume to the sample in the order listed below. Vortex samples for 3 seconds after the addition of each reagent.

	Plasma Volume		
	50 µl	**200 µl**	**400 µl**
Buffer 1*	50 µl	200 µl	400 µl
Protease Solution	2 µl	8 µl	16 µl
Spike-in Mix	4.3 µl	5.2 µl	6.4 µl

* CAUTION: Irritant. Contains guanidine hydrochloride.

4.2. Incubate samples at 25°C for 60 min

5. miRNA capture

NOTE: Work ***one tube at a time.***

5.1. Depending on the amount of plasma used, add the indicated reagent volume to the sample in the order listed below. Vortex samples for 3 seconds after the addition of each reagent.

	Plasma Volume		
	50 µl	**200 µl**	**400 µl**
Buffer 2	11.5 µl	46 µl	92 µl
Probe Mix	6.4 µl	6.4 µl	6.4 µl

5.2. Incubate at 37°C for 60 min

5.3. Add 20 µl magnetic beads (and Buffer 3 if indicated) and vortex each tube for 3 seconds after addition.

	Plasma Volume		
	50 µl	**200 µl**	**400 µl**
Magnetic Beads	20 µl	20 µl	20 µl
Buffer 3	275 µl	none	none

5.4. Incubate at room temperature for 15 minutes

Pulse spin tubes at 325 x g (approximately 3 seconds) to collect the liquid. **Do not allow beads to pellet.**

6. Washing steps

6.1. Place all samples on the magnetic rack for 10 minutes.

NOTE: Work ***one tube at a time.***

6.2. While keeping the tube on the rack, carefully remove the solution from the beads by pipetting from the top of the tube, keeping the pipette tip against the tube wall opposite of the magnetic bead pellet. Be very careful not to disturb the beads.

Discard supernatant.

6.3. Remove the tube from the magnetic rack.

6.4. Add 500 µl room temperature wash buffer and vortex for 3 seconds. Leave tube in regular tube rack.

Repeat steps 6.1 to 6.4 two more times for each tube only 470 µl are removed after final wash (leaving 30 µl buffer with the magnetic beads).

Keep tubes on a bench top rack until all samples are processed.

6.5. Pulse vortex tubes to resuspend the magnetic bead pellet.

6.6. Pulse spin tubes at 325 x g (approximately 3 seconds) to collect the liquid. **Do not allow beads to pellet.**

Leave samples on benchtop rack.

7. miRNA circularization

7.1. Mix the following reagents in a separate tube (same volume for all plasma input volumes). When assaying multiple samples, make a combined master mix and allow 10% overage for pipetting loss. Gently mix components and pulse-spin to collect liquid on bottom. Keep on ice.

Reagent	Volume
Circularization Reaction Mix	5.0 µl
RNAseOUT	0.6 µl
CircLigase II Enzyme	0.4 µl
Nuclease-free Water	4.0 µl
Total	**10.0 µl**

NOTE: Work ***one tube at a time.***

7.2. Place sample tube from step 6.6. on the magnetic rack. When beads have aggregated, solution will be clear.

7.3. While keeping the tube on the rack, carefully remove the solution from the beads by pipetting from the top of the tube, keeping the pipette tip against the tube wall opposite of the magnetic bead pellet. Be very careful not to disturb the beads.

7.4. Immediately after removing wash buffer, remove the tube from the magnetic rack and pipet 10 µl circularization mixture from step 7.1. onto the bead pellet. Pipette up and down 10 times to fully resuspend the beads into the circularization mixture, making sure that there are no beads left on the side of the tube.

7.5. Repeat steps 7.2 to 7.4 with the remaining samples.

7.6. Pulse spin tubes at 325 x g (approximately 3 seconds) to collect the liquid. **Do not allow beads to pellet.**

7.7. Incubate beads in circularization mixture at 60°C for 15 minutes.

8. Reverse transcription

8.1. Prepare the RT master mix while the circularization reaction is proceeding: mix the following reagents in a separate tube. When assaying multiple samples, make a combined master mix 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	40.0 µl
SuperScript II Enzyme	1.6 µl
Nuclease-free Water	8.8 µl
Total	**70.0 µl**

8.2. Remove tubes from 60°C (step 7.7.) and place on magnetic rack.

NOTE: Work ***one tube at a time.***

8.3. After magnetic beads have migrated to the tube wall, carefully pipet supernatant (10 µl) into new, labeled PCR tubes.

8.4. Add 70 µl of RT master mix to each sample (80 µl final volume)

8.5. Mix each sample gently and collect liquid by briefly spinning the tubes at 325 x g.

8.6. Place sample PCR tubes into thermal cycler and run the following parameter values:

Step Type	Temperature	Time
HOLD	42°C	60 min
HOLD	75°C	15 min
HOLD	4°C	∞

***Stopping Point*:** RT reactions may be used directly in qPCR or stored at -20°C.

9. Quantitative RT PCR

9.1. If RT products were frozen, thaw samples and pulse vortex to mix and pulse spin to collect liquid.

9.2. Dilute cDNA (5x for 50 µl samples, 20x for 200 µl samples and 40x for 400 µl samples).

9.3. Thaw 2x iQ SYBR green Master Mix and ROX dyes before use.

NOTE: SYBR green and ROX dyes are **light sensitive.** Perform all steps involving SYBR Green and ROX dyes away from direct light sources and keep tubes in dark when not in use.

9.4. Mix qPCR reagents as listed below.

A separate master mix needs to be prepared for each assayed miRNA. Calculate master mix components for each miRNA by multiplying the reagent volume by the number of samples and by the number of technical repeats (triplicates are recommended for quantitative RT-PCR assays). Allow 10% overage for pipetting loss. Mix gently and spin down the qPCR master mix.

Reagent	Volume
2X iQ SYBR Green Supermix	10.0 µl
PCR Primers (miRNA-specific)	0.6 µl
qPCR Reference Dye (ROX)	0.3 µl
Nuclease-free Water	7.1 µl
Total	**18.0 µl**

9.5. Pipet 18 µl of the appropriate master mix into the well of a qPCR plate.

9.6. Pipet 2 µl cDNA from the RT reaction (after 8.6. is completed) into the appropriate wells of the qPCR plate.

9.7. Seal qPCR plate with optical seal.

9.8. Centrifuge plate at 4000 rpm for 20-30 seconds.

9.9. Place plate in the ABI thermal cycler. Run qPCR using the profile below.
Assay: Standard Curve (Absolute Quantification)
Run mode: Standard 7500 - (if using the ABI 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.

V. Appendix: Overview of Working Steps

Plasma lysis
add to each sample:
Buffer 1
Protease
Spike-in RNA (cel-39)

Incubation: 25°C, 60 min

miRNA capture
add to each sample:
Buffer 2
Probe Mix

Incubation: 37°C, 60 min

miRNA capture, 2
add to each sample:
Magnetic Beads
(Buffer 3)

Incubation: RT, 15 min

Washing steps
3 repeats

Incubation: RT, 10 min
for each washing step

Circularization
add to each sample:
Circularization Buffer
RNAse inhibitor
Circularization Enzyme
Water

Incubation: 60°C, 15 min

Reverse Transcription
add to each sample:
RT Buffer
RT Primer Mix
Super Script II enzyme
Water

Incubation: 42°C, 60 min,
75° C, 15 min

qPCR (for BioRad iQ Supermix)
Combine for each miRNA:
iQ Supermix (BioRad)
H_2O
PCR primer mix
Rox Dye (Life Technologies)
cDNA from RT reaction

Temp.	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

Melting curve profile after 40 cycles

Cycle parameters

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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. The miRNA amplification signal should be 5-6 Cts lower than the no-template control (NTC). A delta Ct of 5 between NTC and miRNA represents that the NTC amplification is only ~3% of the total signal. A delta Ct of 6 means that the NTC contributes only ~1% of the signal.
- Analyze melting curves for PCR products. The profiles of the no-template control (if there is a product) and the miRNA assay should be different. Typically, the NTC melting curves are broader and at a lower temperature than the melting temperature from the miRNA sample, which results from the specific signal and should be one defined peak.

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