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Quantification of circulating microRNA using droplet digital PCR

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We use this protocol and it's working

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Abstract

A protocol for quantification of circulating microRNA using droplet digital PCR with TaqMan assays.
MicroRNAs are purified from platelet-poor plasma using Nucleospin columns
During the purification step samples are spiked with cel-miR-39 as a mean of technical normalization
Normalization are performed by calculating the relative concentration of the target microRNA and the reference.

Guidelines

Blood samples should be obtained using a minimum of venous stasis and with discard of the first 3 mL of blood.
Platelet-poor plasma should be prepared within 2 hours from blood sampling

Materials

MATERIALS

- K2-EDTA containing tubes **Becton Dickinson (BD)** **Catalog #**366643
- Nucleospin®miRNA Plasma **Macherey-Nagal** **Catalog #**740971.50
- TaqMan®MicroRNA Reverse Transcription Kit **Applied Biosystems (ThermoFisher Scientific)** **Catalog #**4366597
- 20X TaqMan MicroRNA Assay **Thermo Fisher Scientific** **Catalog #**4440887
- ddPCR Supermix for probes (no dUTP) **Bio-Rad Laboratories** **Catalog #**1863024
- Automated Droplet Generation Oil for Probes **Bio-Rad Laboratories** **Catalog #**1864110
- DG32™ Automated Droplet Generator Cartridges **Bio-Rad Laboratories** **Catalog #**1864108

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Protocol materials

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Before start

We recommend to use a few samples to test if the volume of spike-in added during microRNA purification is adequate before purifing all your samples.
The concentration of the spike-in and the target miRNA should be within the same range, otherwise adjust the volume of spike-in added during miRNA purification.

Preparation of platelet-poor plasma (PPP)

1 Dual centrifugation

1. use 10 ml of EDTA anticoagulated whole blood
2. centrifugation at 3000 g for 15 minutes (acceleration 5, brake 6, temperature 18 °C)
3. transfer plasma phase to new tube, leaving approximately 1 mL of plasma on top of the buffy coat
4. centrifugation at 3000 g for 15 minutes (acceleration 5, brake 6, temperature 18 °C)
5. transfer plasma phase to cryo tubes, leaving approximately 1 ml of plasma in the bottom of the tube
6. store at -80 °C

Equipment

new equipment	NAME
Hettich centrifuge	BRAND
4706-01	SKU
Rotina 420R	SPECIFICATIONS

Note

Alternatively, a prolonged single centrifugation may be used
1. use 5 ml of EDTA anticoagulated whole blood
2. centrifuge at 3000 g for 30 minutes (acceleration 5, brake 6, temperature 18 °C)
3. transfer plasma phase to cryo-tubes, leaving approximately 0.5 ml of plasma on top of the buffy coat

MicroRNA purification

2 Nucleospin®miRNA Plasma **Macherey-Nagal Catalog #**740971.50

3 Follow the instructions given by the manufacture (se notes before you start):

		NucleoSpin® miRNA Plasma
1 Prepare sample		300 µL plasma or serum* 90 µL MLP Vortex 5 s RT, 3 min
2 Precipitate protein		30 µL MPP Vortex 5 s RT, 1 min 11,000 x *g*, 3 min
3 Transfer supernatant		Transfer clear supernatant to Collection Tube (2 mL, lid)
4 Adjust binding conditions		400 µL isopropanol Vortex 5 s
5 Bind RNA and DNA		Load sample on NucleoSpin® miRNA Column RT, 2 min 11,000 x *g*, 30 s
6 *Optional: Digest DNA*		***Optional:*** 1st 700 µL MW2, 11,000 x *g*, 30 s 2nd 250 µL MW2, 11,000 x *g*, 2 min 50 µL rDNase in Reaction Buffer for rDNase RT, 15 min
7 Wash and dry		1st 100 µL MW1, 11,000 x *g*, 30 s 2nd 700 µL MW2, 11,000 x *g*, 30 s 3rd 250 µL MW2, 11,000 x *g*, 2 min
8 Elute RNA		30 µL RNase-free H_2O RT, 1 min 11,000 x *g*, 1 min

Note

STEP 1: use 300 µL platelet-poor plasma
STEP 4: after addition of isopropanol, add also 5 µL spike-in (cel-miR-39, 2.75×10^{-12} M)
STEP 6: perform the optional DNA digest

Reverse transcription

4 TaqMan®MicroRNA Reverse Transcription Kit **Applied Biosystems (ThermoFisher Scientific) Catalog #**4366597

20X TaqMan MicroRNA Assay **Thermo Fisher Scientific Catalog #**4440887

5 Prepare RT master mix:

	Component	Master mix volume per 15 µL reaction*
	100 mM dNTPs (with dTTP)	0.15 µL
	Multiscribe RT enzyme (50 U/µL)	1 µL
	10x RT buffer	1.5 µL
	RNase inhibitor (20 U/µL)	0.19 µL
	Nuclease free water	to 15 µL in total**

*add 10-20% excess volume
**add water to a total reaction volume of 15 µL (including microRNA and RT-primers, see step 7)

Mix gently and place on ice

Note

Remember to include a RT-negative sample (no template)
It is also a good idea to include a RT-positive sample (a microRNA-sample included in all runs)

6 For each RT reaction, combine RT master mix with 2 µL of purified microRNA in a 0.2 µL polypropylene reaction tube
(the volume of RT master mix is dependent on the number of RT-primers, see step 7)
Keep on ice

7 Prepare RT primer mix:
Add for each RT reaction 0.75 µL 20x RT primer from each microRNA assay set

Example with two microRNA assays:

	Component	Volume per 15 µL reaction*
	20x RT specific primer #1	0.75 µL
	20x RT specific primer #2	0.75 µL
	Total volume	1.5 µL**

*add 10-20% excess volume
**when using two microRNA assays the volume of water needed in step 5 is 8.66 µL for each reaction, and the volume of master mix added in step 6 is 11.5 µL

Mix gently

8 Add the appropiate volume of RT primer mix to the reaction tubes prepared in step 6
Mix gently and incubate on ice for 5 minutes

9 Transfer the reaction tubes to a termocycler
Incubate at 16 °C for 30 min, at 42 °C for 30 min and at 85 °C for 5 min.

10 Continue immediately to the PCR amplification or store the RT-reaction in -20 °C.

Droplet digital PCR

11 20X TaqMan MicroRNA Assay **Thermo Fisher Scientific Catalog #**4440887
ddPCR Supermix for probes (no dUTP) **Bio-Rad Laboratories Catalog #**1863024

12 Prepare ddPCR reaction mix:

	Component	Volume per 20.3 µL reaction*
	2x ddPCR supermix	24 µL
	20x TaqMan microRNA assay	2.4 µL
	Nuclease free water	19 µL
	Total volume	**45.4 µL**

*add 10-20% excess volume

Mix gently

13
1. Dilute each of the RT-reactions (cDNAs) 1:10 with nucleace free water (e.g. 2 µL cDNA + 18 µL water)
2. Combine 45.4 µL of ddPCR reaction mix with 2.6 µL of diluted RT-reaction (cDNA) in a 96 well plate

Droplet generation

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DG32™ Automated Droplet Generator Cartridges **Bio-Rad Laboratories Catalog #**1864108

Automated Droplet Generation Oil for Probes **Bio-Rad Laboratories Catalog #**1864110

Equipment	
new equipment	NAME
AutoDG	BRAND
1864101	SKU
Automated Droplet Generator from BioRad	SPECIFICATIONS

15
1. Transfer 22 µL of the reaction mixture into each of two wells on an empty 96 well plate
2. Heat sealing
3. Transfer plate to the AutoDG
4. The AutoDG transfers 20 µL from each well to a GD32 cartidge for droplet generation
5. Heat sealing of output plate

PCR

16 Transfer plate to termocycler
Incubate in 95 °C for 5 minutes
Proceed with 44 cycles of 95 °C for 15 sec and 60 °C for 60 sec.

Final incubation in 98 ºC for 10 min
Cool to 4 °C

Droplet reading

17

Software

QuantaSoft Software

NAME

http://www.bio-rad.com/en-dk/sku/1864011-quantasoft-software-regulatory-edition?ID=1864011

SOURCE LINK

18 ddPCR analysis is performed with QX100 Droplet Reader and QuantaSoft Software

Normalization

19 Normalization is performed by calculating the relative concentration of target miRNA and cel-miR-39