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Quantification of circulating microRNA using droplet digital PCR

PLOS One

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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

Protocol status: Working
We use this protocol and it's working

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13286

Keywords: Circulating microRNAs, sample preparation, microRNA purification, reverse transcription, ddPCR, TaqMan assays

MATERIALS

MATERIALS

- ✕ K2-EDTA containing tubes **Becton-Dickinson 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**

STEP MATERIALS

- ✕ Nucleospin®miRNA Plasma **Macherey-Nagal Catalog #740971.50**
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BEFORE START INSTRUCTIONS

We recommend to use a few samples to test if the volume of spike-in added during microRNA purification is adequate before purifying 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

	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 ₂ O 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

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

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

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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)

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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

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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 μ L reaction*
20x RT specific primer #1	0.75 μ L
20x RT specific primer #2	0.75 μ L
Total volume	1.5 μ L**

*add 10-20% excess volume

**when using two microRNA assays the volume of water needed in step 5 is 8.66 μ L for each reaction, and the volume of master mix added in step 6 is 11.5 μ L

Mix gently

- 8 Add the appropriate 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 thermocycler
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 nuclease 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 #1864101**

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

Equipment

	NAME
AutoDG	BRAND
1864101	SKU
Automated Droplet Generator from BioRad	SPECIFICATIONS

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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 cartridge 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 °C for 10 min
Cool to 4 °C

Droplet reading

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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