Quantification of circulating microRNA using droplet digital PCR

Helle Glud Binderup,Jonna Skov Madsen,Kim Houlind,Rikke Fredslund Andersen,Claus Lohman Brasen

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.

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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

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.

Materials

K2-EDTA containing tubes 366643 by Becton-Dickinson

Nucleospin®miRNA Plasma 740971.50 by Macherey-nagel

TaqMan®MicroRNA Reverse Transcription Kit 4366597 by Applied Biosystems, Foster City, California, USA

20X TaqMan MicroRNA Assay 4440887 by Thermo Fisher Scientific

ddPCR Supermix for probes (no dUTP) 1863024 by BioRad Sciences

Automated Droplet Generation Oil for Probes 1864110 by BioRad Sciences

DG32™ Automated Droplet Generator Cartridges 1864108 by BioRad Sciences

Protocol

Preparation of platelet-poor plasma (PPP)

Step 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

NOTES

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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

EQUIPMENT

Equipment brand:
Hettich centrifuge
SKU:
4706-01
Specifications:
Rotina 420R

MicroRNA purification

Step 2.

REAGENTS

Nucleospin®miRNA Plasma
740971.50 by Macherey-nagel

Step 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

NOTES

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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

Step 4.

REAGENTS

TaqMan®MicroRNA Reverse Transcription Kit 4366597 by Applied Biosystems, Foster City, California, USA

20X TaqMan MicroRNA Assay 4440887 by Thermo Fisher Scientific

Reverse transcription

Step 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

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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)

Step 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

Step 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

Step 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

Step 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.

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

Droplet digital PCR

Step 11.

REAGENTS

20X TaqMan MicroRNA Assay
4440887 by Thermo Fisher Scientific

ddPCR Supermix for probes (no dUTP) 1863024 by BioRad Sciences

Step 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

Step 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

Step 14.

REAGENTS

DG32™ Automated Droplet Generator Cartridges 1864108 by BioRad Sciences

Automated Droplet Generation Oil for Probes 1864110 by BioRad Sciences

EQUIPMENT
Equipment brand:
AutoDG
SKU:
1864101
Specifications:
Automated Droplet Generator from BioRad

Droplet generation

Step 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

Step 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

Step 17.

SOFTWARE PACKAGE

QuantaSoft Software, 1.7
http://www.bio-rad.com/en-dk/sku/1864011-quantasoft-software-regulatory-edition?ID=1864011

Step 18.

ddPCR analysis is performed with QX100 Droplet Reader and QuantaSoft Software

Normalization

Step 19.

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