Quantification of circulating microRNA using TaqMan Low Density Array (TLDA)

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Abstract

A protocol for quantification of circulating microRNA using TaqMan Low Density Array.
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 is performed using the ΔCt-method

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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 test if the volume of spike-in added during microRNA purification is adequate before purifing all your samples.
When testing the preamplification product, the Ct-values 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

2X TaqMan Universal PCR Master Mix 4318157 by Applied Biosystems, Foster City, California, USA

20X TaqMan MicroRNA Assay 4440887 by Thermo Fisher Scientific

Custom TaqMan®Array MicroRNA Cards 4449135 by Applied Biosystems, Foster City, California, USA

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.

READAGENTS

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

Custom TaqMan®Array MicroRNA Cards 4449135 by Applied Biosystems, Foster City, California, USA

Reverse transcription

Step 5.

Prepare RT master mix:

Component	Master mix volume per 10 µL reaction*
Customs RT primer pool (10X)	1 µL
100 mM dNTPs (with dTTP)	0.27 µL
Multiscribe RT enzyme (50 U/µL)	2 µL
10x RT buffer	1 µL
MgCl2 (25 mM)	1 µL
RNase inhibitor (20 U/µL)	0.1 µL
Nuclease free water	1.63 µL

*add 10-20% excess volume

Mix gently and place on ice

NOTES

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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 7 µL RT master mix with 3 µL of purified microRNA in a 0.2 µL polypropylene reaction tube

Keep on ice

Step 7.
Mix gently and incubate on ice for 5 minutes

Step 8.
Transfer the reaction tubes to a termocycler
Incubate in 40 cycles of 16 °C for 2 min, at 42 °C for 1 min and 50 °C for 1 sec.
Finish with 85 °C for 5 min and cool to 4 ºC.

Step 9.
Continue immediately to the PCR amplification or store the RT-reaction in -20 °C.

Preamplification of cDNA

Step 10.

REAGENTS

Custom TaqMan®Array MicroRNA Cards 4449135 by Applied Biosystems, Foster City, California, USA

TaqMan™ PreAmp Master Mix 4391128 by Applied Biosystems, Foster City, California, USA

Step 11.
Prepare preamplification reaction mix:

Component	Volume per 25 µL reaction*
2x TaqMan PreAmp master mix	12.5 µL
Customs Primers (10X)	2.5 µL

Nuclease free water	5 µL
Total volume	**20 µL**

*add 10-20% excess volume

Mix gently

Step 12.
Combine 20 µL of qPCR reaction mix with 5 µL of RT-reaction (cDNA) in a 96 well plate
Seal plate

Step 13.
Incubate in 95 °C for 10 min, 55 °C for 2 min and 72 °C for 2 min
Proceed with 14 cycles of 95 °C for 15 sec and 60 °C for 4 min.
Finish with 99.9 °C for 10 min and cool to 4 °C

Continue immediately or store at 4 °C for up to 12 hours or at -20 °C for up to 1 week.

Test preamplification product

Step 14.
Use a specific TaqMan assay for the spike-in (Cel-miR-39, assay 000200) and one of the target miRNAs included in the Array to test the preamplification product before loading it on the array.

REAGENTS

20X TaqMan MicroRNA Assay 4440887 by Thermo Fisher Scientific

2X TaqMan Universal PCR Master Mix 4318157 by Applied Biosystems, Foster City, California, USA

Test preamplification product

Step 15.
Prepare qPCR reaction mix (run each sample in doublets):

Component	Volume per 20.3 µL reaction*

2x TaqMan Universal PCR master mix	10 µL
20x TaqMan microRNA assay	1 µL
Nuclease free water	8 µL
Total volume	**19 µL**

*add 10-20% excess volume

Mix gently

Test preamplification product

Step 16.

Combine 19 µL of qPCR reaction mix with 1.3 µL of RT-reaction (cDNA) in a 96 well plate

Seal plate

Test preamplification product

Step 17.

SOFTWARE PACKAGE

Sequence Detection System (SDS), 2.4

https://www.thermofisher.com/search/results?query=sequence+analysis+software&persona=Catalog&navId=10949&refinementAction=true&focusarea=Search+All

Test preamplification product

Step 18.

Transfer plate to ABI Prism 7900HT

Use the SDS software to set up the run

Incubate in 50 °C for 2 min and 95 °C for 10 min

Proceed with 40 cycles of 95 °C for 15 sec and 60 °C for 60 sec.

Test preamplification product

Step 19.

Evaluate the Ct-values obtained

Loading of the array

Step 20.

REAGENTS

Custom TaqMan®Array MicroRNA Cards 4449135 by Applied Biosystems, Foster City, California, USA

Loading of the array

Step 21.

Prepare samples:

Component	Volume per 120 µL reaction
2x TaqMan Universal PCR II mix No AmpErase UNG	60 µL
PreAmplification product	1,2 µL
Nuclease free water	58,8 µL
Total volume	**120 µL**

Loading of the array

Step 22.

Load 100 µL sample-dilution to each port on the array

Centrifuge The array at 1200 rpm for 2 min

Seal the array

Real-time PCR

Step 23.

SOFTWARE PACKAGE

Sequence Detection System (SDS), 2.4

https://www.thermofisher.com/search/results?query=sequence+analysis+software&persona=Catalog&navId=10949&refinementAction=true&focusarea=Search+All

Real-time PCR

Step 24.

Transfer array to ABI Prism 7900HT

Use the SDS software to set up the run

Incubate in 50 °C for 2 min and 94.5 °C for 10 min

Proceed with 40 cycles of 97 °C for 30 sec and 59.7 °C for 60 sec.

Normalization

Step 25.

Normalization is performed using the ΔCt-method ($2^{-\Delta Ct}$)

ΔCt = mean $Ct_{target\ miRNA}$ - mean $Ct_{cel\text{-}miR\text{-}39}$ (mean of triplets)