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

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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 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

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
- 2X TaqMan Universal PCR Master Mix **Applied Biosystems (ThermoFisher Scientific)** **Catalog #**4318157
- 20X TaqMan MicroRNA Assay **Thermo Fisher Scientific** **Catalog #**4440887
- Custom TaqMan®Array MicroRNA Cards **Applied Biosystems (ThermoFisher Scientific)** **Catalog #**4449135

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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.

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

Custom TaqMan®Array MicroRNA Cards **Applied Biosystems (ThermoFisher Scientific) Catalog #**4449135

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

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

Keep on ice

7 Mix gently and incubate on ice for 5 minutes

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.

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

Preamplification of cDNA

10 Custom TaqMan®Array MicroRNA Cards **Applied Biosystems (ThermoFisher Scientific) Catalog #**4449135

TaqMan™ PreAmp Master Mix **Applied Biosystems (ThermoFisher Scientific) Catalog #**4391128

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

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

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

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.

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

2X TaqMan Universal PCR Master Mix **Applied Biosystems (ThermoFisher Scientific) Catalog #**4318157

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

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

Seal plate

17

Software

Sequence Detection System (SDS)

https://www.thermofisher.com/search/results?query=sequence+analysis+software&persona=Catalog&navId=10949&refinementActio

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.

19 Evaluate the Ct-values obtained

Loading of the array

20

Custom TaqMan®Array MicroRNA Cards **Applied Biosystems (ThermoFisher Scientific) Catalog #**4449135

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

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

23

Software

Sequence Detection System (SDS)

https://www.thermofisher.com/search/results?query=sequence+analysis+software&persona=Catalog&navId=10949&refinementActio

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

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)