JUL 05, 2018

OPEN ACCESS

DOI:
[dx.doi.org/10.17504/protocols.io.q62dzge](https://doi.org/10.17504/protocols.io.q62dzge)

External link:
<https://doi.org/10.1371/journal.pone.0201069>

Protocol Citation: Helle Glud Binderup, Jonna Skov Madsen, Kim Houllind, Rikke Fredslund Andersen, Claus Lohman Brasen 2018. Quantification of circulating microRNA using TaqMan Low Density Array (TLDA). **protocols.io** <https://dx.doi.org/10.17504/protocols.io.q62dzge>

MANUSCRIPT CITATION: Binderup HG, Madsen JS, Heegaard NHH, Houllind K, Andersen RF, Brasen CL (2018) Quantification of microRNA levels in plasma – Impact of preanalytical and analytical conditions. PLoS ONE 13(7): e0201069. doi: [10.1371/journal.pone.0201069](https://doi.org/10.1371/journal.pone.0201069)

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

PLOS One

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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 Δ Ct-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 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**

STEP MATERIALS

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

Created: Jun 21, 2018

Last Modified: Jul 05, 2018

PROTOCOL integer ID:
13242

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

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TaqMan™ PreAmp Master Mix Applied Biosystems (ThermoFisher Scientific) Catalog #4391128
20X TaqMan MicroRNA Assay Thermo Fisher Scientific Catalog #4440887
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BEFORE START INSTRUCTIONS

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

	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

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

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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

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
MgCl ₂ (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 thermocycler

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 °C.

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

*add 10-20% excess volume

Mix gently

- 16** Combine 19 μ L of qPCR reaction mix with 1.3 μ L of RT-reaction (cDNA) in a 96 well plate
Seal plate

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Software

Sequence Detection System (SDS)

NAME

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

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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.

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Evaluate the Ct-values obtained

Loading of the array

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

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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
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- 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

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Software

Sequence Detection System (SDS)

NAME

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

SOURCE LINK

- 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 ΔC_t -method ($2^{-\Delta C_t}$)
 $\Delta C_t = \text{mean } C_{t_{\text{target miRNA}}} - \text{mean } C_{t_{\text{cel-miR-39}}} \text{ (mean of triplets)}$