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TEMPLATE for PROTOCOL UNIT

Unit Title

MicroRNA isolation from plasma for Real-Time qPCR array

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Significance Statement

MicroRNA are small non-coding RNA that can be derived from tissues and body fluids including human plasma. As they appear to be very stable in human plasma, microRNA have gained particular interest as valuable biomarkers. Several protocols exist for microRNA isolation and analysis, which makes comparison between studies difficult. Moreover, the yield of microRNA isolated from plasma can differ according to the isolation protocol used.

In an attempt to overcome pre-analytical and analytical issues, our research group optimized a microRNA isolation and preamplification protocol from human plasma for Real-Time (RT) qPCR array purpose (Gevaert et al., 2018). Here, a step-by-step microRNA isolation and quantification protocol with RT qPCR array from human plasma is presented.

ABSTRACT

MicroRNA are short non-coding RNA that regulate gene expression at the post-transcriptional level by mRNA degradation or suppression of translation. Their stability in plasma makes them attractive biomarkers. Since many plasma microRNA isolation protocols exist and the yield can be highly variable, we recently optimized the microRNA isolation and preamplification protocol using the mirVana Paris kit (ThermoFisher, AM1556) for miRNA quantification with TaqMan Low Density Arrays in plasma samples (Gevaert et al., 2018). The original protocol supplied by ThermoFisher was slightly modified. Based on our findings, recommendations are the following: (1) use *Arabidopsis thaliana* (ath) miR-159a as spike-in control; (2) use a 100µL elution volume during RNA isolation and (3) add a preamplification step without dilution of the preamplification product.

In this article we provide a step-by-step microRNA isolation and quantification protocol using human plasma samples for TaqMan Low Density Arrays.

Keywords: microRNA, isolation, plasma, array

UNIT TITLE MicroRNA isolation from plasma for Real-Time qPCR array

INTRODUCTION

MicroRNA (miRNA or miRs) are small single-stranded RNA that act as negative regulators of gene expression at the posttranscriptional level. They inhibit the expression of specific mRNA targets through base pairing between the miRNA 'seed region' and the seed match site in the 3'-untranslated region of their target mRNA, which results in translational repression or degradation of the mRNA. More than 50% of the human protein-coding genes are under miRNA regulatory control. One miRNA may exert inhibitory effects on many mRNA, and one target gene can be regulated by many miRNA (Small and Olson, 2011). Therefore, miRNA have been shown to regulate a wide array of cellular processes such as differentiation, proliferation, apoptosis and angiogenesis (Long et al., 2017; Duygu et al., 2016). MicroRNA can be released upon cell death or apoptosis, they can be packed in intracellular exosomes and microparticles or they can bind to high density lipoprotein cholesterol and Argonaute proteins; these processes facilitate cellular crosstalk (Small and Olson, 2011).

A subset of miRNA is detectable in plasma and is very stable, making them attractive biomarkers (Chen et al., 2008). Therefore, miRNA have emerged as promising new biomarkers in a wide range of pathologies such as cancer and cardiovascular diseases (Small and Olson, 2011; Rupaimoole and Slack, 2017; Vegter et al., 2016). However, accurate quantification of circulating miRNA in plasma remains challenging due to their small size, the relatively low amount in the circulation, the sequence homology with precursor forms and importantly, the lack of reference miRNA (Hergenreider et al., 2012; Stoorvogel, 2012; Dellett and Simpson, 2016). Moreover, it appears that data are difficult to validate and sometimes conflicting between groups due to a lack of methodological consensus (both at the pre-analytical and analytical level), hampering direct comparison of different studies on circulating miRNA. In this protocol, particular attention is paid to the processing and separation of plasma samples, total RNA extraction from plasma samples and further analysis using reverse transcription, preamplification and real-time qPCR for TaqMan Low Density Arrays (TLDA). This protocol is based on previously published results regarding the comparison of different strategies for RNA isolation from plasma, preamplification and dilution of the preamplification product as well as quantification using TaqMan Low Density Arrays (Gevaert et al., 2018). The recommendations of Gevaert et al. on miRNA isolation and quantification are the following: (1) use *Arabidopsis thaliana* (ath) miR-159a as spike-in control; (2) use a 100µL elution volume during RNA isolation and (3) add a preamplification step without dilution of the preamplification product. We chose to extract miRNA from plasma samples since this avoids contamination with platelet-derived miRNA. Serum is obtained after activation of platelets and thus may contain platelet-derived miRNA, therefore EDTA is the preferred anticoagulant to collect blood samples for PCR-based miRNA profiling (Lombardi et al., 2016).

The following five basic protocols provide step by step guidance for miRNA isolation and quantification protocol for arrays (TLDA) using human plasma samples, starting with sample processing and plasma separation, followed by total RNA isolation, reverse transcription and preamplification reaction, real-time PCR reaction solution preparation and ending with array loading and running.

BASIC PROTOCOL 1

SAMPLE PROCESSING AND PLASMA SEPARATION

All laboratory investigations start with correct collection and processing of plasma samples. The aim is to collect blood plasma while minimizing release of DNA and RNA from blood cells. Plasma is the fraction of blood that is cell-free and thus contains no white blood cells (WBCs), red blood cells (RBCs) or platelets, and that contains DNA and RNA. The plasma has to be eligible for miRNA profiling, therefore collection in EDTA-treated tubes is recommended (Lombardi et al., 2016).

Materials

- 21-gauge needle, tourniquet and waste tube
- Whole-blood samples collected in EDTA-treated tubes (BD Vacutainer catalogue number: 367525)
- Centrifuge with swinging-bucket rotor
- 500µl vials (Sarstedt, catalogue number: 72730006)
- Pipette tips (Eppendorf, catalogue numbers: 0030 000.854, 0030 000.870, 0030 000.919) and pipette
- Laboratory gloves (WM supplies, catalogue numbers: 10-0181 (S), 10-0182 (M))
- Biological safety hood

Protocol steps—Step annotations

Sample processing

1. Whole-blood should be taken in the morning after an overnight fast, and collected in EDTA-treated blood collection tubes.
 - Apply the same conditions to all patients in order to minimize pre-analytical variation in miRNA profile. Indicate whether subjects should refrain from physical exercise and nicotine, etc. at least 12 hours before blood drawing.
 - EDTA is the preferred anticoagulant for PCR-based miRNA profiling, see section on background information.
2. Use a 21 gauge needle and the EDTA Vacutainer system.
3. Apply a tourniquet just before puncture (after taking all materials and disinfection), do not leave it longer than 1 minute (make a note of difficult blood draws).
4. Puncture the antecubital vein, discard the first 3 ml of blood (use a waste tube).
5. After the EDTA tube is filled, it must be inverted at least 5 times.
6. Keep on room temperature and transfer all tubes immediately to the lab.
7. Proceed with “plasma separation” as soon as possible. The EDTA tubes should be centrifuged within 30 minutes after collection to avoid contamination with DNA and RNA released from blood cells.

Plasma separation

8. Centrifuge the EDTA-treated tubes within 30 minutes after collection at 1,500x *g*, room temperature, in a swinging-bucket rotor for 15 minutes.
 - This results in separation of the whole blood into three layers: plasma (top), buffy coat, RBCs (bottom).

9. Prepare labelling of the 500µl vials.
10. Wear laboratory gloves. Remove the upper $\frac{3}{4}$ of plasma (this avoids disturbing the separated buffy coat and RBCs) using disposable pipette tips and make 500µl aliquots in pre-labelled vials under the biological safety hood.
11. Freeze the labelled vials at -80°C immediately or proceed to RNA isolation immediately.
In our laboratory the stability of miRNA in plasma samples stored up to 10 years at -80°C has been proven.

BASIC PROTOCOL 2

EXTRACTION OF TOTAL RNA FROM PLASMA FOR QUANTIFICATION USING TLDA

It is of utmost importance to safeguard the isolated RNA from contamination with ribonucleases, as this may influence the integrity of RNA and comprise downstream applications. Therefore, frequent use of RNase inhibitors to clean the lab bench and laboratory gloves and the use of RNase-free pipette tips is recommended.

This protocol is based on the mirVana™ PARIS™ Kit protocol for total RNA isolation (Applied Biosystems), which was only slightly modified (note, the mirVana™ PARIS™ Kit protocol includes a protocol for total RNA and for small RNA isolation; here the total RNA isolation protocol was used).

Materials

- Laboratory gloves (WM supplies, catalogue numbers: 10-0181 (S), 10-0182 (M))
- Fume hood
- 70% ethanol (VWR, catalogue number: 20821.310)
- RNase inhibitor cleaning agent (example RNaseZAP™ RNase Decontamination Wipes, Thermo Scientific, catalogue number AM9786 (100 sheets) or AM9788 (300 sheets))
- RNase-free pipette tips with aerosol-resistant filter (example ThermoFisher Scientific ART™ pipette tips, catalogue numbers: 10078950, 10045460) and pipettes
- Heating pad
- Nuclease-free water (Ambion, catalogue number: AM9937)
- Microcentrifuge (temperature-controlled)
- PCR-clean Eppendorf tube 2ml (Ambion, catalogue number: AM12475); 2 tubes per sample
- mirVana™ PARIS™ RNA and Native Protein Purification Kit content and storage (ThermoFisher Scientific catalogue number AM1556)
 - 30 ml miRNA Wash Solution 1 (room temperature)
 - 50 ml miRNA Wash Solution 2/3 (room temperature)
 - 80 Collection Tubes (room temperature); 2 per sample
 - 40 Filter Cartridges (room temperature); 1 per sample
 - 25 ml Cell Disruption Buffer (4°C)
 - 25 ml 2X Denaturing Solution (4°C)
 - 2 × 25 ml Acid-Phenol:Chloroform (4°C)
 - 5 ml Elution Solution (-20°C, 4°C, or room temperature)
- n PCR-clean Eppendorf tubes per sample where $n = (\text{number of aliquots}) - 1$. If you want 4 aliquots, you need 3 tubes. You can choose the size of the tube (commonly 1.5ml, catalogue number Ambion AM12475)
- 1x 500µl plasma vial per sample
- Small box of ice
- β-mercaptoethanol (Sigma-Aldrich, catalogue number: M6250)
- 100% ethanol (VWR, catalogue number: 20821.310)
- Spike-in control Ath-miR-159a (1.27×10^{-6} nmol/ µl working solution, ThermoFisher Scientific, catalogue number 4464066)
- Vortex
- RNA bench
- Waste tube

CAUTIONS

- Wear laboratory gloves and long sleeves at all times to prevent contamination with nucleases on the skin and to prevent skin burns (acid-phenol:chloroform and denaturing solution cause severe skin burns).
- Work under a fume hood (β -mercaptoethanol, ethanol and chloroform fumes). Turn on the fume hood 10 minutes before starting.

NOTE

- Use RNase-free pipette tips with aerosol-resistant filter.

Protocol steps—*Step annotations*

Equipment preparation

1. Clean the lab bench with 70% ethanol and RNase inhibitor cleaning agent. Clean everything you bring under the fume hood with RNase inhibitor. Clean your gloves with RNase inhibitor before starting.
2. Turn the heating pad on, set temperature at 95°C. Heat a 2ml tube filled with nuclease-free water to 95°C.
3. Cool the microcentrifuge to 4°C.
4. Label the tubes
 - For use during the protocol:
 - 2 PCR-clean 2ml Eppendorf tubes per sample
 - 2 collection tubes per sample (mirVana PARIS kit)
 - 1 filter cartridge per sample (mirVana PARIS kit)
 - For final aliquots
 - n PCR-clean Eppendorf tubes per sample, where $n = (\text{number of aliquots}) - 1$. If you want 4 aliquots, you need 3 tubes. You can choose the size of the tube (commonly 1.5ml)

Protocol for total RNA isolation

Step 5-14: under the fume hood, microcentrifuge at 4°C

5. Thaw the 500 μ l plasma vial on ice.
6. Centrifuge the thawed plasma vial for 10 minutes at 16,000x g in a cooled microcentrifuge (4°C).
7. Meanwhile, prepare all solutions under the fume hood. These prepared solutions can be stored after use and can be reused for future isolations (caveat: expiration date).
 - 2X Denaturing Solution: see topic “Reagents and Solutions”
 - miRNA Wash Solution 1: see topic “Reagents and Solutions”
 - miRNA Wash Solution 2/3: see topic “Reagents and Solutions”
 - 100% ethanol: bring to room temperature.
8. Mix 400 μ l of plasma with 400 μ l 2x Denaturing Solution at room temperature in a PCR-clean Eppendorf tube 2ml (1 tube per sample) under the fume hood. After mixing, incubate on ice for 5 minutes.
9. Add the spike-in normalization under the fume hood, pipette up and down to mix with plasma:

- 20fmol ath-miR-159a = 16µl of a 1.27×10^{-6} nmol/µl working solution (preparation is described in “Reagents and Solutions”).
10. Add 816µl of Acid-Phenol: Chloroform without touching the surface of the solution (to avoid spike-in removal with the pipette tip) under the fume hood.
 11. *Make sure to sample Acid-Phenol: Chloroform from the lower level of the bottle (upper level is solvent). Close the tubes in between the steps to minimize evaporation of ethanol-containing mixtures and drying of the filter cartridges.*
 12. Vortex for 30-60 seconds.
 13. Centrifuge for 30 minutes at 16,000x g in a cooled microcentrifuge (4°C). Carefully take out the sample when centrifuging is finished, keeping it upright.
 14. Carefully transfer the upper aqueous phase (max. 500-600µl) to a fresh 2ml PCR-clean Eppendorf tube (1 tube per sample) under the fume hood. Record the volume of this aqueous phase.
This is a crucial step, be very careful NOT to touch the bottom phase, NOR the white cloudy substance that can be present in the interphase. This will ruin your sample.

Step 15-36 at RNA bench, use dedicated RNase-free pipettes (to avoid contamination), heat the microcentrifuge to 25°C

15. Move to the RNA bench.
16. Add ‘1.25x the volume of the upper aqueous phase’ of 100% ethanol to the aqueous phase.
Example: if the volume of the upper aqueous phase is 500µl, then the volume of 100% ethanol that has to be added is $1.25 \times 500\mu\text{l} = 625\mu\text{l}$
17. For each plasma sample, place a filter cartridge into a new collection tube.
18. Pipet the lysate/ethanol mixture onto the filter.
The maximum volume of the filter is 700µl, do not exceed this volume.
19. Centrifuge for 30 seconds at 10,000x g in the microcentrifuge at 25°C.
If not all mixture has passed through the filter, continue until all mixture is filtered.
20. Discard the flow-through. Re-use the collection tube.
21. If necessary (lysate/ethanol mixture >700µl), repeat step 14 and 15.
22. Apply 700µl of miRNA Wash Solution 1 to the filter cartridge.
23. Centrifuge for 15 seconds at 10,000x g in the microcentrifuge at 25°C.
24. Discard the flow-through and re-use the collection tube.
25. Apply 500µl of miRNA Wash Solution 2/3 to the filter cartridge.
26. Centrifuge for 15 seconds at 10,000x g in the microcentrifuge at 25°C.
27. Discard the flow-through and re-use the collection tube.
28. Apply 500µl of miRNA Wash Solution 2/3 to the filter cartridge.
29. Centrifuge for 15 seconds at 10,000x g in the microcentrifuge at 25°C.
30. Discard the flow-through.
31. Centrifuge the “filter cartridge + collection tube” for 1 minute at 10,000x g at 25°C.
32. Transfer the filter cartridge into a new collection tube.
33. Apply 100µl of preheated nuclease-free water (95°C) to the centre of the filter, close the tube immediately.
34. Centrifuge for 30 seconds at 10,000x g in the microcentrifuge at 25°C to recover total RNA.
35. Aliquot the eluate immediately in pre-labelled PCR-clean Eppendorf tubes.
We prefer to aliquot 20µl per vial because only 3µl eluate is needed per reverse transcription reaction and each vial can be thawed only once (degradation of miRNA with repetitive freeze-thaw cycles).

36. Store the aliquots immediately at -20°C or proceed with the reverse transcription reaction immediately.

BASIC PROTOCOL 3

REVERSE TRANSCRIPTION REACTION

In an RT reaction, a reverse transcriptase enzyme is used to generate a copy DNA strand from an RNA template. In this part of the protocol, it is important to prepare a sufficient amount of RT reaction mix to avoid pipetting of small volumes (<1µl volumes) since there is a large margin of error on pipetting these volumes. The protocol is based on the Megaplex™ Pools With Preamplification protocol for miRNA Expression Analysis (Applied Biosystems), which was only slightly modified.

Materials

- Laboratory gloves (WM supplies, catalogue numbers: 10-0181 (S), 10-0182 (M))
- 70% ethanol (VWR, catalogue number: 20821.310) and RNase inhibitor cleaning agent
- RNase-free pipette tips with aerosol-resistant filter (example ThermoFisher Scientific ART™ pipette tips, catalogue numbers: 10078950, 10045460) and pipettes
- 1.5ml PCR-clean Eppendorf tube (Axygen Scientific, catalogue number: MCT150LC)
- MicroAmp 8-Tube Strip and MicroAmp Optical Strip Caps or 96-well MicroAmp Optical Reaction Plate and MicroAmp Clear Adhesive Film.
 - In our experiments we used the Tube Strips (BIORAD, catalogue number: TBS0211).
- Small box of ice
- Megaplex™ RT primers (Human Pool A V2.1, 10X, for 50 reactions) and MgCl₂ (25mM), store at -15 to -25°C (ThermoFisher Scientific, catalogue number 4399966)
- TaqMan™ MicroRNA Reverse Transcription Kit, store at -15 to -25°C (ThermoFisher Scientific, catalogue number 4366596 for 200 reactions, 4366597 for 1000 reactions)
 - dNTPs with dTTP (100mM)
 - 10X RT buffer
 - RNase Inhibitor (20 U/µl)
 - MultiScribe™ Reverse Transcriptase (50 U/µl)
- Isolated total RNA (20µl per sample)
- Nuclease-free water (Ambion, catalogue number: AM9937)
- Microcentrifuge at room temperature
- Ice pad
- Centrifuge with swinging-bucket rotor at room temperature
- Thermal cycler: in our experiments, a CFX96 thermal cycler (BioRad, California, USA) was used

CAUTIONS

- Wear laboratory gloves and long sleeves at all times to prevent contamination with nucleases on the skin.
- Work at the RNA bench.

NOTE

- Use RNase-free pipette tips with aerosol-resistant filter.

Protocol steps

Equipment preparation

1. Clean the lab bench with 70% ethanol and RNase inhibitor. Clean everything you bring to the lab bench with 70% ethanol and with RNase inhibitor. Clean your gloves with RNase inhibitor before starting.

2. Label the tubes
 - For use during the protocol:
 - 1 PCR-clean 1.5ml Eppendorf tube
 - For final aliquots
 - MicroAmp 8-Tube Strip and MicroAmp Optical Strip Caps or 96-well MicroAmp Optical Reaction Plate and MicroAmp Clear Adhesive Film. One tube or well per sample.

Prepare the reverse transcription (RT) reaction mix

3. Thaw the following on ice:
 - a. Megaplex™ RT primers
 - b. TaqMan™ MicroRNA Reverse Transcription Kit
 - c. MgCl₂
 - d. Isolated total RNA (20µl per sample)
4. Combine the ingredients listed under Table “1”.in a 1.5-ml PCR-clean Eppendorf tube:
Table 1: RT reaction mix preparation
Close the tubes in between the steps to minimize possible contamination.
5. Invert the tube six times to mix, then centrifuge the tube briefly.
6. In an 8-Tube Strip (or a 96-well MicroAmp Optical Reaction Plate), pipette 4.5µl of the RT reaction mix into each tube (or well, 1 tube or well per sample), on an ice pad.
7. Add 3µl total RNA (or 3µl of water for the No Template Control reactions) into each tube or well containing the RT reaction mix, on an ice pad.
8. Seal the tubes or plate using MicroAmp Optical Strip Caps or MicroAmp Clear Adhesive Film, respectively. Then invert the tubes or plate six times to mix. Spin briefly (at room temperature) in a centrifuge with swinging-bucket rotor.
Do not use MicroAmp Optical Adhesive Film to seal the plate.
9. Incubate the tubes or plate on ice for 5 minutes.

Perform Megaplex Reverse Transcription

10. Use a thermal cycler. In our experiments, a CFX96 thermal cycler (BioRad, California, USA) was used.
11. Set up the run method using the following conditions:
 - a. Ramp speed or mode: 9700 using Std or Max ramp speed. 7900HT using Std ramp speed.
 - b. Reaction volume: 7.5µl (if .5 not possible, enter 8µl)
 - c. Thermal-cycling conditions:
Table 2: Thermal-cycling conditions for reverse transcription reaction
 - d. Load, then run the strip or plate
 - i. If you are running the plate on a 7900HT System and sealing the plate with MicroAmp Clear Adhesive Film (PN 4306311), then place a MicroAmp Optical Film Compression Pad (PN 4312639) on top of the plate.
 - e. When finished, store the cDNA at -20°C (the cDNA can be stored at -15 to -25°C for maximum 72h) or continue with preamplification and qPCR.
In our experiments, degradation of cDNA was observed when stored for 1 week at -20°C.

BASIC PROTOCOL 4

PREAMPLIFICATION

In this step, preamplification of cDNA results in an increase in the amount of cDNA for gene expression analysis using arrays. In our optimizing experiments for microRNA arrays, it appeared that preamplification of the cDNA was an essential step in the protocol to obtain a larger quantity of amplified wells (Gevaert et al., 2018). The protocol is based on the Megaplex™ Pools With Preamplification protocol for miRNA Expression Analysis (Applied Biosystems), which was only slightly modified.

Materials

- Laboratory gloves (WM supplies, catalogue numbers: 10-0181 (S), 10-0182 (M))
- 70% ethanol and DNase inhibitor cleaning agent (example DNA AWAY™ Surface Decontaminant, Thermo Scientific, catalogue number 7010)
- RNase-free pipette tips with aerosol-resistant filter (example ThermoFisher Scientific ART™ pipette tips, catalogue numbers: 10078950, 10045460) and pipettes
- 1.5ml PCR-clean Eppendorf tube (Axygen Scientific, catalogue number: MCT150LC)
- MicroAmp 8-Tube Strip and MicroAmp Optical Strip Caps or 96-well MicroAmp Optical Reaction Plate and MicroAmp Clear Adhesive Film.
 - In our experiments we always used the Tube Strips (BIORAD, catalogue number: TBS0211)
- Small box of ice
- Megaplex™ PreAmp primers (Human Pool A V2.1 for 50 reactions, 10X) store at -15 to -25°C (ThermoFisher Scientific, catalogue number 4399233)
- Microcentrifuge at room temperature
- RT product: cDNA tube or plate
- TaqMan™ PreAmp Master Mix 2X, store at 2 to 8°C (ThermoFisher Scientific, catalogue nr. 4488593 for 200 reactions, nr. 4391128 for 40 reactions)
- Nuclease-free water (Ambion, catalogue number: AM9937)
- Ice pad
- Centrifuge with swinging-bucket rotor at room temperature
- Thermal cycler: in our experiments, a CFX96 thermal cycler (BioRad, California, USA) was used

CAUTIONS

- Wear laboratory gloves and long sleeves at all times to prevent contamination with nucleases on the skin.
- Work at the DNA bench.

NOTE

- Use RNase-free pipette tips with aerosol-resistant filter.

Protocol steps

Equipment preparation

1. Clean the lab bench with 70% ethanol and DNase inhibitor. Clean everything you bring to the lab bench with 70% ethanol and with DNase inhibitor. Clean your gloves with DNase inhibitor before starting.
2. Label the tubes

- For use during the protocol:
 - 1 PCR-clean 1.5ml Eppendorf tube
- For final aliquots
 - 8-Tube Strip and MicroAmp Optical Strip Caps or 96-well MicroAmp Optical Reaction Plate and MicroAmp Clear Adhesive Film. One tube or well per sample.

Prepare the preamplification reaction mix

3. Thaw the following on ice:
 - a. Megaplex™ PreAmp Primers (10X), mix by inverting 6 times, spin briefly.
 - b. cDNA tube or plate
4. Mix the TaqMan™ PreAmp Master Mix (2X) by swirling the bottle.
5. Combine the ingredients listed under “Table 3” in a 1.5-ml PCR-clean Eppendorf tube:
Table 3: Preamplification reaction mix preparation
Close the tubes in between the steps to minimize possible contamination.
6. Invert the tube 6 times to mix, then centrifuge the tube briefly.
7. In a MicroAmp 8-tube strip or a 96-well MicroAmp Optical Reaction Plate, pipette 2.5µl of each RT product into its corresponding tube or well on an ice pad. Make sure that no RT product remains in the pipette tip.
8. Dispense 22.5µl of PreAmp reaction mix into each MicroAmp Optical 8-Tube Strip or into each well of the 96-well plate containing the RT product on an ice pad. Make sure to add the PreAmp reaction mix without touching the RT product (to avoid that droplets of RT product stick to the pipette tip), so dispense it while lightly touching the pipette tip against the side of the tube or well.
9. Seal the tubes or plate using MicroAmp Optical Strip Caps or MicroAmp Clear Adhesive Film, then invert the tubes or plate 6 times to mix. Spin briefly in a centrifuge with swinging-bucket rotor.
10. Incubate the tubes or plate on ice for 5 minutes.

Perform the preamplification

11. In our experiments, a CFX96 thermal cycler (BioRad, California, USA) was used.
12. Set up the run method using the following conditions:
 - a. Ramp speed or mode: 9700 using Std ramp speed.
 - b. Reaction volume: 25µl
 - c. Thermal-cycling parameters:
Table 4: Thermal-cycling conditions for preamplification reaction
13. Load, then run the strip or plate.
14. Do not dilute the product. Place the preamplification product on ice and immediately continue with the real-time PCR reaction (BASIC PROTOCOL 5).

BASIC PROTOCOL 5

Real-time PCR reaction: solution preparation, array loading and running

In this step, the DNA polymerase amplifies the preamplified cDNA. The targets are detected in real-time through cleavage of the TaqMan probe by the polymerase 5'-3' exonuclease activity. The protocol is based on the Megaplex™ Pools With Preamplification protocol for miRNA Expression Analysis (Applied Biosystems), which was only slightly modified.

Materials

- Laboratory gloves (WM supplies, catalogue numbers: 10-0181 (S), 10-0182 (M))
- 70% ethanol and DNase inhibitor cleaning agent (example DNA AWAY™ Surface Decontaminant, Thermo Scientific, catalogue number 7010)
- RNase-free pipette tips with aerosol-resistant filter (example ThermoFisher Scientific ART™ pipette tips, catalogue numbers: 10078950, 10045460) and pipettes
- 1.5ml PCR-clean Eppendorf tube per sample (Axygen Scientific, catalogue number: MCT150LC)
- Preamplification product
- Small box of ice
- Centrifuge with swinging-bucket rotor at room temperature
- TaqMan™ Universal PCR Master Mix No AmpErase UNG 2X, store at 2 to 8°C (ThermoFisher Scientific, catalogue number 4324018 for 200 reactions, also available for 400, 1000 or 2000 reactions)
- Nuclease-free water (Ambion, catalogue number: AM9937)
- Microcentrifuge (room temperature)
- TaqMan™ Array Human MicroRNA A cards v2.0, store at 4°C (ThermoFisher Scientific, catalogue number 4398965), 1 array per sample
- Low Density Array Sealer (Applied Biosystems, 7900HT TaqMan™ Low Density Array Hardware Upgrade Kit catalogue number 4329012)
- Scissors
- Real-Time PCR System, we used the 7900HT Fast Real-Time PCR system (Applied Biosystems, California, USA)

CAUTIONS

- Wear laboratory gloves and long sleeves at all times to prevent contamination with nucleases on the skin.
- Work at the DNA bench.

NOTE

- Use RNase-free pipette tips with aerosol-resistant filter.

Protocol steps

Equipment preparation

1. Clean the lab bench with 70% ethanol and DNase inhibitor. Clean everything you bring to the lab bench with 70% ethanol and with DNase inhibitor. Clean your gloves with DNase inhibitor before starting.
2. Label the tubes
 - 1 PCR-clean 1.5ml Eppendorf tube per sample

Prepare the PCR reaction mix

3. Place the preamplification product from BASIC PROTOCOL 4 on ice. Mix by inverting 6 times, then centrifuge the tube or plate briefly.
4. Mix the TaqMan™ Universal PCR Master Mix by swirling the bottle.
5. Combine the ingredients listed under “Table 5” in a 1.5ml PCR-clean Eppendorf tube per sample:

Table 5: PCR reaction mix preparation

Close the tubes in between the steps to minimize possible contamination.

6. Invert the tube 6 times to mix, then centrifuge the tube briefly in the microcentrifuge.
7. Place the tube on ice and continue with microRNA array loading and running.

MicroRNA array loading and running

For detailed information on microRNA array loading, sealing and running, we refer to the Applied Biosystems TaqMan Low Density Array User Bulletin (PN 4371129).

8. Dispense 100µl of the PCR reaction mix into each port of the TaqMan MicroRNA array, using the reverse pipetting technique.
9. Centrifuge the array 2 x 1 minute in a centrifuge with swinging bucket rotor at room temperature; make sure to correctly place the array in the centrifuge (see User Bulletin for correct placement and settings). Verify whether filling is complete; the amount of fluid remaining in the fill reservoirs should be uniform and consistent over the reservoirs.
10. Seal the array using the sealer, trim the fill reservoirs from the TaqMan Array using scissors (use the edge of the TaqMan Array's carrier as a guide).
11. Run the array on the Real-Time PCR system.

- a. If you cannot proceed immediately with running the array, wrap aluminum foil around the array and place it in the refrigerator (4 to 8 °C). Proceed with running the array within 6h after loading.

We observed a decrease in number of amplified wells and higher Cq values with arrays that were kept at room temperature for 1h. Arrays run within 6h and kept in the refrigerator all had a similar, good performance.

12. Import the SDS setup file (SDS.txt) located on the Information CD provided with the arrays:
 - b. Start the SDS v2.2 or later software.
 - c. In the main menu, select File → New
 - d. In the New Document dialog box, select the following from the drop-down menu:
 - i. Relative Quantification ($\Delta\Delta Ct$)
 - ii. 384-well TaqMan Low Density Array
 - e. In the main menu, select File → Import to open the new document
 - f. In the Open dialog box, navigate to the Setup.txt file specific for the array being run and click Import.
 - g. Save as an SDS 7900 Template (.sdt) file
13. Run the array using the 384-well TaqMan Low Density Array default thermal-cycling conditions.

REAGENTS AND SOLUTIONS

Working solution Ath-miR-159a 1.27×10^{-6} nmol/ μ l

ThermoFisher Ath-miR-159a concentration 1.9×10^{-5} nmol/ μ l

As spike in control, we need 16 μ l per sample of a 1.27×10^{-6} nmol/ μ l working solution.

Example

- If 20 samples $\rightarrow 16\mu\text{l} \times 20 \text{ samples} + \text{extra for pipetting errors} = 400\mu\text{l}$
- $\frac{19 \times 10^{-6} \text{ nmol}/\mu\text{l}}{x} = 1.27 \times 10^{-6} \text{ nmol}/\mu\text{l} \rightarrow x = 15$
- 400 μ l: 15 = 26.7 μ l

$\rightarrow$ For a total volume of 400 μ l, we need 26.7 μ l Ath-miR 159a 1.9×10^{-5} nmol/ μ l + 373 μ l nuclease-free water

$\rightarrow$ This results in a 400 μ l solution with an Ath-miR-159a concentration of 1.27×10^{-6} nmol/ μ l

$\rightarrow$ This solution should be stored at -80°C for a maximum of 1 year.

Preparation of denaturing solution and wash solutions:

- 2X Denaturing Solution: add 375 μ l β -mercaptoethanol, mix well and place a check mark in the box on the label to indicate that it has been added.
 - The 2X Denaturing Solution may solidify at 4°C and should be warmed at 37°C before use with occasional agitation for 5–10 min, or until it is completely in solution. It may be stored at room temperature for up to 1 month (avoid solidification).
- miRNA Wash Solution 1: add 21ml 100% ethanol, mix well and place a check mark in the box on the label to indicate that it has been added.
- miRNA Wash Solution 2/3: add 40ml 100% ethanol, mix well and place a check mark in the box on the label to indicate that it has been added.
 - A precipitate may form in the Wash Solution 2/3 bottle over the next several days as excess EDTA falls out of solution. Leave these crystals in the bottle when removing Wash Solution for use.

COMMENTARY

Background Information

In 2001, Lagos-Quintana et al. first reported on the existence of miRNA (Lagos-Quintana et al., 2001). Ever since, more than 70.000 articles on this topic have been published, of which more than 3.000 on circulating miRNA. Since 2007, miRNA quantification using arrays have come into play (Si et al., 2007); this technique allowed to quantify large amounts of miRNA (around 400 miRNA per array) in a timeframe of several hours. As it appeared that circulating miRNA are stable and it requires only a minimally invasive technique to collect blood samples, their capacities as diagnostic biomarkers have emerged over the past few years and they have been studied in many pathologies such as cancer and cardiovascular diseases (Small and Olson, 2011; Rupaimoole and Slack, 2017; Vegter et al., 2016). With this great scientific interest, many protocols for isolation, quantification and interpretation were developed, which contributed to the heterogeneity in studies on circulating miRNA and (pre-)analytical problems.

To minimize pre-analytical problems, we chose for optimization of the miRNA profiling protocol from plasma samples, quantification using TaqMan microRNA arrays (Gevaert et al., 2018) and to provide a step-by-step protocol.

Plasma is the cell-free blood component that is derived after platelet activation inhibition using EDTA. By doing this, contamination with platelet-derived miRNA that are released from platelet granules with platelet activation, is prevented. Contrary to this, serum is obtained after activation of platelets and thus may contain platelet-derived miRNA. It has been proven that citrate and heparin interfere with enzyme activity in PCR-based assays. Moreover, the volume of citrate used in citrate-treated tubes may slightly differ and influence the RNA isolation and quantification. Therefore, EDTA is the preferred anticoagulant for PCR-based miRNA profiling (Lombardi et al., 2016). In addition, it is well known that repetitive freeze-thaw cycles are detrimental for the miRNA stability and leads to degradation of miRNA (Glinge et al., 2017).

In this protocol, the mirVana PARIS kit for total RNA *isolation* and the TaqMan Array Human MicroRNA A Card v2.0 were used; both are commercially available since +/-10 years, with the first publications citing this technique in 2007 (Si et al., 2007). Historically, many techniques and methods have been used to isolate total RNA such as chemical extraction; this technique is not well suited for the isolation of very small RNA due to desalting and alcohol precipitation concentration procedures. Another method for extraction is immobilization on glass (the so called solid-phase extraction), using this technique salt or salt and alcohol are used to decrease the affinity of RNA for water and to increase the affinity for the solid support. This is suitable for large RNA, but is not effective in recovering small RNA.

Using the mirVana PARIS kit for total RNA isolation, the 2X Denaturing Solution and the Acid-Phenol:Chloroform extraction purifies the RNA and removes most of the DNA (Chomczynski and Sacchi, 1987). This kit has been shown to be effective for purification of RNA enriched for small or for large RNA. The TaqMan Array Human MicroRNA A Card v2.0 contains 384 TaqMan® MicroRNA dried probes and primers, which enables profiling of 377 human miRNA; these include 3 endogenous controls and 1 non-human negative control. When searching for new miRNA, other, more recent techniques such as RNA sequencing are gaining more interest, since these techniques allow quantification of *more* and *new* miRNA both at the same time, compared to arrays.

Critical Parameters and troubleshooting

Sample processing and plasma separation

- Apply the same conditions to all patients that undergo blood sampling and indicate whether subjects should refrain from physical exercise, alcohol, nicotine, caffeine, etc. at least 12 hours before blood drawing, this is to minimize variation in miRNA profile due to diurnal variation in miRNA and binding to proteins.
- Process the samples within 30 minutes after collection to avoid degradation of miRNA prior to plasma separation.
- Use EDTA-treated blood collection tubes, this is the preferred anticoagulant for PCR-based miRNA profiling.

Total RNA extraction from plasma samples for quantification using arrays

- Avoid repetitive freeze-thaw cycles of plasma, this causes degradation of miRNA.

- When pipetting the upper aqueous solution, be very careful NOT to touch the bottom phase, NOR the white cloudy substance that can be present in the interphase. This will ruin your sample.

Array loading

- Make sure that filling is complete and that the array is correctly sealed. Incomplete filling or incorrect sealing may result in abnormally high Cq values or no amplification of wells.

In general

- Wear laboratory gloves, clean the lab bench and everything you bring on the lab bench and clean your gloves with 70% ethanol and RNase or DNase inhibitor, and use RNase-free pipette tips with aerosol-resistant filter to prevent contamination with nucleases on the skin, DNA and RNase or DNase.
- Adhere to the expiration dates of reagents, storage duration and temperatures of freezing.
- Close the tubes in between the steps to minimize possible contamination, drying and evaporation.
- Carefully transfer the total RNA/RT product/preamplification product to the tube or well, avoid pipetting errors (remaining droplets of total RNA/RT product/preamplification product in pipette tip). This may result in abnormally high Cq levels since the input quantity is too low.
- Use ice pads when indicated, this is to avoid early enzyme activation.
- Enter the thermal-cycling conditions correctly. When you do not respect the temperatures, durations and cycles, this will ruin your sample.
- Always make note of suboptimal conditions or handlings, this will help you to explain unexpected results.

Troubleshooting → combined with critical parameters

Statistical Analyses

Raw Cq values were calculated in SDS software v.2.4 using automatic baseline and threshold settings. Cq values that were undetermined or >35 were replaced by “Cq = 36” for further analysis. Relative miRNA levels were expressed as $2^{-\Delta Cq}$. Data were analyzed using R version 3.4.3. The geNorm algorithm was obtained from the *NormqPCR* package in R {Perkins:2012ug}. For detailed information on statistical analysis, we refer to our recent publication “MicroRNA profiling in plasma samples using qPCR arrays: recommendations for correct analysis and interpretation” (Gevaert et al., 2018).

Understanding Results

For interpretation of the results, we refer to “MicroRNA profiling in plasma samples using qPCR arrays: recommendations for correct analysis and interpretation” (Gevaert et al., 2018).

Table 6 Example of expected yield of microRNA quantification in a HFrEF patient using the protocol described in this article.

Time Considerations

For 1 sample:

- Blood sampling: 10 minutes
- Plasma separation: 25 minutes
- Extraction of total RNA from plasma: 2 hours 15 minutes
- Reverse transcription reaction: 4 hours
- Preamplification: 2 hours 30 minutes
- Real-time PCR solution preparation: 40 minutes
- Array loading + running: 2 hours 30 minutes

Total: 750 minutes = 12,5 hours

When you have collected all your plasma samples, we recommend to start with RNA isolation of all your samples and to store them at -80°C. Since arrays have to be run one by one, you can easily perform 3 arrays on one day. Therefore, we recommend performing the reverse transcription of max. 9 samples on day 1, and doing the preamplification reaction and preparation of the real-time PCR solution and array loading + running on day 2 (for 3 samples), day 3 (again preamplification, PCR solution, array loading and running for 3 samples) and day 4 (again for 3 samples). If you do this on 4 consecutive days, you will always respect the expiration dates of your products.

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CONFLICTS OF INTEREST

We have no conflicts of interest to declare.

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KEY REFERENCE NA

INTERNET RESOURCES

<https://www.thermofisher.com/>

On this website materials can be ordered and the protocol for array loading and running can be found.

FIGURE LEGENDS NA

TABLES

Table 1: Reverse transcription reaction mix preparation

RT reaction mix components	Volume for 1 to 5 samples (μl)*	Volume for 10 samples (μl)*
Megaplex™ RT primers (10X)	4.5	9.0
dNTPs with dTTP (100mM)	1.13	2.25
10X RT buffer	4.5	9.0
MgCl ₂ (25mM)	5.06	10.12
RNase Inhibitor (20 U/μl)	0.56	1.12
Nuclease-free water	1.13	2.25
MultiScribe™ Reverse Transcriptase (50 U/μl)	8.44	16.88
Total	25.32	50.62

* Includes min. 12.5% excess for volume loss from pipetting

RT= reverse transcription

Table 2: Thermal-cycling conditions for reverse transcription reaction

Stage	Temperature (°C)	Time
Cycle (40 Cycles)	16	2 minutes
	42	1 minutes
	50	1 second
Hold	85	5 minutes
Hold	4	∞

Table 3: Preamplication reaction mix preparation

PreAmp reaction mix components	Volume for 1 sample (μl)*	Volume for 10 samples (μl)*
TaqMan™ PreAmp Master Mix (2X)	14.1	140.62
Megaplex™ PreAmp Primers (10X)	2.8	28.13
Nuclease-free water	8.4	84.37
Total	25.3	253.12

* Includes min. 12.5% excess for volume loss of pipetting.

Table 4: Thermal-cycling conditions for preamplication reaction

Stage	Temperature (°C)	Time
Hold	95	10 minutes
Hold	55	2 minutes
Hold	72	2 minutes
Cycle (12 cycles)	95	15 seconds
	60	4 minutes
Hold*	99.9	10 minutes
Hold	4	∞

*Required for enzyme inactivation.

Table 5: PCR reaction mix preparation

Component	Volume for one array (μl)*
TaqMan™ Universal PCR Master Mix No AmpErase UNG 2X	450
Preamplification product	9
Nuclease-free water	441
Total	900

*Includes 12.5 excess for volume loss from pipetting.

Table 6: Example of expected yield of microRNA quantification in a HFrEF patient using the protocol described in this article.

Protocol		Amplified wells		Cq values ≤35		Mean Cq		Normalization microRNA Cq			
RNA elution (μL)	Preamplification dilution	Number	Percentage	Number	Percentage	Raw	Global	ath-miR-159a	RNU4 4	RNU4 8	U6
100	1:1	188	48.96	173	45.05	30.12	29.65	24.58	Under	34.60	31.10

HFrEF= Heart failure with reduced ejection fraction, Cq= quantification cycle, N/A= not available on plate, TLDA= TaqMan Low Density Array, Undet= not amplified on plate, Raw= mean of raw Cq values, Global= mean of global mean Cq values.