RNADIAGON

Excellence in research and development of non-coding RNA DIAGnostics in ONcology

H2020-MSCA-RISE-2018

D1.1 Standard Operating Protocol (SOP)

Work Package:	WP1
Task:	
Deliverable due date:	31/07/22
Responsible partner:	MU
Editors:	Jiří Šána
Deliverable number:	D1
Deliverable type:	Report
Dissemination level:	Public
First Created:	13/07/22
Last Updated:	30/07/22
Version:	1.0

STANDARD OPERATING PROTOCOL (SOP) DEFINING APPROPRIATE METHODS AND APPROACHES IN PREANALYTICAL MANAGEMENT OF BLOOD SAMPLES, RNA ISOLATION, SMALL NCRNA DETECTION AND BIOINFORMATICS ANALYSIS

Contents

Purpose

Global profiling of small non-coding RNA (ncRNA), particularly microRNA (miRNA), by next-generation sequencing (NGS) in blood plasma and sequencing data validation using RT-qPCR has great potential as this non-invasive approach allows the discovery of new important biomarkers for the diagnosis, prognosis or staging of tumors.

1 Preanalytical Phase

1.1 Patient Preparation and Blood Collection

1.1.1 Patient Preparation

Proper preparation of the patient regarding fasting needs as well as careful phlebotomy procedures are essential to ensuring accurate laboratory testing results. General practice is to have the patient fast twelve hours prior to blood draw. Blood collection is done typically in the morning or, if the patient is to undergo surgery, blood is drawn before the procedure.

1.1.2 Blood Collection

1. During the actual collection, the patient is invited to clench his fist, but repeated "pumping" is not allowed.
2. The quality of the venous system in the elbow socket is checked, for example, especially regarding healed burns, conditions after breast ablation, hematomas, parenteral therapy (always choose the opposite arm), inserted cannulas. Small veins can be highlighted, for example, by massaging the arm from the wrist to the elbow, short taps with the index finger on the site of sampling, application of a warm agent (around 40 °C for 5 minutes), lowering the arm along the edge of the bed or chair.
3. Disinfection of the puncture site is carried out; after disinfection, the skin must be allowed to dry, both to prevent haemolysis of the sample and to eliminate the burning sensation at the site. After disinfection, further palpation of the site is unacceptable!
4. Blood is drawn into containers with the addition of anticoagulant (anti-clotting) agents – typically EDTA Na2 or sodium citrate.

1.2 Blood transport

The transport of the material must be gentle and fast. Care should be taken to avoid mechanical damage from shaking the tube, which can cause haemolysis. If the blood sample is transported immediately after collection to the laboratory, room temperature is usually sufficient for transport. For longer transport (more than 30 minutes) and due to external climatic conditions, it is preferable to send the material in a cooler or on ice. If blood cannot be transported to the laboratory within the required time, it is preferable to send plasma to the laboratory.

1.3 Blood Processing to Obtain Plasma

Plasma is obtained by centrifugation of non-clotting blood (2000 × g / 10 minutes / 4 °C). The plasma should be separated as soon as possible, at the latest within 1 hour of collection. The

sealed blood syringes/tubes are placed in a rotor where centrifugal force is used to accelerate the sedimentation of higher density substances. Centrifugation of blood is always carried out in perfectly closed tubes (avoiding aerosol formation or contamination of the sample). Longer centrifugation times or increased centrifugation overload may lead to partial or complete haemolysis.

1.4 Plasma Storage

Plasma is stored at a temperature of at least −80 °C which is recommended to maintain long-term integrity of biomarkers.

2 Analytical Phase

2.1 Isolation of Total RNA Enriched for Small RNA From Plasma Samples

Method principle

The plasma is lysed by a combination of organic extraction and a chaotropic agent with a phenol-guanidine solution, for example, Trizol reagent followed by chloroform. Combining organic extraction and cell disruption with a chaotropic agent contributes to high yields of RNA. Lysis and organic extraction are followed by RNA precipitation with alcohol and isolation using the QIAGEN miRNeasy Serum/Plasma Kit, which is based on the solid phase immobilization of RNA on a column, followed by washing with alcohol-based wash solution and elution with a low ionic strength solution.

Reagents

- QIAGEN miRNeasy Serum/Plasma Kit, QIAGEN, cat. No: 217004
- QIAzol Lysis Reagent, QIAGEN, cat. No.: 79306
- Chloroform:Isoamyl alcohol
- Absolute ethanol (99.9%)

Laboratory Equipment

- tabletop microcentrifuge for 1.5 / 2 ml tubes, with cooling
- fume hood

Procedure

1. Thaw plasma on ice. Thawing takes approximately 1.5 hours. Keep samples on ice until the step 6.

2. Cool down the centrifuge (CFG) to 4 °C. Set the temperature, close the lid and push FAST TEMP. Cooling takes approximately 15 minutes.
3. Prepare two 50 ml falcons – one for the absolute ethanol, the other for the freshly mixed 80% ethanol.
4. *When opening a new kit, be sure to add absolute ethanol to the wash solutions – the amount required is indicated on each bottle. After topping up on each vial, indicate that the volume has been replenished and the solution is ready for use.*
5. Prepare 3 × 1.5 ml tube, 1 × collection tube without lid, 1 × collection tube without lid and with filtration colon, 1 × collection tube with longer lid (part of the kit) – per sample, mark all the tubes properly.
6. Transfer 250 µl plasma into 1.5 ml Eppendorf tube. Centrifuge for 5 minutes at 1000 × g and 4 ° C to remove impurities.
7. Pipet 200 µl of plasma into a new 1.5 ml Eppendorf tube (carefully remove without touching the pellet).
8. To 200 µl of plasma add 750 µl of QIAzol. Vortex.
9. Incubate 5 min at room temperature (RT).
10. Add 200 µl of chloroform to each sample. Vortex for at least 15 seconds
11. Incubate for 2 min at RT.
12. Centrifuge at 12000 × g for 15 min at 4 ° C.
13. Set centrifuge to RT (20–25 °C).
14. Carefully pipet the upper aqueous phase into 2 ml Eppendorf tube (even if the volume is greater than 200 µl, use max. 200 µl tip, and take extreme caution – do not disrupt the interphase and the lower phase).
15. If the phases do not separate and you get only thick pinkish layer on top of the magenta organic phase, put the samples into −20 °C freezer overnight (not more than 24 hours) and continue again from the step 11.
16. Add 1.5 × volumes of ethanol. Mix by pipetting.
17. Pipet 650 µl into the Rneasy Mini Spin Column, label carefully.
18. Centrifuge 30 sec at RT / 8000 × g, remove flowthrough.
19. Repeat step 15–16 until you have filtered the entire sample volume, before applying the sample to the filter, always mix by pipetting.

20. Wash once with 700 µl of RWT, centrifuge for 30 sec at RT / 8000 × g, remove the flowthrough.
21. Wash with 500 µl of RPE. 30 sec at RT / 8000 × g, remove the flowthrough.
22. Wash with 500 µl of 80% ethanol. 30 sec at RT / 8000 × g, remove the flowthrough.
23. Place the column into a new tube and centrifuge for 2 min at RT / 8000 × g.
24. Change the column to the new Non-Stick Rnase-free Microfuge Tube and carefully add 20 µl of Dnase / Rnase-free water directly to the center of the column (do not touch anything with the tip, point to the middle of the column to cover its entire surface).
25. Incubate for 10 min at RT. Centrifuge 1 min at RT / 12000 × g. Place tubes with purified RNA on ice.
26. Store purified RNA at −80 ° C.

Since the final product concentration is too low and not directly measurable accurately by available spectrophotometric or fluorescence methods, QC is not performed in this case. To prepare miRNA cDNA libraries using QIAseq miRNA cDNA Library Kit, a single fixed volume of RNA is usually used, see 2.2.1.

2.2 Preparation of miRNA cDNA Libraries

Method principle

Optimized reaction chemistry in the QIAseq miRNA Library Kit enables robust, miRNA-specific libraries while minimizing reaction biases and eliminating adapter dimers. Unique Molecular Indices (UMIs) tag each miRNA at an early stage, eliminating PCR and sequencing bias.

In an unbiased reaction, adapters are ligated sequentially to the 3' and 5' ends of miRNAs. Subsequently, universal cDNA synthesis with UMI assignment, cDNA cleanup, library amplification, and library cleanup are performed. Proprietary methodology using modified oligonucleotides virtually eliminates the presence of adapter–dimers in the sequencing library, effectively removing a major contaminant often observed during sequencing.

Reagents

- QIAseq miRNA Library Kit, QIAGEN, cat. No: 33150, 33150
- QIAseq miRNA Index IL, QIAGEN, cat. No.: 331592, 33159, 3315
- Chloroform:Isoamyl alcohol

- Absolute ethanol (99.9%)

Laboratory Equipment

- tabletop microcentrifuge
- PCR box
- thermocycler
- magnetic rack
- Eppendorf tubes
- PCR strips

Procedure

The library preparation using QIAseq miRNA Library Kit is performed according to the manufacturer's protocol available.

2.2.1 3' Ligation

1. Thaw template RNA on ice. Gently mix, briefly centrifuge to collect residual liquid from the sides of the tubes, and return to ice.
2. Prepare reagents required for the 3' ligation reactions. Thaw QIAseq miRNA NGS 3' Adapter, QIAseq miRNA NGS 3' Buffer, 2x miRNA Ligation Activator, and Nucleasefree Water at room temperature (15–25ºC). Mix each solution by flicking the tubes. Centrifuge the tubes briefly to collect any residual liquid from the sides of the tubes and keep at RT. Remove QIAseq miRNA NGS RI and QIAseq miRNA NGS 3' RNA Ligase from the −30 to −15ºC freezer just before use, and place on ice. Return both enzymes to the freezer immediately after use.
3. Dilute the QIAseq miRNA NGS 3’ Adapter 1:5 using nuclease-free water. Briefly centrifuge, mix by pipetting up and down 12 times and centrifuge briefly again.
4. On ice, prepare the 3’ ligation reaction (QIAseq miRNA NGS 3’ Adapter 1 µl – diluted, QIAseq miRNA NGS RI 1 µl, QIAseq miRNA NGS 3’ Ligase 1 µl, QIAseq miRNA NGS 3’ Buffer 2 µl, 2x miRNA Ligation Activator 10 µl, Template RNA 5 µl) - Total volume 20 µl. Briefly centrifuge, mix by pipetting up and down 15 to 20 times and centrifuge briefly again.
5. Incubate for 1 h at 28ºC.
6. Incubate for 20 min at 65ºC.

7. Hold at 4ºC
8. Proceed immediately to 5’ Ligation Protocol.

2.2.2 5' Ligation

1. Dilute the QIAseq miRNA NGS 5’ Adapter 1:2.5 using nuclease-free water. Briefly centrifuge, mix by pipetting up and down 12 times and centrifuge briefly again.
2. On ice, prepare the 5’ ligation reaction (3’ ligation reaction 20 µl, Nuclease-free Water 15 µl, QIAseq miRNA NGS 5’ Buffer 2 µl, QIAseq miRNA NGS RI 1 µl, QIAseq miRNA NGS 5’ Ligase 1 µl, QIAseq miRNA NGS 5’ Adapter 1 µl – diluted) – total volume 40 µl.
3. Incubate for 30 min at 28ºC.
4. Incubate for 20 min at 65ºC.
5. Hold at 4ºC.
6. Proceed immediately to Protocol: Reverse Transcription.

2.2.3 Reverse Transcription

1. Prepare reagents required for the reverse transcription reactions. Thaw QIAseq miRNA NGS RT Initiator, QIAseq miRNA NGS RT Buffer and QIAseq miRNA NGS RT Primer at room temperature (15–25ºC). Mix by flicking the tube. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes and keep at room temperature. QIAseq miRNA NGS RI and QIAseq miRNA NGS RT Enzyme should be removed from the –20ºC freezer just before preparation of the master mix, and placed on ice. Both enzymes should be returned to the freezer immediately after use.
2. Add 2 µl QIAseq miRNA NGS RT Initiator to each tube. Briefly centrifuge, mix by pipetting up and down 15 to 20 times and centrifuge briefly again.
3. Incubate tubes for 2 min 75°C, 2 min 70°C, 2 min 65°C, 2 min 60°C, 2 min 55°C, 5 min 37°C, 5 min 25°C, ∞ 4°C.
4. Dilute the QIAseq miRNA NGS RT Primer 1:5 using nuclease-free water.
5. On ice, prepare the reverse transcription reaction (5’ ligation reaction + QIAseq miRNA NGS RT Initiator 42 µl, QIAseq miRNA NGS RT Primer 2 µl, Nuclease-free Water 2 µl, QIAseq miRNA NGS RT Buffer 12 µl, QIAseq miRNA NGS RI 1 µl, QIAseq miRNA NGS RT Enzyme 1 µl) – total volume 60 µl. Briefly centrifuge, mix by pipetting up and down 12 times and centrifuge briefly again.
6. Incubate for 1 h at 50ºC.

7. Incubate for 15 min at 70ºC.
8. Hold at 4ºC.

Proceed to Protocol: Preparation of QIAseq miRNA NGS Beads.

2.2.4 Preparation of QIAseq miRNA Beads

1. Thoroughly vortex QIAseq Beads and QIAseq miRNA NGS Bead Binding Buffer to ensure beads are in suspension and homogenously distributed. Do not centrifuge the reagents.
2. Carefully add 400 µl of QIAseq Beads (bead storage buffer is viscous) to a 2 ml microfuge tube. This amount of beads is sufficient to perform Protocol: cDNA Cleanup and the cleanup associated with library amplification for one sample. Briefly centrifuge and immediate separate beads on a magnet stand
3. When beads have fully migrated, carefully remove and discard the supernatant.
4. Remove the tube from the magnet stand, and carefully pipet (buffer is viscous) 150 µl of QIAseq miRNA NGS Bead Binding Buffer onto the beads. Thoroughly vortex to completely resuspend the bead pellet. Briefly centrifuge and immediately separate beads on a magnet stand.
5. When beads have fully migrated, carefully remove and discard the supernatant.
6. Remove the tube from the magnet stand and carefully pipet 400 µl of QIAseq miRNA NGS Bead Binding Buffer onto the beads (buffer is viscous). Thoroughly vortex to completely resuspend the bead pellet. Preparation of the QMN Beads is now complete. If the beads will not be used immediately, store beads on ice or at 4°C.
7. Proceed to Protocol: cDNA Cleanup.

2.2.5 cDNA Cleanup

1. Ensure the QMN Beads are thoroughly mixed at all times. This necessitates working quickly and resuspending the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads again.
2. Centrifuge the tubes/plates containing the cDNA reactions
3. Add 143 µl of QMN Beads to tubes/plates containing the cDNA reactions. Vortex for 3 sec and centrifuge briefly.
4. Incubate for 5 min at room temperature.

5. Place the tubes/plates on a magnet stand for ~4 min (or until beads have fully migrated).
6. Discard the supernatant and keep the beads.
7. With the beads still on the magnet stand, add 200 µl of 80% ethanol. Immediately remove and discard the ethanol wash.
8. Repeat the wash by adding 200 µl of 80% ethanol. Immediately remove and discard the second ethanol wash.
9. With the beads still on the magnetic stand, air dry at room temperature for 10 min.
10. With the beads still on the magnetic stand, elute the DNA by adding 17 µl of nuclease-free water to the tubes/plates. Subsequently close/cover and remove the tubes/plates from the magnetic stand.
11. Carefully pipet up and down until all the beads are thoroughly resuspended, briefly centrifuge and incubate at room temperature for 2 min.
12. Return the tubes/plates to the magnetic stand for ~2 min (or until beads have fully migrated).
13. Transfer 15 µl of eluted DNA to new tubes/plates.
14. Proceed to Protocol: Library Amplification. Alternatively, the completed cDNA cleanup product can be stored at −30 to −15°C in a constant temperature freezer.

2.2.6 Library Amplification Using Tube Indices

1. Prepare reagents required for the library amplification reactions. Thaw QIAseq miRNA NGS Library Buffer, QIAseq miRNA NGS ILM Library Forward Primer and required index primers. Mix by flicking the tube. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes. HotStarTaq DNA Polymerase should be removed from the –20ºC freezer just before preparation of the master mix, and placed on ice. HotStarTaq DNA Polymerase should be returned to the freezer immediately after use.
2. On ice, prepare the library amplification reaction (cDNA Cleanup product 15 µl, QIAseq miRNA NGS Library Buffer 16 µl, HotStarTaq DNA Polymerase 3 µl, QIAseq miRNA NGS ILM Library Forward Primer 2 µl, QIAseq miRNA NGS ILM IPD1 through IPD48 2 µl, Nuclease-free water 42 µl) – total volume 80 µl. Briefly centrifuge, mix by pipetting up and down 12 times and centrifuge briefly again.

3. Program the thermal cycler (Hold 15 min 95°C, 22 Cycles (Denaturation: 15 sec, 95°C Annealing: 30 sec 60°C Extension: 15 sec 72°C), Hold 2 min 72°C, Hold ∞ 4°C).
4. Place the library amplification reaction in the thermal cycler and start the run.
5. Add 75 µl of QMN Beads to tubes
6. Briefly centrifuge the 80 µl library amplification reactions, and transfer 75 µl to the tubes containing the QMN Beads. Vortex for 3 sec and briefly centrifuge.
7. Incubate for 5 min at room temperature
8. Place tubes on a magnet stand for approximately 4 min (or until beads have fully migrated).
9. Keep the supernatant, and transfer 145 µl of the supernatant to new tubes. Discard the tubes containing the beads.
10. To the 145 µl supernatant, add 130 µl of QMN Beads. Vortex for 3 sec and briefly centrifuge.
11. Incubate at room temperature for 5 min.
12. Place the tubes on a magnet stand until beads have fully migrated.
13. Discard the supernatant and keep the beads.
14. With the beads still on the magnet stand, add 200 µl of 80% ethanol. Immediately remove and discard the ethanol wash.
15. Repeat the wash by adding 200 µl of 80% ethanol. Immediately remove and discard the second ethanol wash.
16. With the beads still on the magnetic stand, air dry at room temperature for 10 min.
17. With the beads still on the magnetic stand, elute the DNA by adding 17 µl of nuclease-free water to the tubes. Subsequently close and remove the tubes from the magnetic stand.
18. Carefully pipet up and down until all beads are thoroughly resuspended; briefly centrifuge and incubate at room temperature for 2 min.
19. Place the tubes on the magnetic stand for ~2 min (or until beads have cleared).
20. Transfer 15 µl of eluted DNA to new tubes. This is the miRNA Sequencing Library.
21. Proceed to Quality Control. Alternatively, the completed cDNA cleanup product can be stored at −30 to −15°C in a constant temperature freezer.

2.3 miRNA Library Presequencing QC

Method Principle

Qubit Fluorometers detects fluorescent dyes that are specific to the target of interest – cDNA. These fluorescent dyes emit only when bound to the target molecules, even at low concentrations. Both the Qubit is order of magnitude more sensitive than UV absorbance, which measures anything absorbing at 260 nm—DNA, RNA, protein, free nucleotides, or excess salts.

DNA ScreenTape analysis provides a fast, automated, and flexible DNA electrophoresis solution for sample quality control, e.g in next-generation sequencing workflows.

Reagents

- TapeStation D1000 ScreenTape, Agilent, cat. No.: 5067-5583
- TapeStation D1000 Ladder, Agilent, cat. No.: 5067-5583
- Qubit™ 1X dsDNA High Sensitivity, Invitrogen, cat. No.: Q33230, Q33231

Laboratory Equipment

- TapeStation System, Agilent
- Invitrogen™ Qubit™ 4 Fluorometer, Invitrogen
- tabletop microcentrifuge
- strips

Procedure

2.3.1 Qubit dsDNA HS Assay

1. Prepare the Qubit® working solution by diluting the Qubit® dsDNA HS Reagent 1:200 in Qubit® dsDNA HS Buffer. Use a clean plastic tube each time you prepare Qubit® working solution.

   Add 190 µL of Qubit® working solution to each of the tubes used for standards.
2. Add 10 µL of each Qubit® standard to the appropriate tube, then mix by vortexing 2–3 seconds. Be careful not to create bubbles
3. Add Qubit® working solution to individual assay tubes so that the final volume in each tube after adding sample is 200 µL.

4. Add each sample to the assay tubes containing the correct volume of Qubit® working solution, then mix by vortexing 2–3 seconds. The final volume in each tube should be 200 µL.
5. Allow all tubes to incubate at room temperature for 2 minutes.
6. Proceed to "Reading standards and samples"; follow the procedure appropriate for your instrument.
7. Record the measured concentration.

2.3.2 Agilent TapeStation System D1000 Screentape

1. Launch the 2200 TapeStation Controller Software.
2. Load High Sensitivity D1000 ScreenTape device and loading tips into the 2200 TapeStation instrument.
3. Allow reagents to equilibrate at room temperature for 30 min.
4. Vortex mix before use 3 If running ladder, prepare by mixing 2 µL High Sensitivity D1000 Sample Buffer with 2 µL High Sensitivity D1000 Ladder.
5. Prepare sample by mixing 2 µL High Sensitivity D1000 Sample Buffer with 2 µL DNA sample.
6. Spin down, then vortex using IKA vortexer and adaptor at 2000 rpm for 1 min.
7. Spin down to position the sample at the bottom of the tube.
8. Load samples into the 2200 TapeStation instrument.
9. Select the required samples on the 2200 TapeStation Controller Software.
10. Click Start and specify a filename with which to save your results.
11. After the analysis, insert the electronical ladder. A miRNA-sized library is approximately 180 bp.

2.4 Global Analysis of miRNA Levels in Plasma

Method Principle

Illumina sequencing technology leverages clonal array formation and proprietary reversible terminator technology:

Cluster Generation: Sequencing templates are immobilized on a proprietary flow cell surface designed to present the DNA in a manner that facilitates access to enzymes while ensuring high stability of surface bound template and low non-specific binding of fluorescently

labelled nucleotides. Solid-phase amplification (bridge amplification) creates up to 1 000 identical copies of each single template molecule in proximity.

Sequencing by Synthesis: The technology uses four fluorescently labelled nucleotides to sequence the tens of millions of clusters on the flow cell surface in parallel. During each sequencing cycle, a single labelled deoxynucleoside triphosphate (dNTP) is added to the nucleic acid chain. The nucleotide label serves as a terminator for polymerization, so after each dNTP incorporation, the fluorescent dye is imaged to identify the base and then enzymatically cleaved to allow incorporation of the next nucleotide. Since all four reversible terminator-bound dNTPs (A, C, T, G) are present as single, separate molecules, natural competition minimizes incorporation bias.

Reagents

- NextSeq 500/550 High Output Kit v2.5 (75 Cycles), Illumina, cat. No.: 20024906
- Tris-HCl
- NaOH
- nuclease-free water

Laboratory Equipment

- NextSeq 500/550 System, Illumina
- tabletop microcentrifuge
- Eppendorf tubes (DNA low-bind type)
- PCR strips

2.4.1 Dilution and Pooling of cDNA Libraries

1. Calculate the c [nmol] od the libraries using formula c[nM] = (c [ng/µL]/660*bp))/10^6.
2. Dilute each library to 4 nM/1 µL with nuclease-free water.
3. Pool together all libraries using 2 µL of each 4nM library.
4. Measure concentration of the pool using Qubit dsDNA HS Assay and length of the cDNA library pool using Tapestation High Sensitivity D1000 ScreenTape.

2.4.2 Denaturation of cDNA Libraries

1. Combine 5 µL of library and 5 µl of freshly diluted 0.2 N NaOH in a microcentrifuge tube.

2. Vortex briefly and then centrifuge at 280 × g for 1 minute.
3. Incubate at room temperature for 5 minutes.
4. Add 5 μL of 200 mM Tris-HCl, pH 7.
5. Vortex briefly and then centrifuge at 280 × g for 1 minute.
6. Add the 985 μL prechilled HT1 to the tube of denatured libraries. The result is a 20 pM denatured library.
7. Vortex briefly and then centrifuge at 280 × g for 1 minute.
8. Place the 20 pM libraries on ice until you are ready to proceed to final dilution.
9. Dilute the denatured 20 pM library solution to 1.8 pM as follows.
10. Mix the denatured library solution (117 μl) and prechilled HT1 (1183 μl). The total volume is 1.3 ml at 1.8 pM.
11. Add 2 μl of PhiX control.
12. Proceed to single read, 80 cycles sequencing using NextSeq 500/550 High Output Kit v2.5 and NextSeq 500/550 instrument.

2.5 Bioinformatical analysis

2.5.1 Sequencing Data Analysis

After completing the sequencing run, the data are evaluated by the bioinformatician. The raw sequencing images from Illumina NextSeq 550 are converted to the fastq format using bcl2fastq (version 2.20.0). Raw reads are quality checked with the FastQC package (version 0.11.9), providing a first simple quality control check on raw sequence data. The Kraken package (Reaper version 16.098) identified adapter sequences that were trimmed using Cutadapt (version 3.3) while tolerating a 10% error rate. Collapsing of adapter-trimmed reads is performed by exploiting unique molecular identifiers (UMIs) with FASTX-Toolkit (version 0.0.14). Subsequently, reads are quality trimmed using Cutadapt, and these shorter than 15bp are removed from the dataset. Reads originating from snoRNAs, snRNAs, rRNAs, tRNAs, piRNAs, and YRNAs (reference sequences downloaded from Ensembl GRCh38, piRNAdb version 1.8, piRBase version 3.0, and RNAcentral release 20) are identified using Bowtie (version 1.3.0) and removed. The remaining potential miRNA reads are again quality checked and mapped against the miRBase (version 22) using miraligner - seqbuster (version 3.2) and then quantified by R package IsomiRs (version 1.22.1). The acquired miRNA counts are statistically analyzed, including normalization for library depth, carried out in R (version 4.0.4) with the DESeq2 package (version 1.30.1).

2.5.2 Endogenous Control

For each experiment, it is necessary to find a suitable endogenous control to normalize the data from RT-qPCR. Based on the results from the NGS or, for example, from the literature, we measure the expression of genes that could be suitable endogenous controls on the training set of samples. GenEx software is used for finding the most appropriate endogenous control for qRT-PCR normalization. Endogenous control is selected by two methods, GeNorm and Normfinder. These methods differ from each other by the algorithm used. Ideally, one should use both ways and select endogenous control based on their overlap. The M-value sorts the results of the GeNorm tool; the lower the M-value, the more suitable gene is for endogenous control.

1. Prepape the Excel file with only one sheet with Ct values.
2. Import the values, so the measured potential endogenous controls are in columns and samples in rows.
3. Name everything correctly and replace commas with decimal points.
4. Open the GenEx software, click Load file, and select Excel in the Files of type field (xls, xlsx).
5. Find the created Excel file and click on Open.
6. There are two icons in the Ref Gene tab - the first shows the GeNorm method, and the second shows NormFinder.
7. Click the GeNorm icon, and the Control Panel appears. Run the analysis.
8. Click the second icon - NormFinder, and in the Control Panel, click Run again. In this case, the genes are ranked by SD (standard deviation) and Acc. SD (accumulated standard deviation).
9. Compare the results of both methods; search for an intersection between GeNorm and NormFinder.

2.6 Validation of miRNA levels in plasma using RT-qPCR

Method principle

The Real-Time PCR (RT-PCR) method is based on a classical polymerase chain reaction, but unlike classical PCR, it can continuously monitor DNA increments during each cycle using fluorescence probes that detect the amount of PCR product by increasing its fluorescence activity. The indisputable advantage of RT-PCR is its high specificity, sensitivity, reproducibility, and, finally, the ability to detect even a very small number of molecules.

Since RNA cannot serve as a template for RT-PCR, it first has to be transcribed into complementary DNA (cDNA) by reverse transcription (RT). The main problem in quantification of miRNA is their short length (ca. 22 nt), which does not allow the use of conventional RT primers. Therefore, in the reverse transcription of miRNA, Applied Biosystems uses a special stem-loop RT primer gene that has the ability to distinguish single-nucleotide-related miRNAs. The primer binds to the 3 'end of the miRNA molecule and its hairpin structure prevents nonspecific hybridization of the primer with another RNA and further stabilizes the miRNA / DNA duplexes.

Reagents

- TaqMan® MicroRNA Reverse Transcription Kit, ThermoFisher Scientific, cat. No.: 4366596 or 4366597
- TaqMan® MicroRNA Assays ThermoFisher Scientific, cat. No.: 4427975
- TaqMan Universal Master Mix II, no UNG, ThermoFisher Scientific, cat. No.: 4440048
- nuclease-free water
- nuclease-free water

Laboratory Equipment

- thermocycler
- centrifuge with rotor suitable for 96/384-well plates
- Real-Time qPCR machine - QuantStudio™ 12K Flex Real-Time PCR System (Applied Biosystems) or Light Cycler® 480 Instrument II (Roche Life Science)
- 96/384-well plate suitable for Real-Time qPCR machines
- optical sealing foil suitable for Real-Time qPCR machine
- PCR strips

2.6.1 Reverse Transcription – cDNA synthesis

1. Thaw RT reagents and RNA samples on ice.
2. In a 1.5 ml tube, prepare master mix (MM) for reverse transcription according to the following table (always prepare MM for X + 5 samples, where X = number of samples analyzed):

Master mix for RT reaction in 10 µl		
Master mix for RT	1 sample (default volume) (µl)	1 sample (reduced reaction volume) (µl)
100mM dNTPs (with dTTP)	0.10	0.05
MultiScribe Reverse Transcriptase, 50 U µl (red cap)	0.67	0.335
10x Reverse Transcription Buffer	1.00	0.5
Rnase Inhibitor, 20 U/uL	0.13	0.065
Nuclease-free watter	2.77	1.80
RT primer	2.00	1.00
Total	**6.67**	**3.75**

Ad 1: Default volume of each reagent is state in the left column; in the right column are volumes of the reagents for reduced reaction volume of 5 µl total including sample. Reduced reaction volume leads to significant saving of reagents with a negligible effect on the qPCR result.

Ad 2: RT primer can be added to the master mix or can be added separately to each sample.

Ad 3: A separate master mix must be prepared for each primer when measuring several targets in one sample.

3. Mix slightly and centrifuge, place on ice.
4. Prepare 0.2 ml strips (according to the number of samples) and pipete 4.67 µl MM (or 3.75 µl in case of the reduced volume) into each well.
5. Add 3.33 µl (or 1.25 µl in case of reduced volume) of the sample to each tube, mix gently a centrifuge briefly.
6. Incubate for 5 minutes on ice.
7. Set the thermocycler according to the table:

temperature	time
hold 16 °C	30 min
hold 42 °C	30 min
hold 85 °C	5 min
hold 4 °C	∞

8. Run the reverse transcription.
9. Upon completion, continue with qPCR or store the product at −20 °C.

2.6.2 Quantitative Real Time PCR

1. Prepare a PCR master mix according to the table (each duplicate sample + Non-template control + Interplate control:

Reagents	1 sample (µl)	96+4 samples extra	1 sample (reduced vol.)
Universal no UNG MM	10.00	1000	7.50
Water	7.67	767	5.75
Assay	1.00	100	0.75
Total	**18.67**	**1867**	**14.00**

2. Pipette 18.67 µl of MM (14 µl in case of reduced volume) for miRNA, 19 µl for gene expression to 96-well PCR plates.
3. Add 1.33 µl of the sample to each well for miRNA, 1 µl for gene expression in duplicates (1 µl in case of reduces volume).
4. Into two wells add no-template controls, ie water instead of sample.
5. If you have a sample set of more than 47 samples, use "interplate control", ie on all plates with the same sample set pipette the same one sample.
6. Cover the plate with adhesive foil to avoid drying out of the extreme wells.
7. Briefly centrifuge, insert the plate into the machine.
8. Start the software, start a new experiment and name the appropriate wells.
9. Set PCR amplification conditions (reaction volume 20 µl or 15 µl) and start the run:

step	*not necessary if no UNG	Enzyme activation	PCR 40 cycles	
	HOLD	HOLD	denaturation	annealing/extension
Temperature	50 °C	95 °C	95 °C	60 °C
Time	2 min	10 min	15 s	60 s

10. Download the qPCR data. For data evaluation, see 2.7.

2.7 Validation Data Evaluation – qPCR Data Normalization

If used and necessary, data are normalized on interplate control. This normalization removes bias between multiple measured PCR plates detecting the same miRNA. The Ct values are normalized on endogenous control, and the miRNA expression levels are calculated using the 2-ΔCt method.

2.8 Validation Data Evaluation – Statistical Analysis

The software GraphPad Prism 7.0 is used for the analysis and visualization of expression values. The first step is to determine the normality of the data, i.e., whether the data distribution is symmetric or asymmetric. Based on this, it is decided to use parametric or non-parametric tests. The decision to use a parametric or non-parametric test is complex, requiring

critical thinking and perspective. This decision should not be automated. Biological data never follow a Gaussian distribution precisely because a Gaussian distribution extends infinitely in both directions. But many kinds of biological data follow a bell-shaped distribution that is approximately Gaussian.

2.8.1 Non-parametric tests

For non-parametric paired samples, the statistical differences between the levels of detected miRNA in tumor and (adjacent) non-tumor tissues are evaluated by the non-parametric Wilcoxon matched-pairs signed rank test. Mann-Whitney U-test is a non-parametric test used to analyze the differences in miRNA expression levels between the patient and control group.

In GraphPad prism, in the opening dialog box, choose a Column layout, and import the data, so each set is in a separate column. In the case of paired samples, it is necessary to have matched values on the same row (option Enter paired or repeated measures data - each subject on a separate row). However, in other cases, the number of values in the columns doesn´t have to be the same, and some fields will remain blank. Continue with the Analyze, and in the Analyze data window, select Column analyses, and choose t-tests (and non-parametric tests).

2.8.1.1 Mann-Whitney U-test

To complete the Mann-Whitney test, choose the option Unpaired; No. Use a non-parametric test.; then a Mann-Whitney test. Compare ranks. For best data visualization, select box plot with Median for error line settings.

2.8.1.2 Wilcoxon matched-pairs signed rank test

To perform the Wilcoxon matched-pairs signed rank test, select Paired and No. Use a non-parametric test. Choose option Before-after and Symbols & lines when adding a graph.

2.8.2 Parametric tests

In GraphPad prism, in the opening dialog box, choose a Column layout, and import the data, so each set is in a separate column. In the case of paired samples, it is necessary to have matched values on the same row. In the unpaired t-test, the number of values in the columns doesn´t have to be the same. Continue with the Analyze, and in the Analyze data window, select Column analyses, and choose t-tests (and non-parametric tests).

2.8.2.1 Unpaired t-test

Two-sample tests compare two independent groups (e.g., patients vs. control, tracking a particular feature in men and women). The unpaired t-test compares the difference between

means with the standard error of the difference, computed by combining the standard errors of the two groups.

In the next window, choose from the menu Unpaired t-test, Unpaired, Yes. Use parametric test; and based on SD either, Unpaired t-test. Assume both populations have the same SD or Unpaired t-test with Welch's correction. Do not assume equal SDs. And confirm with OK. In the plotted graph, change the type to Scatter plot and plot Mean with SD.

2.8.2.2 Paired t-test

In the opening tab, select Column, Enter/Import data: Enter paired or repeated measures data - each subject on a separate row. Enter the data, so each group is placed in a separate column, and each value must have a second paired value. In the Analysis tab, select Analyze and t-tests (and non-parametric tests) from the Column analyses menu. In the next step, check the Paired test, Paired t-test, and confirm with OK. In addition to the significance level, the output is also a graph (in the navigation panel).

2.8.2.3 ROC analysis

ROC analysis (Receiver Operating Characteristic) is a statistical test evaluating the relationship between true and false positivity. The samples can be classified according to the appropriate discriminatory cut-off value using the ROC curves, representing the dependence between sensitivity and 1-specificity. We evaluate the area under the ROC curve (Area Under the Curve), which shows the efficiency of the diagnostic test. A test with an AUC above 0.75 can be considered a good discriminatory test.

Import the data in a column layout (Column), then select Analyze, and in the Analyze data window, select Column analyses, and choose ROC curve.

2.8.2.4 Kaplan-Meier Analysis

Kaplan-Meier analysis is used for the non-parametric estimate of the survival probability of patients through survival times in the presence of censored observations. In practice, we will use the results obtained from the ROC analysis, which divided the patients according to the degree of expression of the chosen miRNA. The Kaplan-Meier survival curves show a possible correlation with the prognosis of the disease.

In the starting panel, select Survival and enter the survival time (in months or another time unit) in the column designated for the X-axis coordinates. The censored data (OS censored) ex. 0,1; is entered in the other columns selected for the Y-axis while maintaining the distribution according to the miRNA expression rate. Continue through Analyze> Survival

analyzes> Survival curve. To visualize data, choose a graph with survival curves Staircase with ticks starting at 100 %.