thrombomiR®

microRNA Biomarkers of Platelet Function

thrombomiR® kit

Wet Lab Instruction Manual v4.0

October 2021

KT-021-TR

TAmiRNA

stability for life.

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Further information and technical notes can be found at **www.tamirna.com/thrombomir**

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1

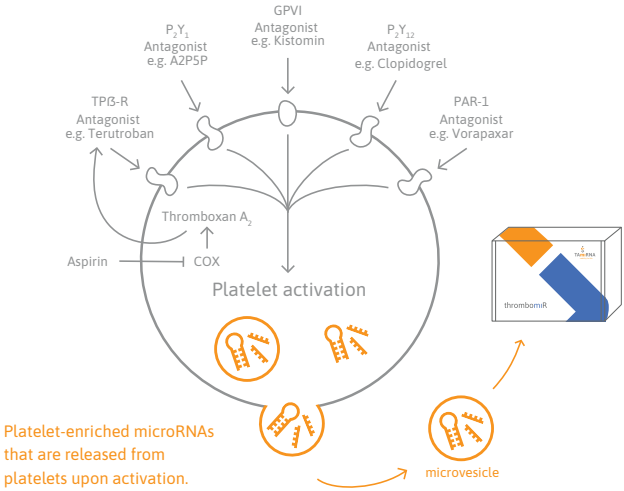
Product Summary

Intended-Use

The thrombomiR® kit has been developed to standardize the quantification of selected microRNA biomarker candidates for platelet function. The thrombomiR® kit is intended for research-use only, not for diagnosis, prevention or treatment of a disease. The clinical utility of the thrombomiR® kit is currently investigated in clinical trials.

The thrombomiR® kit provides users with a highly standardized method to determine the levels of 11 informative microRNAs in human platelet poor plasma (PPP) samples. It alleviates the task of selecting and optimizing analytical methods, data pre-processing and data normalization. It provides standardized plasma concentrations for microRNAs with a known association for anti-platelet therapy montitoring, risk assessment of cardiovascular events or early diagnosis of type 2 diabetes.

Figure 1
thrombomiR® – mode of action.



The selection of microRNAs for the thrombomiR® kit was **based on a series of prospective and cross-sectional studies**, which aimed to screen the levels of microRNAs in plasma of patients with acute coronary syndroms, anti-platelet therapy and type 2 diabetes. The thrombomiR® kit enables an in vivo measure of platelet function, independently of the activation pathway (Figure 1).

- Bye A, et al. Circulating microRNAs predict future fatal myocardial infarction in healthy individuals - The HUNT study. 2016 J Mol Cell Cardiol.
- Kaudewitz D, et al. Association of MicroRNAs and YRNAs With Platelet Function. 2016 Circ Res.
- Mayr M, et al. MicroRNAs within the continuum of postgenomics biomarker discovery. 2013 Arterioscler Thromb Vasc Biol.
- Willeit P, et al. Circulating microRNAs as novel biomarkers for platelet activation. 2013 Circ Res.
- Willeit P, et al. Circulating MicroRNA-122 Is Associated With The Risk of New-Onset Metabolic Syndrome And Type-2-Diabetes. 2016 Diabetes.
- Zampetaki A, et al. Prospective study on circulating MicroRNAs and risk of myocardial infarction. 2012 J Am Coll Cardiol.
- Zampetaki A, et al. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. 2010 Circ Res.
- Sunderland N, et al. MicroRNA Biomarkers and Platelet Reactivity: The Clot Thickens. 2017 Circ. Res.

Table 1
List of microRNAs included in the thrombomiR® kit

Based on the findings from these studies, the following list of microRNAs was selected for the thrombomiR® kit:

miRNA ID	platelet enrichment	platelet function	other cardiovascular functions	main cellular origin in plasma	validated pathways/targets
hsa-miR-126-3p	+++	platelet activation		platelets, megakaryocytes & endothelial cells	VEGF signaling: SPRED1 and PIK3R2/p85-β↓ Vascular inflammatory pathways: VCAM-1↓
hsa-miR-223-3p	+++	aggregation and granule secretion		platelets & megakaryocytes	P2Y12 receptor↓ RPS6KB1/HIF-1a signaling pathway
hsa-miR-197-3p	+++	platelet activation		platelets	
hsa-miR-191-5p	+++	platelet activation		platelets & endothelial cells	
hsa-miR-24-3p	++	platelet activation	monocyte differentation	platelets & endothelial cells, monocytes	PDGF-BB signaling: GATA2, PAK4↓: Vascularity, cardiac function, and infarct size after myocardial infarction
hsa-miR-21-5p	++	platelet biogenesis	inhibits cell growth in VSMCs	vascular smooth muscle cells, endothelial cells, cardiac fibroblasts, and cardiomyocytes, platelets	PTEN, BMPR2, WWP1, WASp
hsa-miR-28-3p	++	megakaryocyte differentiation↓		platelets & hematopoietic cells	
hsa-miR-320a	++		insulin signaling, angiogenesis, progression of retinopathy	platelets & endothelial cells	Survivin, VEGF
hsa-miR-150-5p	+	platelet activation, megakaryocyto-poiesis↑	insulin signaling, angiogenesis	leukocytes, megakaryocytes & monocytes	c-Myb, VEGF-a, HIF-1a
hsa-miR-27b-3p	+	megakaryocyte differentiation	angiogenesis, vascular disease and vascular aging, progression of retinopathy	platelets & vasculature	PPARγ, SMAD7
hsa-miR-122-5p	–		fatty acid and cholesterol synthesis in hepatocytes	liver tissue	multiple genes required for hepatocyte differentiation and fatty acid synthesis

Kit Components

The following components are required for the measurement of 11 informative microRNAs and 5 quality controls in human PPP samples:

- Serum/Plasma RNA extraction kit
- thrombomiR® chemistry, including spike-ins, RT chemistry and miGreen Mix
- primer coated thrombomiR® 96- or 384-well qPCR plates

Figure 2
Overview of required components

Serum/Plasma RNA extraction kit, RT

thrombomiR® chemistry, including spike-ins, RT chemistry and miGreen Mix, -20°C

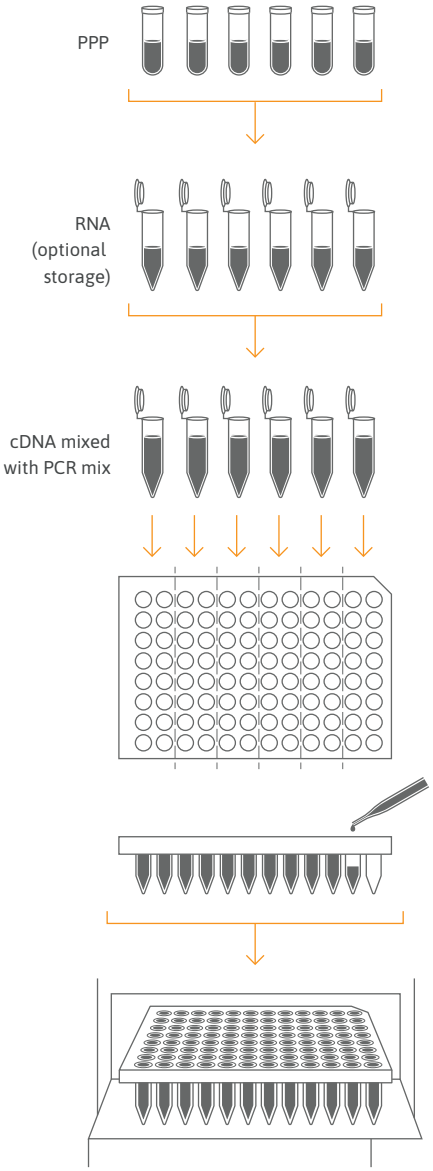
primer coated thrombomiR® 96- or 384-well qPCR plates, RT/-20°C

This combination of kits enables the measurement of microRNAs in PPP in a single day.

The entire workflow consists of three main steps:

1. RNA extraction
2. Reverse transcription to cDNA
3. PCR amplification

Figure 3
Workflow summary



1 RNA extraction (1.5h)

Extract RNA using the Serum/Plasma RNA extraction kit

2 cDNA synthesis (1.5h)

Prepare cDNA using the thrombomiR® kit reagents

3 Prepare PCR Mix

Dilute cDNA and mix with miGreen Mix

4 Real-time PCR amplification (2.5h)

Distribute PCR mix into wells on the ready-to-use thrombomiR® plate and start qPCR run

5 Data analysis

Export data for further analysis, data pre-processing, normalization and statistical analysis

Kit Technology

RNA Extraction

The Serum/Plasma RNA extraction kit enables the isolation of microRNA, from a minimum of 200 µl of sample. The phenol-free protocol uses spin column technology without the need for a vacuum pump. It allows analysis of extracellular vesicle RNA through lysis of the vesicles. The kit is designed to isolate high quality microRNA in amounts sufficient for qPCR analysis using the thrombomiR® kit.

The workflow consists of 5 simple steps:

1. Lysis of biofluid components
2. Precipitation and removal of proteins
3. Precipitation of RNA using isopropanol and column loading
4. Washing
5. Elution

In the first part of the RNA isolation process, membranized particles/cells are lysed using the provided lysis solution. Proteins are precipitated using the precipitation solution and the supernatant (including RNA) is mixed with isopropanol for precipitation. This solution is loaded onto a spin-column, where a resin binds RNA in a manner that depends on ionic concentrations. Thus, microRNA will bind to the column, while the residual proteins will be removed in the flow-through or retained on the top of the resin. The bound microRNA is then washed with the provided wash solutions in order to remove any remaining impurities, and the purified microRNA is eluted with RNase free water.

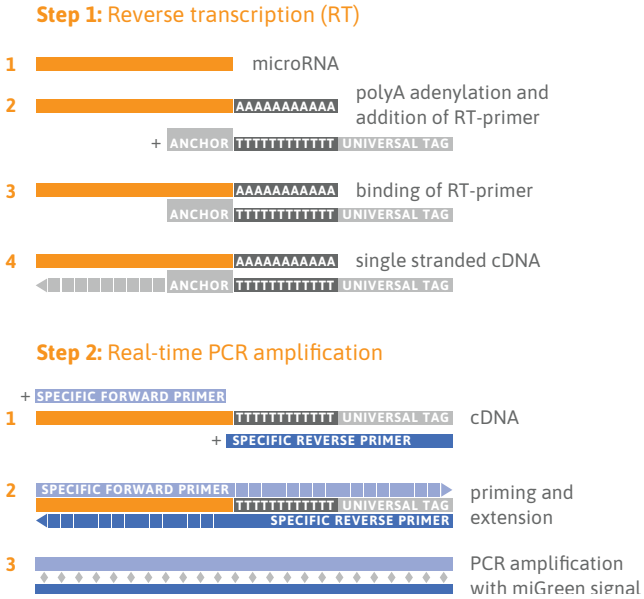
Reverse Transcription Quantitative PCR Detection

A universal reverse transcription (RT) reaction is performed, as shown in Figure 4 (Step 1), which means that all microRNA species are converted into complementary DNA (cDNA) at the same time. This enables parallel quantitative PCR (qPCR) detection of different microRNA sequences in one cDNA sample using the thrombomiR® test plate.

Universal RT is achieved by first adding a poly-A tail to the mature microRNA template (Step 1). Complementary cDNA is synthesized using a poly-T-primer with a 3' degenerate anchor and a 5' universal tag.

During qPCR, the cDNA is then amplified using microRNA-specific and LNA™-enhanced forward and reverse primers using SYBR® Green for detection of double-stranded DNA (Step 2).

Figure 4
Reverse transcription and PCR amplification



LNA™ Technology

Locked Nucleic Acids (LNA™) are chemically modified nucleotides, which offer substantially increased affinity for its complementary strand, compared to traditional DNA or RNA oligonucleotides. This results in unprecedented sensitivity and specificity and makes LNA™ oligonucleotides ideal for the detection of microRNAs, due to their short length and varying content of G-C and A-T bases. Without LNA™, the heterogeneous hybridization properties could result in unspecific and low efficient primer binding and compromise data quality.

Read more about the technology at www.exiqon.com/lna-technology

Storage and Stability

The thrombomiR® chemistry will be shipped on dry ice and **must be stored at -20°C**. The thrombomiR® qPCR plates will be shipped at ambient temperature and **must be stored at -20°C**. The Serum/Plasma RNA extraction kit is shipped at ambient tempature. Store the spin columns immediately at 2-8°C. Under these conditions, all components are stable until the expiry date on the package or vial.

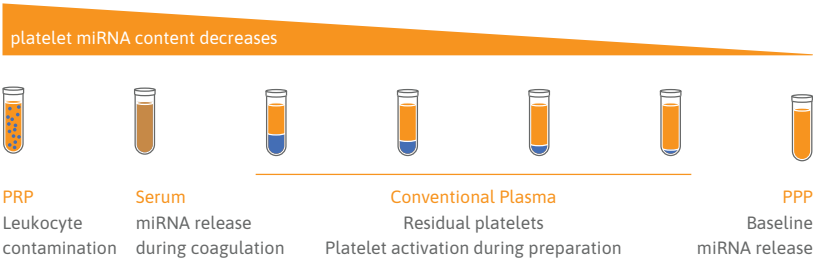
2

Important Pre-Analytical Considerations

Choice of Biofluid

TAmiRNA has used platelet-poor plasma (PPP) incubated with prostacyclin to inhibit platelet activation during centrifugation throughout its clinical program for the discovery of microRNA biomarkers for platelet function. Therefore, we recommend to use PPP for any experimental study using the thrombomiR® kit. The protocol for collection of PPP samples is provided on page 20 of this manual.

Figure 5
Platelet miRNA content in different blood components



Pre-analytical Standardization

Conditions during PPP processing might affect the detection of microRNAs using qPCR. Therefore, we strongly recommend to standardize protocols for pre-analytical processing and PPP collection. The following points should be incorporated in the pre-analytical study protocols:

- Patient variation: ensure overnight fasting prior to blood collection. Circadian rhythm, activity and diet are known to influence the levels of circulating microRNA content in patients.
- Use standardized needles and blood collection tubes. We recommend to use 21 gauge needles for blood collection. Ensure that only the specified blood collection materials are used during the entire study.
- Blood collection must be performed by a person that is well trained and familiar with the study protocol. Gloves must be worn at all times when handling specimens.

This includes amongst others removal of the rubber stopper from the blood tubes, centrifugation, pipetting, disposal of contaminated tubes, and clean-up of any spills. Tubes, needles, and pipets must be properly disposed of in biohazard containers, in accordance with institutional requirements.

- Universal precautions and OSHA (Occupational Safety and Health Administration) and institutional requirements (http://www.osha.gov/SLTC/biologicalagents/index.html) should be followed, including gloves, eye protection or working in a biosafety cabinet for blood processing.
- Centrifugation protocols for obtaining PPP from whole blood must be standardized and followed strictly (see "Platelet-Poor Plasma (PPP) Collection" on page 19 for more details).
- Hemolysis (visible as red-colored biofluid) must be recorded for all samples used (see "Quality Control" on page 15 for more details on how to detect hemolysis in your samples).
- Plasma samples can contain substances that inhibit the RT-qPCR reaction. A prominent inhibitor is heparin. Therefore, the use of lithium-heparin as an anticoagulant must be avoided. Presence of heparin in the sample usually results in higher variability in cDNA spike-ins (see page 15 on "Quality Control"). If presence of heparin cannot be avoided, heparinase treatment of extracted total RNA is an option. Please get in touch with us to request our heparinase treatment protocol.
- After thawing of collected PPP samples, ensure that samples are kept cool (on ice or 4°C) at all times and avoid frequent freeze-thaw cycles. Low temperature is essential for RNA stability and sample matrix.
- For handling of PPP as well as RNA/DNA we strongly advise to use RNase-free filter tips and nuclease-free microcentrifuge tubes with optimized surface properties to prevent adsorption of nucleic acids ("low binding").

Storage and Stability of PPP, RNA and DNA

All samples should be stored in nuclease-free plastic tubes with minimized absorption rates for nucleic-acids "low binding". To avoid freeze-thaw cycles the generation of aliquots of PPP samples is recommended. We recommend aliquot volumes of 225 µL serum.

PPP and RNA samples must be stored at -80°C for long term storage and kept on ice at all times during working procedures.

At TAmiRNA, we have successfully used PPP samples for microRNA analysis, which have been stored at -80°C for 15 years. In case the kit is intended to be used for PPP samples that are older than 15 years, we recommend to conduct a feasibility study to assess the detection rates of microRNAs compared to fresh samples.
Total RNA samples should be stored in nuclease-free low-binding tubes for not more than 6 months prior to analysis.

Undiluted cDNA must be stored at -20°C and can be used for up to 5 weeks after initial storage. Avoid more than 5 freeze/thaw cycles of frozen RNA and undiluted cDNA samples.

Working with RNA

RNases are very stable and robust enzymes that degrade RNA. Before working with RNA, it is recommended to create an RNase-free environment following the precautions below:

- The RNase-free working environment should be located away from microbiological work stations.
- Clean, disposable gloves should be worn at all times when handling reagents, samples, pipettes, disposable tubes, etc.
- It is recommended that gloves are changed frequently to avoid contamination.
- Designated solutions, tips, tubes, lab coats, pipettes, etc. should be prepared for RNA work only.
- All solutions that will be used should be prepared using molecular biology grade nuclease-free water.
- Clean all surfaces with commercially available RNase decontamination solutions.
- When working with purified RNA samples, ensure that they remain on ice.
- Spin down all reaction and sample tubes before opening.
- Use filter barrier pipette tips to avoid aerosol-mediated contamination.

It is recommended to establish and maintain designated areas for PCR setup, PCR amplification, and DNA detection of PCR products, due to the risk of contaminating reagents and mastermixes with amplified DNA.

The isolation of RNA and the reaction steps preceeding real-time PCR should be performed in rooms or areas, which are separated from areas where PCR experiments are performed in order to avoid contamination with amplified DNA. Use separate clean lab coats for RNA sample preparation, cDNA synthesis and when setting up PCR reactions or handling PCR products. Avoid bringing and opening tubes with amplified PCR products into the PCR setup area.

Quality Control

Synthetic spike-in Controls

In general, spike-in controls are used to monitor the efficiency and correct result of every workstep in the experiment. They can be used to identify outliers due to the presence of inhibiting factors or incorrect handling.

Uniform Cq-values obtained for the spike-ins demonstrate successful and homogenous RNA isolation, reverse transcription and qPCR for the samples. Synthetic spike-ins do not reveal the RNA content and quality in the biological sample.

RNA spike-in – The synthetic RNA spike-in is added to the sample during RNA extraction. It is used to monitor RNA extraction efficiency. The sequence has been designed to not match eukaryotic genomes and can therefore be applied across all species.

cDNA spike-in – The synthetic cDNA spike-in is added to the extracted RNA during reverse transcription into cDNA. It is used as a control for reverse transcription efficiency. It shares the natural microRNA sequence from C. elegans, which is not found in mammalian species. Reverse transcription efficiency is known to introduce the highest technical variance to RT-qPCR data.

PCR spike-in – This synthetic DNA template together with primers is spotted at a fixed position on every thrombomiR® test plate. The PCR spike-in is used to monitor PCR efficiency and to detect the presence of PCR inhibitors in samples.

The results obtained for all three spike-ins should be carefully analyzed using the thrombomiR® analysis toolkit. It should be used to identify potential outliers, and to exclude samples from subsequent normalization and statistical analysis. Spike-ins can be used for calibration of Cq-data of informative microRNAs to remove technical variance. More information can be found in technote TN05 at https://www.tamirna.com/technical-notes/.

Hemolysis

Hemolysis can be a major cause of variation in PPP microRNA levels due to contamination with cellular RNA.

The presence of hemolysis should be assessed visually for each sample. In addition, hemolysis can be assessed using the hemolysis-index, which is based on the relative expression of miR-451a-5p compared to miR 23a-3p. An increase in miR-451a-5p relative to miR-23a-3p indicates the presence of hemolysis in human PPP samples. The thrombomiR® software app will automatically calculate and report the Hemolysis Index.

Another option to determine hemolysis is the measurement of the absorbance peak of free haemoglobin by assessing free haemoglobin using a spectrophotometer such as NanoDrop™. Human PPP samples are classified as being hemolyzed if the absorption at 414 nm is exceeding 0.2. However, the presence of small amounts of cellular contamination in PPP samples is not readily detectable by visual or spectrophotometric means.

RNA Yield

Determination of RNA yield from 200 µL PPP is not possible by optical spectrophotometry or NanoDrop™ due to the lack in sensitivity of the method. We therefore recommend to assess RNA yield and extraction efficiency using synthetic spike-in controls. In addition, the entire analytical protocol precisely specifies to fluid volumes throughout the entire workflow (see “Lab Protocol” on page 17). The thrombomiR® kit uses 200 µL PPP for RNA extraction and 2 µL RNA for reverse transcription into cDNA. This is the optimum sample input in order to avoid inhibition of the reverse transcription reaction due to inhibitors that are co-extracted with total RNA. Excess amounts of total RNA in the reverse transcription reaction have been shown to lead to a non-linear quantification of microRNAs as well as a poor call rate.

3

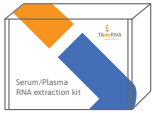
Lab Protocol

Essential components

1 Serum/Plasma RNA extraction kit

This box contains enough reagents to extract RNA from 50 PPP samples.

1x	Lysis Buffer	20 mL
1x	Protein Precipitation Buffer	8 mL
1x	Wash Buffer 1	15 mL*
1x	Wash Buffer 2	11 mL **
1x	RNase-free water	10 mL
1x	spin columns	50
1x	Collection tube 1.5 mL	50
1x	Collection tube 2 mL	50



* Add 2 volumes of 96%-100% Ethanol
** Add 4 volumes of 96%-100% Ethanol

2 thrombomiR® chemistry

This box contains spike-in controls, all reagents for cDNA synthesis, as well as the miGreen Mix

1x	RNA spike-in Mix	dried
1x	cDNA spike-in Mix	dried
1x	5x RT Buffer	0.1 mL
1x	10x RT Enzyme Mix	0.05 mL
3x	RNase-free water	1.5 mL
4x	2x miGreen Mix	1 mL
1x	Glycogen (5 mg/ml)	0.125 mL

3 Primer coated thrombomiR® qPCR plates

Depending on the qPCR cycler

8x	primer coated 96 well qPCR plates	
2x	primer coated 384 well qPCR plates	

Consumables and Instruments Not Supplied by TAmiRNA

- 99 % Ethanol, pro analysis
- Nuclease-free PCR tubes
- Nuclease-free, low nucleic acid binding tubes (1.5 mL)
- Nuclease-free, filter pipette tips
- Sealing foils for PCR plates, transparent, suitable for qPCR
- Heating block or PCR cyler for RT
- Vortexer
- Calibrated pipettes
- Centrifuge for <2 mL tubes and multiwell plates
- ROX reference dye for ABI cycler

Table 3
Plate format and PCR cycler

Plate format and PCR cycler

Plate Format 96-well	**Cycler**
A	Applied Biosystems® models 5700, 7000, 7300, 7500, 7700, 7900HT, ViiA™ 7 (96-well block), QuantStudio 12K Flex (96-well), QuantStudio 3/5/7 (96-well Standard Block), QuantStudio 6K Flex (96-well); Bio-Rad® models iCycler®, iQ™5, MyiQ™, MyiQ2; Bio-Rad/MJ Research Chromo4™; Eppendorf® MasterCycler® ep realplex models 2, 2s, 4, 4s; Stratagene® models Mx3005P®, Mx3000P®; Takara: TP-800
C	Applied Biosystems models 7500 (Fast block), 7900HT (Fast block), StepOnePlus™, ViiA 7 (Fast block), QuantStudio 12K Flex (96-well Fast Block), QuantStudio 3/5/7 (96-well Fast Block), QuantStudio 6K Flex (96-well Fast)
D	Bio-Rad CFX96™; Bio-Rad/MJ Research models DNA Engine Opticon®, DNA Engine Opticon 2; Stratagene Mx4000®
F	Roche® LightCycler® 480 (96-well block)
Plate Format 384-well	**Cycler**
E	Applied Biosystems models 7900HT (384-well block), ViiA 7 (384-well block), QuantStudio 12K Flex (384-well), QuantStudio 3/5/7 (384-well Standard Block), QuantStudio 6K Flex (384-well); Bio-Rad CFX384™
G	Roche® LightCycler® (384-well block)

Platelet-Poor Plasma (PPP) Collection

The thrombomiR® workflow requires 200 µL PPP.

PPP collection is ideally performed after overnight fasting in the morning hours between 8 am and 10 am. This can reduce biological variance in microRNA levels due to activity and diet. We recommend to use 21 gauge needles and vacutainer tubes (ETDA or citrate, BD vacutainer®) for blood collection.

Filled tubes should be mixed gently. The first step is the preparation of platelet-rich plasma (PRP). Therefore collection tube should be centrifuged at 1,000 g for 10 minutes at 4°C with low brake for cell depletion.

After centrifugation the clot is located at the bottom of the tube, and the PRP is on top of the clot. Remove the PRP layer, be careful not to disturb the clot. Transfer the top three-quarters of plasma into labeled nuclease-free (1.5 mL) tubes using nuclease free filter tips. Aliquote volume is recommended for the thrombomiR® kit.

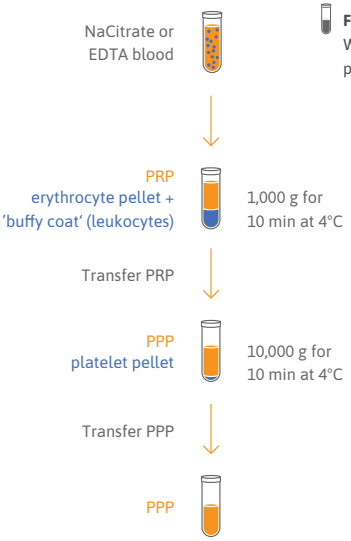


Figure 6
Workflow for the preparation of PPP

Note: Never pour off plasma; pouring off plasma directly from the draw tube will introduce excess cells to the specimen. To remove plasma, start from the top, gently draw specimen into pipette as you go further down tube. Leaving approximately 0.5 mL of plasma will insure that you do not disturb the buffy coat and cell layer.

To prepare PPP centrifuge tubes at 10,000 g for 10 minutes at 4°C. Transfer the supernatant, PPP, into new labeled nuclease-free (1.5 mL) tubes.

Check that all aliquot vial caps are secure and that all vials are labeled. Place all aliquots upright in a specimen box or rack in an -80°C or colder freezer. All specimens should remain at -80°C or colder prior to analysis or shipping. The sample aliquots should not be thawed prior to analysis or shipping.

Protocol

The RNA extraction has been **standardized to a volume of 200 µL PPP** as starting material to ensure high RNA yield and prevent inhibition of downstream PCR applications.
If less than 200 µL PPP is available we recommend to fill available PPP up to 200 µL total volume using RNase-free water. Keep in mind that lower sample input might lead to a reduced sensitivity and yield.

RNA Extraction

Important points before starting

- Lysis Buffer and Wash Buffer 1 may form a precipitate upon storage. If necessary, redissolve by warming and then place at room temperature.
- Equilibrate buffers at room temperature before starting the protocol.
- Prepare 80% ethanol for Step 9c.
- All steps should be performed at room temperature. Work quickly!

Important Note:
when your are performing the thrombomiR® analysis for the first time, we highly recommend to start with a pilot analysis. Only a small subset of your samples for this pilot analysis and analyze your data using the thrombomiR® data analysis application see page 29. Once you have confirmed that sample quality and data quality are OK, proceed to the full analysis.

This protocol is designed for human PPP

Notes

Step 1
Preparation of Wash Buffer 1 and Wash Buffer 2
First time use only

Add 2 volumes of >99 % ethanol to Wash Buffer 1 and 4 volumes of >99 % ethanol to Wash Buffer 2.
Note: The label on the bottle has a box that should be checked to indicate that the ethanol has been added.

Step 2
Reconstitution of lyophilized spike-in controls (RNA spike-in and cDNA spike-in)
First time use only

- Spin down vials before use by centrifugation at 3,000 g for 30 sec at room temperature.
- Resuspend the spike-ins by adding 80 µL nuclease-free water.
- Mix by vortexing and spin down. Store on ice for 20 min.
- Mix by vortexing again and aliquot in low bind tubes. (20 µL aliquots are recommended).
- Store at -20°C.

Step 3
Thaw PPP samples and glycogen

After thawing on room temperature, centrifuge the samples at 12,000 g for 5 min at 4°C to pellet any debris and insoluble components and to reduce effect of inhibitors/nucleases.
Thaw glycogen on RT and store on ice.

Step 4
PPP lysis

Transfer exactly 200 µL PPP to a new 1.5 mL tube. If using less than 200 µL, fill up to 200 µL with RNase-free water. Ensure equal volumes of all samples. Add 1 µL RNA spike-in to 60 µL Lysis Buffer and mix it with the 200 µL PPP. Vortex for 5 sec and incubate for 3 min at room temperature. When processing multiple samples vortex immediately after addition of Lysis Buffer. For multiple samples prepare a mastermix including 1 extra Rxn.

Important note: The RNA spike-in must be mixed with the Lysis Buffer before mixing with the sample – if added directly to the sample it will be rapidly degraded.

Step 5
Protein precipitation

Add 20 µL of Protein Precipitation Buffer. Vortex for >20 sec and incubate for 3 min at room temperature. When processing multiple samples vortex immediately after addition of Protein Precipitation Buffer. The solution should become a milky suspension. Centrifuge for 3 min at 12,000 g at room temperature.

		Notes
Step 6 **Transfer supernatant**	Transfer exactly 200 µL of the clear supernatant (aqueous phase) into a new collection tube (1.5 mL, with lid) and add 2 µL glycogen (5 mg / mL). Vortex and spin down.	
Step 7 **Adjust binding conditions**	Add 200 µL Isopropanol. Vortex for 5 sec	
Step 8 **Load column**	Place a Spin Column in a collection tube and load the entire sample onto the column. Centrifuge for 30 sec at 8,000 g at room temperature. Discard flow-through and place column back into the collection tube.	
Step 9a **Wash and dry**	Add 700 µL Wash Buffer 1 to the spin column. Centrifuge for 15 sec at 8,000 g at room temperature. Discard flow-through and place column back into the collection tube.	
Step 9b **Wash and dry**	Add 500 µL Wash Buffer 2 to the spin column. Centrifuge for 15 sec at 8,000 g at room temperature. Discard flow-through and place column back into the collection tube.	
Step 9c **Wash and dry**	Add 500 µL 80% ethanol to the spin column. Centrifuge for 2 min at 8,000 g at room temperature. Place the spin column in a new collection tube (without lid). Centrifuge column with open lid for 5 min at 12,000 g at room temperature to dry the membrane completely.	
Step 10 **Elute**	Place the spin column in a new low bind collection tube (1.5 mL). Add 30 µL RNase free water directly onto the membrane of the spin column. Incubate for 1 min at room temperature. Close the lid and centrifuge for 1 min at 12,000 g at room temperature.	
Step 11 **Storage**	Store the RNA sample immediately at -80°C or proceed to cDNA synthesis (Step 13).	

cDNA Synthesis

Keep samples, reagents and reactions on ice (or at 4°C) at all time.

Notes

Step 12
Thaw total RNA

Thaw total RNA (from step 11) on ice.

Step 13
Prepare reagents

Thaw 5x RT reaction buffer and nuclease free water and put on ice. Immediately before use, remove the enzyme mix from the freezer, mix by flicking the tube and place on ice. Spin down all reagents.

Step 14
Prepare cDNA synthesis mix

If performing cDNA synthesis on multiple RNA samples, prepare a mastermix including 1 extra reaction.

Reagent (Volumes in µL)	10 µL Rxn
5x Buffer	2
cDNA spike-in	0.5
Nuclease-free water	4.5
Enzyme mix	1
Total Volume Mix	8

Pipet 2 µL RNA template in each tube and add 8 µL cDNA mastermix. Mix by pipetting and spin down.

Step 15
Incubate and heat inactivate

Incubate the reaction at 42°C for 60 min. Heat-inactivate the reverse transcriptase at 95°C for 5 min. Immediately cool to 12°C.

Step 16
Storage

Transfer the undiluted cDNA into nuclease-free low bind tubes and freeze at -20°C for up to 5 weeks.

qPCR Amplification

The thrombomiR® test plate contains 16 different microRNA or QC primer sets. Six samples can be measured on one 96-well plate. Two columns are used per sample. 24 samples can be measured on one 384-well plate, using one column per sample.

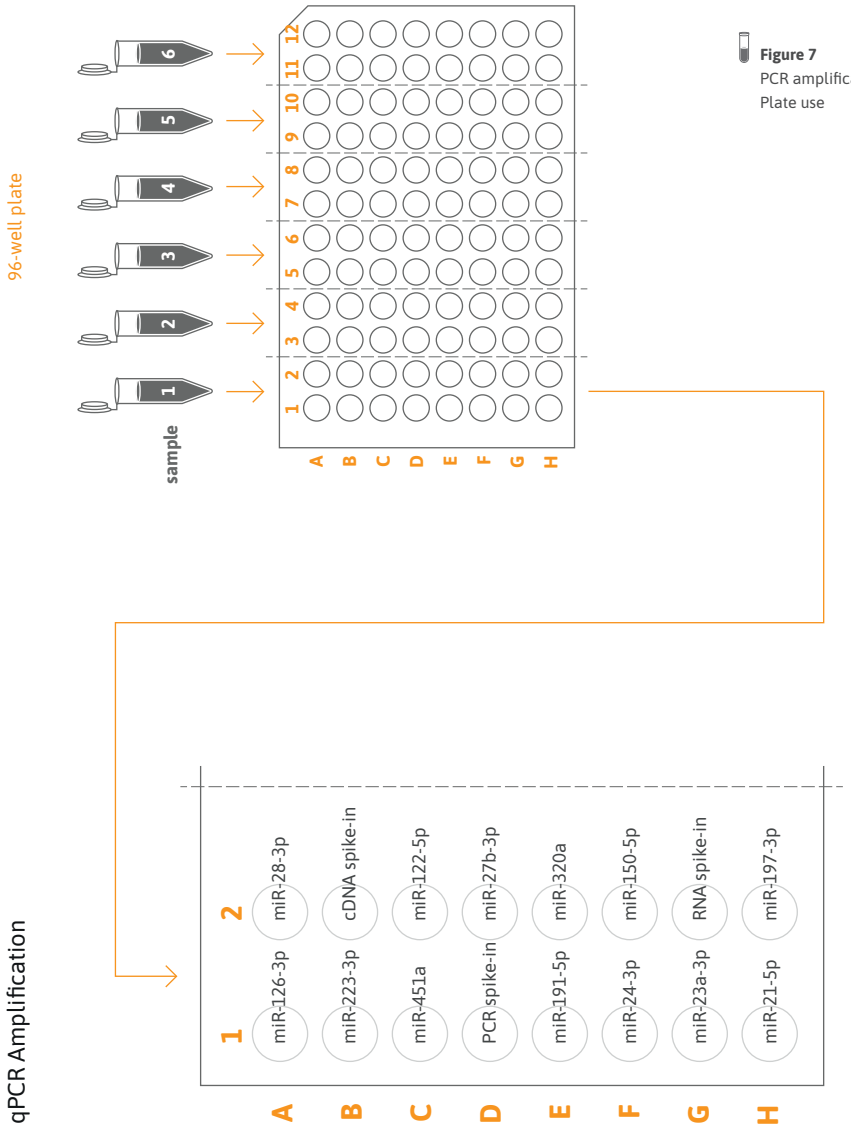
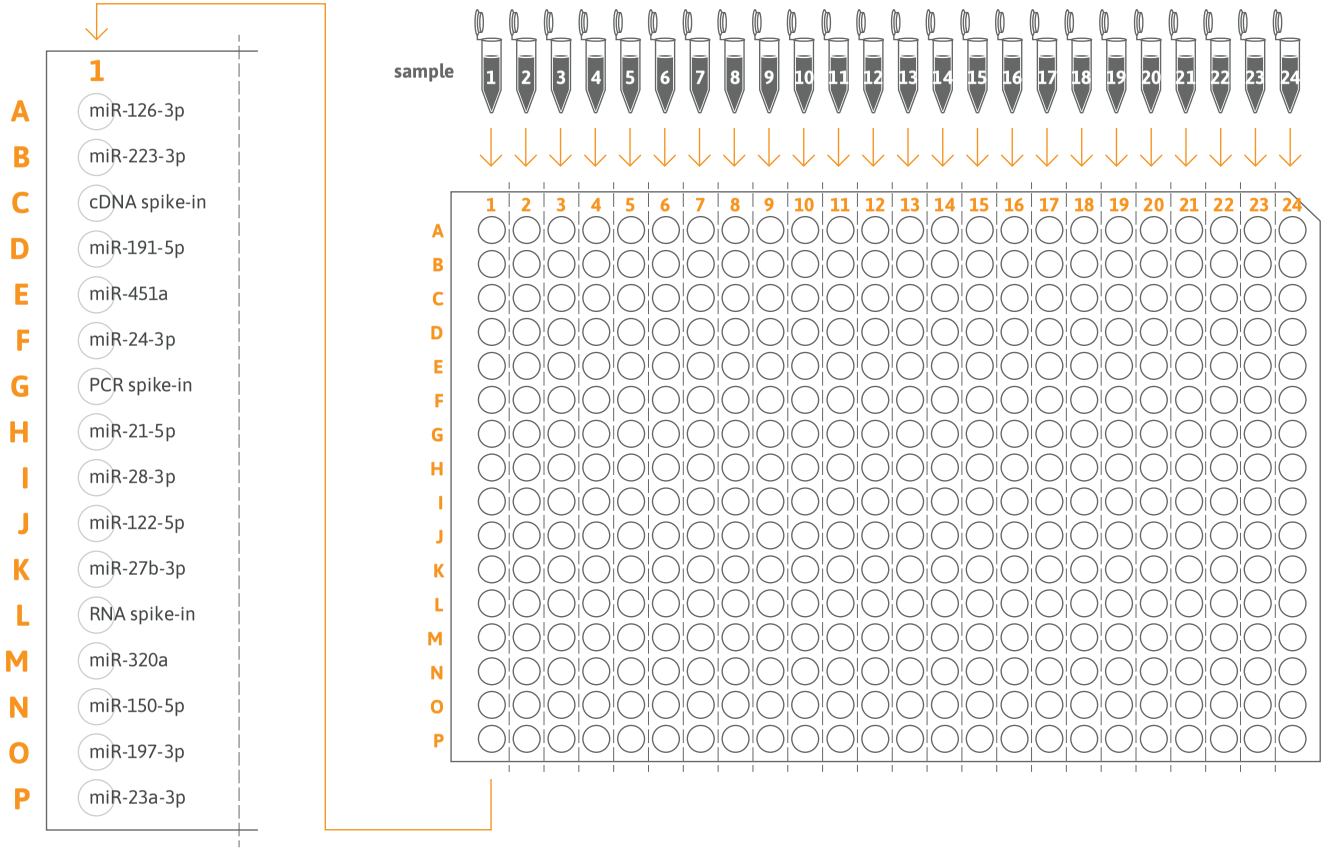


Figure 7
PCR amplification
Plate use

qPCR Amplification



Notes

Step 17
Thaw reagents

Thaw cDNA (from step 16) and miGreen master mix on ice for 15–20 minutes. Keep reagents on ice all the time. Before use mix the Master mix by pipetting up and down.

Note: If you using an ABI cycler please skip to page 26.

Notes

Step 18
Mix cDNA with water and qPCR Master mix

Mix 1.8 µL cDNA with 88.2 µL nuclease free water, then add 90 µL miGreen Master mix (in total 180 µL). Mix by pipetting up and down, spin down to collect the liquid at the bottom. Repeat this step for all samples.

Step 19
Prepare thrombomiR® plate

Add 10 µL reaction mixture (from step 18) (cDNA, NFW, Master mix) to each of the 16 wells. Seal the plate with the appropriate optical sealing. Incubate at 4°C for a minimum of 1 hour.

Note: The plate can be stored up to 16 hours at 4°C protected from light.

Step 20
Perform qPCR (Roche LC 480)

Before running the qPCR, spin plate at 1,000 g for 90 sec. Perform qPCR and melting curve analysis as shown below. Settings have been optimized for the Roche Light Cycler® 480 II instruments.

Step	Setting
Polymerase activation/ denaturation	95°C , 2 min, ramp-rate 4.4°C/sec
Amplification – 45 cycles	Analysis mode: Quantification 95°C, 10 sec, ramp 4.4°C/sec 56°C, 60 sec, ramp 2.2°C/sec Acquisition mode: single
Melting curve	Analysis mode: Melting curve 95°C, 10 sec, ramp 4.4°C/sec 55°C, 60 sec, ramp 2.2°C/sec 99°C, acquisition mode: Continuous, ramp 0.11°C/sec, acquisition per °C: 5

If using an Applied Biosystems Instrument, following step must be adapted:

Notes

Step 18.1

qPCR master mix

	Low ROX	High ROX
miGreen mastermix	90	90
Nuclease Free water	87.3	79.2
ROX	0.9	9
cDNA	1.8	1.8

ROX dye is required at the following concentrations:
Low concentration of ROX dye (200x): Applied Biosystems 7500 and ViiA 7 and QuantStudio Instruments.
High concentration of ROX dye (20x): ABI PRISM® 7000, Applied Biosystems 7300 and 7900.

Step 19.1

Prepare thrombomiR® plate

Add 10 µL reaction mixture (from step 18.1) (cDNA, NFW, master mix) to each of the 16 wells. Seal the plate with the appropriate optical sealing. Incubate at 4°C for a minimum of 1 hour.

Note: The plate can be stored up to 16 hours at 4°C protected from light.

Step 20.1

Perform qPCR

Step	Setting
Polymerase activation/ denaturation	95°C, 2 min, maximal/fast mode
Amplification – 40 cycles	95°C, 10 sec, maximal/fast mode 56°C, 60 sec, maximal/fast mode
Melting curve analysis:	60–95°C

Data Analysis

Data analysis is performed using our proprietary software application platform at https://thrombomir.tamirna.com. After purchase of the thrombomiR® kit or service, you will automatically receive a username and password to access the application

The application requires the upload of raw fluorescence data in text-file format, which can be exported from all supported qPCR instruments (see list on page 19 of this manual). The application will automatically call Cq-values using the second-derivative maximum method. Based on Cq-values from spike-in controls (please read information on page 15), hemolysis controls, and thrombomiRs a quality check will be performed. All samples that have passed the QC will be used to provide raw and normalized data for all thrombomiRs. Figure 7 below provides an example of a thrombomiR® 96-well plate run with 12 high quality samples: equal RNA extraction efficiency and absence of inhibition results in very little variation in spike-in controls.

Figure 8
A) QC plot generated from a high quality thrombomiR® experiment using the software application. All three spike-ins show comparable levels across the samples that are within the expected range

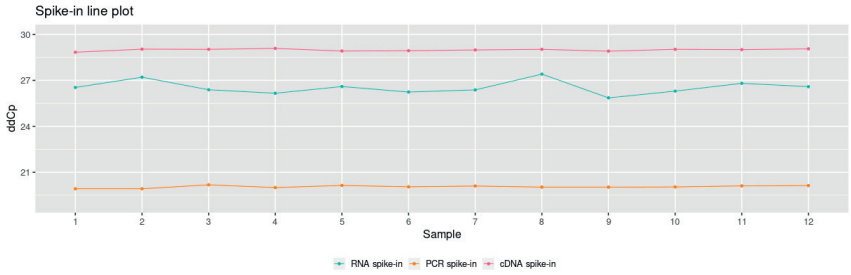


Figure 8

B) QC plot of an experiment where 2 samples show elevated levels for RNA and cDNA spike-in indicating inhibition of the reverse transcription.

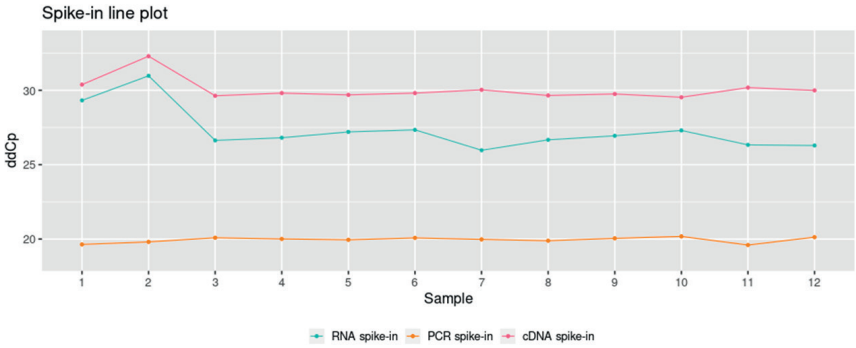


Table 4

Quality control results interpretation

thrombomiR®: Quality control results interpretation

Scenario	RNA Spike-In	cDNA Spike-In	PCR Spike-in	Hemo lysis	thrombomiRs	Result	Interpretation	Action
1	Cq <30	Cq <32	Cq <23	ratio <7	all 11 miRs detected	Valid	RNA extraction, RT-qPCR OK, no hemolysis, sample is intact	Perform scoring and generate report
2	Cq <30	Cq <32	Cq <23	ratio >7	n.r.	Invalid	Sample error: Hemolysis	If possible, draw a new sample.
3	Cq <30	Cq <32	Cq <23	n.r.	< 11 miRs detected	Invalid	Sample error: low quality plasma sample	If possible, draw new blood sample
5	Cq >30	Cq >32	Cq <23	n.r.	< 11 miRs detected	Invalid	Sample error: reverse transcription failed due to inhibition	Draw new blood sample. In case of heparin contamination consider heparinase treatment.
4	Cq >30	Cq <32	Cq <23	n.r.	< 11 miRs detected	Invalid	Technical error: RNA extraction has failed	Repeat RNA extraction
6	Cq <30	Cq >32	Cq <23	ratio <7	all 11 miRs detected	Invalid	Technical error: cDNA spike-in control	Use new cDNA Spike-In aliquot
7	Cq >30	Cq <32	Cq <23	n.r.	all 11 miRs detected	Invalid	Technical error:RNA spike-in control	Use new RNA Spike-In aliquot and repeat RNA extraction
8	Cq >30	Cq >32	Cq >23	n.r.	< 11 miRs detected	Invalid	Technical error: PCR reaction has failed	Repeat PCR and exchange PCR mastermix

n.r.: result is not relevant for judging the run quality

Troubleshooting

RNA Isolation

Poor RNA Recovery

Column has become clogged	In most cases this can happen when recommended amounts of starting materials were exceeded. For most biofluids this is unlikely to occur. However, because of the variety of biological samples the amount of starting material may need to be decreased below the recommended levels if the column shows signs of clogging. See also "Clogged Column" below
An alternative elution solution was used	For maximum RNA recovery it is recommended to elute the RNA with the RNase-free water supplied with this kit.
RNA content	The RNA content in PPP is low therefore the concentration measurement of the purified RNA (e.g. spectrophotometric or with fluorescent dyes) is not accurately possible. The protocol is optimized using fixed volumes.

Clogged Column

Temperature too low	Ensure that the centrifuge and solutions remain at room temperature (18 - 25°C) throughout the procedure. Temperatures below 15°C may result in salt precipitates that may clog the columns. If salt precipitation is present, heat the solution to 30°C until completely redissolved and let the solutions cool to room temperature before use.

Degraded RNA

RNase contamination	RNases may be introduced when working with the samples. Ensure that proper procedures are followed when working with RNA. Please refer to "Working with RNA" at the beginning of this manual
Procedure not performed quickly enough	In order to maintain the integrity of the RNA, it is important that the procedure be performed quickly.

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Improper storage of the purified RNA	For short term storage RNA samples may be stored at -20°C for a few days. It is recommended that samples be stored at -70°C for longer term storage **Tip! If possible, snap freeze your RNA in liquid nitrogen before storage in the freezer. Avoid repeated freeze/ thaw-cycles by freezing aliquots of your RNA.**
Enzymes used may not be RNase-free	In order to prevent possible problems with RNA degradation ensure that enzymes used upstream of the isolation process are RNase-free.

RNA does not perform well in downstream applications

Salt or Ethanol carryover	Traces of salt and ethanol from the binding step can interfere with downstream applications. Therefore, Step 6 (Wash) is important for the quality of your RNA sample To avoid remaining salts please make sure that the RNA bound to the column is washed 3 times with the provided Wash Solution and ensure that the dry spin is performed, in order to remove traces of ethanol prior to elution.
Inhibitors	Some individual serum samples can contain inhibitors. Using spike-ins that control every step of the protocol inhibitors can be easily detected. Samples that contain inhibitors must be excluded from the analysis.

cDNA and qPCR Amplification

Notes

Problem	Suggestion
No fluorescent signal is detected during the PCR	Confirm that the PCR setup was correct by checking the signal obtained for the PCR spike-in control.
No fluorescent signal detected during the PCR, but the spike-in „UniSp3 IPC" gives a valid signal.	Check that the filter in the qPCR cycler was set to either miGreen or FAM/FITC Check that the optical read is at the correct step of the qPCR cycles.

Related Services

TAmiRNA offers a broad range of high quality RNA services performed by experts according to GLP standards, including RNA isolation, next generation sequencing and qPCR analysis. Read more about there services at
www.tamirna.com/small-rna-sequencing-services/

Related Products

TAmiRNA also offers research-use kits for novel microRNA biomarkers in bone quality. Read more about these products at www.tamirna.com.

Further Reading

Below you find a list of publications, which describe the identification and utility of thrombomiRs.

1. Bye A, et al. Circulating microRNAs predict future fatal myocardial infarction in healthy individuals - The HUNT study. 2016 J Mol Cell Cardiol.

2. Kaudewitz D, et al. Association of MicroRNAs and YRNAs With Platelet Function. 2016 Circ Res.

3. Mayr M, et al. MicroRNAs within the continuum of postgenomics biomarker discovery. 2013 Arterioscler Thromb Vasc Biol.

4. Willeit P, et al. Circulating microRNAs as novel biomarkers for platelet activation. 2013 Circ Res.

5. Willeit P, et al. Circulating MicroRNA-122 Is Associated With The Risk of New-Onset Metabolic Syndrome And Type-2-Diabetes. 2016 Diabetes.

6. Zampetaki A, et al. Prospective study on circulating MicroRNAs and risk of myocardial infarction. 2012 J Am Coll Cardiol.

7. Zampetaki A, et al. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. 2010 Circ Res.

8. Sunderland N, et al. MicroRNA Biomarkers and Platelet Reactivity: The Clot Thickens. 2017 Circ. Res.

Further reading on quality controls for circulating microRNA experiments

1. TAmiRNA TechNote TN-05. Quality controls and best practices for analyzing microRNAs in cell-free biofluids by RT-qPCR. April 2019

2. Mussbacher M, Krammer TL, Heber S, Schrottmaier WC, Zeibig S, Holthoff HP, et al. Impact of Anticoagulation and Sample Processing on the Quantification of Human Blood-Derived microRNA Signatures. Cells. 2020 Aug 18;9(8):1915.

3. Blondal T, et al. Assessing sample and miRNA profile quality in serum and plasma or other biofluids. 2013 Methods

4. Shah JS, et al. Comparison of methodologies to detect low levels of hemolysis in serum for accurate assessment of serum microRNAs. 2016 PLoS One

5. Mestdagh P, et al. Evaluation of quantitative miRNA expression platforms in the microRNA quality control (miRQC) study. 2014 Nat Methods

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