thrombomıR™

microRNA Biomarkers of Platelet Function





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

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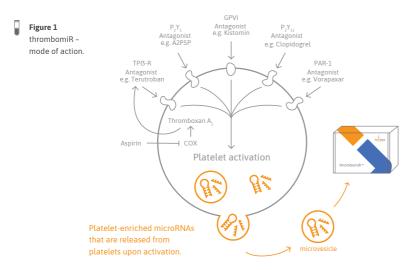
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Product Summary

Intended-Use

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



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.
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- 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, megakaryocy- to-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	PPARy, 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

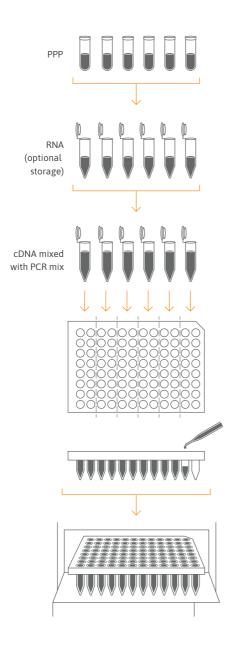


primer coated thrombomiR[™] 96- or 384-well qPCR plates, RT/-20°C

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



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 μ l 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 thrombomiRTM 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.

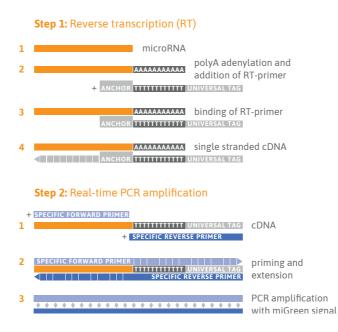
Reverse Transcription Quantitative PCR Detection

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).





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LNA[™] Technology

Locked Nucleic Acids (LNATM) 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 LNATM oligonucleotides ideal for the detection of microRNAs, due to their short length and varying content of G-C and A-T bases. Without LNATM, 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 RT 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.



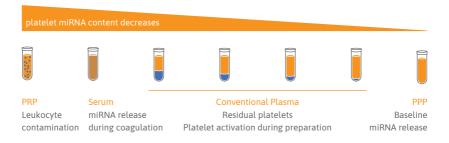
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Important Pre-Analytical Considerations

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.

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).
- 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 μ L 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 thrombomiRTM 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 technical notes at www.tamirna.com.

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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 μ L PPP is not possible by optical spectrophotometry or NanoDropTM 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 thrombomiRTM kit uses 200 μ L PPP for RNA extraction and 2 μ L 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.

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

lx	RNA spike-in Mix	dried
lx	cDNA spike-in Mix	dried
lx	5x RT Buffer	0.1 mL
lx	10x RT Enzyme Mix	0.05 mL
Зx	RNase-free water	1.5 mL
4x	2x miGreen Mix	1 mL
lx	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 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® Mas- terCycler® ep realplex models 2, 2s, 4, 4s; Stratagene® models Mx3005P®, Mx3000P®; Takara: TP-800
С	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)

Plate format and PCR cycler

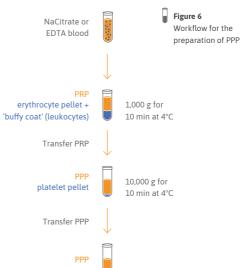
Platelet-Poor Plasma (PPP) Collection

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.



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!

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

This protocol is designed for human PPP

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 serum 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. When using citrate plasma add 60 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. Centrifu- ge for 3 min at 12,000 g at room temperature.
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.
	Reconstitution of lyophilized spike-in controls (RNA spike-in) First time use only Step 3 Thaw PPP samples and glycogen Step 4 PPP lysis Step 5 Protein precipitation

		Notes
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. Centrifu- ge 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. Centrifu- ge 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 tem- perature 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 (from step 11) on ice.	
Thaw total RNA		

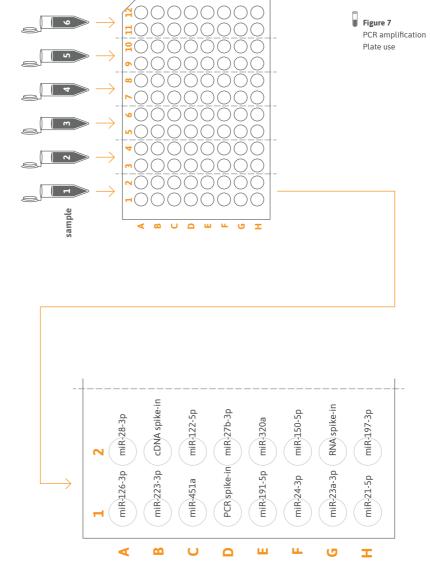
Step 13 Prepare reagents	Thaw 5x RT reaction buffer and nuclease free wa put on ice. Immediately before use, remove the e mix from the freezer, mix by flicking the tube and on ice. Spin down all reagents.				
Step 14 Prepare cDNA synthesis mix	 If performing cDNA synthesis on multiple RNA samples, prepare a mastermix including 1 extra reaction. Reagent (Volumes in µL) 10 µL Rxn 				
	5x Buffer cDNA spike-in Nuclease-free water Enzyme mix Total Volume Mix	2 0.5 4.5 1 8 each tube and add 8 µL cDN.			
Step 15 Incubate and heat inactivate	 Incubate the reaction at 42 Heat-inactivate the reverse 5 min. Immediately cool to 	e transcriptase at 95°C for			
Step 16 Storage	Transfer the undiluted cDN tubes and freeze at -20°C f	IA into nuclease-free low bind or 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.

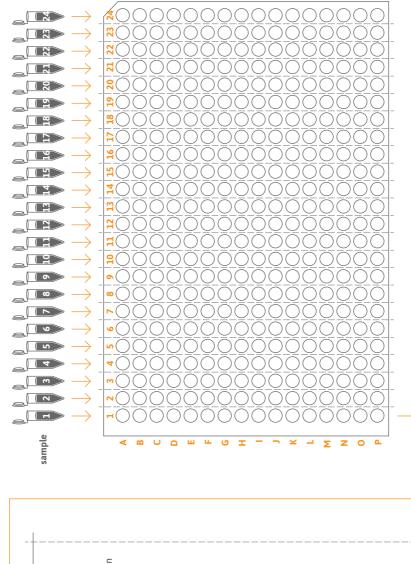
96-well plate

qPCR Amplification



Lab Protocol

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384-well plate

 Step 17
 Thaw cDNA (f

 Thaw reagents
 ice for 15-20 r

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.

Notes

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

			Notes
Step 18		/ith 88.2 μL nuclease free water, then	
Mix cDNA with	•	n Master mix (in total 180 µL). Mix by	
water and qPCR		lown, spin down to collect the liquid at	
Master mix	the bottom. Repea	at this step for all samples.	
Step 19	Add 10 uL reaction	n mixture (from step 18) (cDNA, NFW,	·
Prepare		ch of the 16 wells. Seal the plate with	
thrombomiR™	the appropriate of	otical sealing. Incubate at 4°C for a	
plate	minimum of 1 hou	r.	
	Note: The plate ca protected from lig	n be stored up to 16 hours at 4°C ht.	
	~		
	_ •		
	0	e qPCR, spin plate at 1,000 g for 90 sec.	
Perform qPCR	Perform qPCR and	I melting curve analysis as shown	
Perform qPCR	Perform qPCR and below. Settings ha	l melting curve analysis as shown ve been optimized for the Roche Light	
<mark>Step 20</mark> Perform qPCR (Roche LC 480)	Perform qPCR and	l melting curve analysis as shown ve been optimized for the Roche Light	
Perform qPCR	Perform qPCR and below. Settings ha	l melting curve analysis as shown ve been optimized for the Roche Light	
Perform qPCR	Perform qPCR and below. Settings ha Cycler® 480 II instr	melting curve analysis as shown ve been optimized for the Roche Light uments.	
Perform qPCR	Perform qPCR and below. Settings ha Cycler® 480 II instr Step	melting curve analysis as shown ve been optimized for the Roche Light uments.	
Perform qPCR	Perform qPCR and below. Settings ha Cycler® 480 II instr Step Polymerase	melting curve analysis as shown ve been optimized for the Roche Light uments.	
Perform qPCR	Perform qPCR and below. Settings ha Cycler® 480 II instr Step Polymerase activation/	melting curve analysis as shown ve been optimized for the Roche Light uments.	
Perform qPCR	Perform qPCR and below. Settings ha Cycler® 480 II instr Step Polymerase activation/ denaturation	I melting curve analysis as shown ve been optimized for the Roche Light numents. Setting 95°C , 2 min, ramp-rate 4.4°C/sec	
Perform qPCR	Perform qPCR and below. Settings ha Cycler® 480 II instr Step Polymerase activation/ denaturation Amplification	I melting curve analysis as shown ve been optimized for the Roche Light numents. Setting 95°C , 2 min, ramp-rate 4.4°C/sec Analysis mode: Quantification	
Perform qPCR	Perform qPCR and below. Settings ha Cycler® 480 II instr Step Polymerase activation/ denaturation Amplification	I melting curve analysis as shown ve been optimized for the Roche Light numents. Setting 95°C , 2 min, ramp-rate 4.4°C/sec Analysis mode: Quantification 95°C, 10 sec, ramp 4.4°C/sec	
Perform qPCR	Perform qPCR and below. Settings ha Cycler® 480 II instr Step Polymerase activation/ denaturation Amplification	I melting curve analysis as shown ve been optimized for the Roche Light uments. Setting 95°C , 2 min, ramp-rate 4.4°C/sec Analysis mode: Quantification 95°C, 10 sec, ramp 4.4°C/sec 60°C, 60 sec, ramp 2.2°C/sec	
Perform qPCR	Perform qPCR and below. Settings ha Cycler® 480 II instr Step Polymerase activation/ denaturation Amplification - 45 cycles	I melting curve analysis as shown ve been optimized for the Roche Light uments. Setting 95°C , 2 min, ramp-rate 4.4°C/sec Analysis mode: Quantification 95°C, 10 sec, ramp 4.4°C/sec 60°C, 60 sec, ramp 2.2°C/sec Acquisition mode: single	
Perform qPCR	Perform qPCR and below. Settings ha Cycler® 480 II instr Step Polymerase activation/ denaturation Amplification - 45 cycles	I melting curve analysis as shown ve been optimized for the Roche Light uments. Setting 95°C , 2 min, ramp-rate 4.4°C/sec Analysis mode: Quantification 95°C, 10 sec, ramp 4.4°C/sec 60°C, 60 sec, ramp 2.2°C/sec Acquisition mode: single Analysis mode: Melting curve	
Perform qPCR	Perform qPCR and below. Settings ha Cycler® 480 II instr Step Polymerase activation/ denaturation Amplification - 45 cycles	I melting curve analysis as shown ve been optimized for the Roche Light uments. Setting 95°C, 2 min, ramp-rate 4.4°C/sec Analysis mode: Quantification 95°C, 10 sec, ramp 4.4°C/sec 60°C, 60 sec, ramp 2.2°C/sec Acquisition mode: single Analysis mode: Melting curve 95°C, 10 sec, ramp 4.4°C/sec	
Perform qPCR	Perform qPCR and below. Settings ha Cycler® 480 II instr Step Polymerase activation/ denaturation Amplification - 45 cycles	I melting curve analysis as shown ve been optimized for the Roche Light uments. Setting 95°C, 2 min, ramp-rate 4.4°C/sec Analysis mode: Quantification 95°C, 10 sec, ramp 4.4°C/sec 60°C, 60 sec, ramp 2.2°C/sec Analysis mode: Melting curve 95°C, 10 sec, ramp 4.4°C/sec 55°C, 60 sec, ramp 2.2°C/sec	

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

Notes

es		~		
	Step 18.1		Low ROX	High ROX
	qPCR master mix	miGreen masterm	ix 90	90
		Nuclease Free wat	ter 87.3	79.2
		ROX	0.9	9
		cDNA	1.8	1.8
		ROX dye is required	d at the following concer	ntrations:
		Low concentration	of ROX dye (200x): Appli	ed Biosyster
		7500 and ViiA 7 and	d QuantStudio Instrumer	nts.
		High concentratior	n of ROX dye (20x): ABI PI	RISM® 7000
		Applied Biosystems	s 7300 and 7900.	
		Add 10 µL reaction	mixture (from step 18.1)	(cDNA, NFV
	Prepare		h of the 16 wells. Seal th	
	thrombomiR™	the appropriate op	tical sealing. Incubate at	4°C for a
	plate	minimum of 1 hour		
		Note: The plate car protected from ligh	n be stored up to 16 hour nt.	rs at 4°C
	Step 20.1	✓ Step	Setting	
	Perform qPCR	Polymerase	95°C, 2 min, maximal	/fast mode
		activation/		
		denaturation		
		Amplification –	95°C, 10 sec, maxima	l/fast mode
		40 cycles	56°C, 60 sec, maxima	l/fast mode
				it inouc
		Melting curve	60–95°C	i, i use inoue

Data Analysis

A data analysis application (thrombomiR™ analysis toolkit) is available for all our customers. Download links will be provided upon purchase of our kits. Please refer to the respective TechNote at our website at www.tamirna.com/TechNotes.

Troubleshooting

RNA Isolation

Poor RNA Recovery		Notes
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 recom- mended 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 concen- tration measurement of the purified RNA (e.g. spectro- photometric 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. Tem- peratures 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 sam- ples. Ensure that proper procedures are followed when working with RNA. Please refer to "Working with RNA" at the beginning of this manual	
Procedure not perfor- med quickly enough	In order to maintain the integrity of the RNA, it is impor- tant that the procedure be performed quickly.	

 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 degrada- tion ensure that enzymes used upstream of the isolation process are RNase-free.
 RNA does not perform	m 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	Check that the filter in the qPCR cycler was set to either miGreen or FAM/FITC
	"UniSp3 IPC" gives a valid signal.	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/croservices/micrornangsandqpcrservice.html

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

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

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

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Patents for the in-vitro diagnostic application of microRNAs for diagnosis of platelet function and platelet related disorders have been granted in the European Union (EP Pat No 20120788630, P528981DE, P528981GB).

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