osteomiR™

Biomarkers of Bone Quality

osteomiR™ Test Kit

Wet Lab Instruction Manual v1.0

August 2016 for products

KT-010-OT, KT-011-OT

TAmiRNA

stability for life.

Tables

Figures

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

1 | Product Summary

2 | Important Pre-Analytical Considerations

3 | Lab Protocol

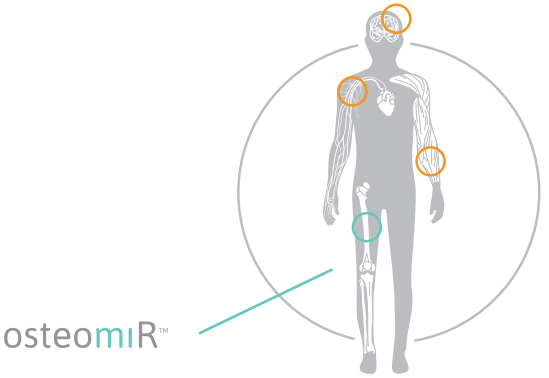
1

Product Summary

Intended-Use

The osteomiR™ test kit is an optimized one-stop-shop solution to detect and quantify selected microRNA biomarker candidates for fracture-risk in postmenopausal and diabetic osteoporosis. The osteomiR™ kit is validated for research-use only, not for diagnosis, prevention or treatment of a disease. The clinical utility of the osteomiR™ kit is currently investigated in clinical trials.

The osteomiR™ kit provides users with a highly standardized method to determine the levels of 11 informative microRNAs in human serum samples. It alleviates the task of selecting and optimizing analytical methods, data pre-processing and data normalization. It provides standardized serum concentrations for microRNAs with a known association to bone cell function, bone remodelling and fracture risk.



The selection of microRNAs for the osteomiR™ kit was **based on several clinical discovery studies**, which aimed to screen the levels of microRNAs in serum of patients with established primary, secondary or idiopathic osteoporosis.

- Weilner S, Skalicky S, Salzer B, Keider V, Wagner M, Hildner F, et al. Differentially circulating miRNAs after recent osteoporotic fractures can influence osteogenic differentiation. Bone 2015;79:43–51.
- Weilner S, Schraml E, Wieser M, Messner P, Schneider K, Wassermann, Klemens Micutkova L, et al. Secreted microvesicular miR-31 inhibits osteogenic differentiation of mesenchymal stem cells. Aging Cell 2016:1–11.

- Hackl M, Heilmeier U, Weilner S, Grillari J. Circulating microRNAs as novel biomarkers for bone diseases – Complex signatures for multifactorial diseases? Mol Cell Endocrinol 2015 432:83-95.
- Heilmeier U, Hackl M, Skalicky S, Weilner S, Schroeder F, Vierlinger K, et al. Serum microRNAs Are Indicative of Skeletal Fractures in Postmenopausal Women with and without Type 2 Diabetes and Influence Osteogenic and Adipogenic Differentiation of Adipose-Tissue Derived Mesenchymal Stem Cells In Vitro. J Bone Miner Res 2016.
- Kocijan R, Muschitz C, Geiger E, Skalicky S, Baierl A, Dormann R, Plachel F, Feichtinger X, Heimel P, Fahrleitner-Pammer A, Grillari J, Redl H, Resch H, Hackl M. Circulating microRNA signatures in patients with idiopathic and postmenopausal osteoporosis and fragility fractures. J Clin Endocrinol Metab. 2016 Aug 2

Table 1
List of microRNAs and quality controls included in the osteomiR™ kit

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

microRNA ID	miRBase Accession	Plate Positions	Assay Purpose
hsa-miR-214-3p	MIMAT0000271	A1, A3, A5, A7, A9, A11	osteomiR
hsa-miR-31-5p	MIMAT0000089	B1, B3, B5, B7, B9, B11	osteomiR
hsa-miR-451a	MIMAT0001631	C1, C3, C5, C7, C9, C11	hemolysis indicator
UniSp3 IPC	artificial sequence	D1, D3, D5, D7, D9, D11	PCR control
hsa-let-7b-5p	MIMAT0000063	E1, E3, E5, E7, E9, E11	osteomiR
hsa-miR-550a-5p	MIMAT0004800	F1, F3, F5, F7, F9, F11	osteomiR
hsa-miR-23a-3p	MIMAT0000078	G1, G3, G5, G7, G9, G11	hemolysis control
hsa-miR-199b-5p	MIMAT0000263	H1, H3, H5, H7, H9, H11	osteomiR
hsa-miR-188-3p	MIMAT0004613	A2, A4, A6, A8, A10, A12	osteomiR
cel-miR-39-3p	MIMAT0000010	B2, B4, B6, B8, B10, B12	cDNA spike-in
hsa-miR-203a	MIMAT0000264	C2, C4, C6, C8, C10, C12	osteomiR
hsa-miR-335-5p	MIMAT0000765	D2, D4, D6, D8, D10, D12	osteomiR
hsa-miR-127-3p	MIMAT0000446	E2, E4, E6, E8, E10, E12	osteomiR
hsa-miR-155-5p	MIMAT0000646	F2, F4, F6, F8, F10, F12	osteomiR
UniSp4	artificial sequence	G2, G4, G6, G8, G10, G12	RNA spike-in
hsa-miR-29b-3p	MIMAT0000100	H2, H4, H6, H8, H10, H12	osteomiR

Kit Design

The osteomiR™ kit provides all necessary reagents for the measurement of 11 informative microRNAs and 5 quality controls in human serum samples:

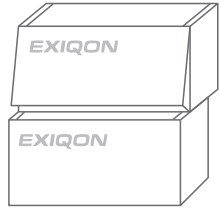
- RNA extraction from 200 µL serum using the miRCURY™ RNA Isolation Kit-Biofluids from Exiqon
- cDNA Synthesis reagents and ready-to-use osteomiR™ 96-well test plates
- ExiLENT SYBR® Green master mix from Exiqon.

Figure 1
Kit components

Kit Components

miRCURY™ RNA Isolation Kit – Biofluids, 50 rxns

osteomiR™ test kit, including microRNA panels, spike in and RT reagents, 48 rxns



ExiLENT SYBR® Green master mix, 2.5 ml (2 boxes required)

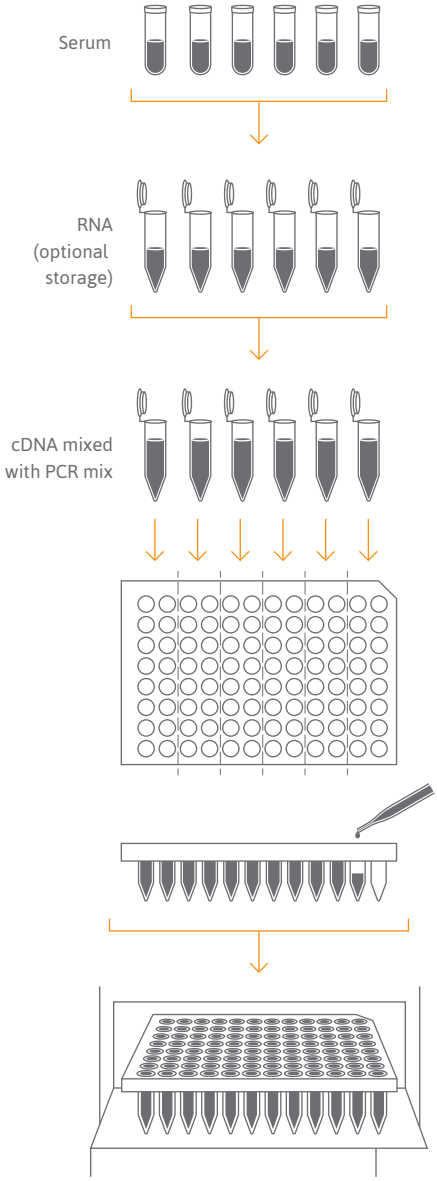
There are no additional reagents required for using the osteomiR™ kit.
The kit enables the measurement of microRNAs in serum in a single day.

The entire workflow consists of three main steps:

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

Figure 2
osteomiR™ workflow

Workflow
osteomiR™ kit workflow summary



1 RNA extraction (1.5h)

Extract RNA using the miRCURY™ RNA Isolation Kit – Biofluids from Exiqon

2 cDNA synthesis (1.5h)

Prepare cDNA using the osteomiR™ kit reagents

3 Prepare PCR Mix

Dilute cDNA and mix with Exilent SYBR® Green master mix

4 Real-time PCR amplification (2.5h)

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

5 Data analysis

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

Kit Technology

TAmiRNA has partnered with Exiqon Inc. (www.exiqon.com) for the manufacturing of the osteomiR™ kit. Exiqons technology is based on proprietary reagents that have been specifically optimized for the detection of microRNAs. The technology has been adapted for the osteomiR™ kit to offer the best performance for circulating microRNA detection.

RNA Extraction

Exiqon's miRCURY™ RNA Isolation Kit for biofluids provides a rapid method for the isolation and purification of RNA smaller than 1000 nucleotides from serum and plasma. The purification is based on spin column chromatography using a proprietary resin as the separation matrix. Small RNAs are separated from other cellular components such as proteins. The kit is designed to isolate high quality microRNA in amounts sufficient for qPCR analysis using the osteomiR™ 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, only the 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.

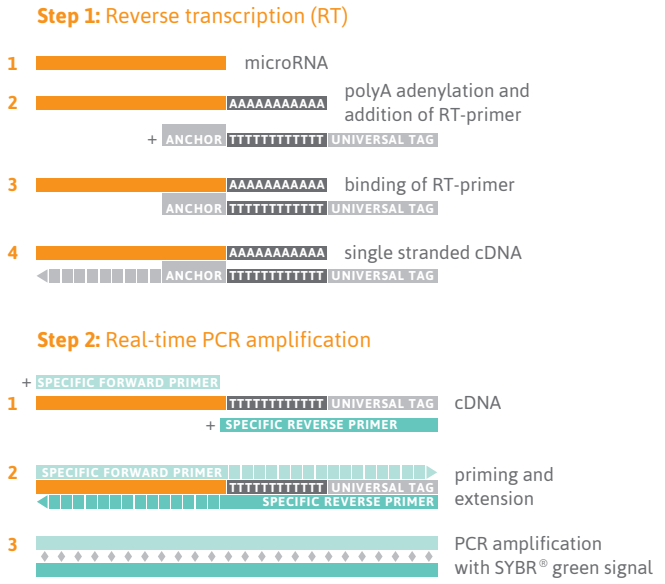
Reverse Transcription Quantitative PCR Detection

A universal reverse transcription (RT) reaction is performed, as shown in Figure 3 (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 osteomiR™ 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 3
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 miRCURY™ RNA Isolation Kit will be shipped and can also be stored tightly sealed at room temperature. The osteomiR™ test kit and ExiLENT SYBR® Green master mix will be shipped on dry ice and **must be store at -20°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 serum throughout its clinical development program for the discovery of microRNA biomarkers for bone quality. Therefore, we recommend to use serum for any experimental study using the osteomiR™ kit. However, the kit has been shown to enable proper detection of osteomiRs also in EDTA-plasma samples.

Pre-analytical Standardization

Conditions during serum or plasma processing might affect the detection of microRNAs using qPCR. Therefore, we strongly recommend to standardize protocols for pre-analytical processing and serum 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 serum 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.

- Coagulation and centrifugation protocols for obtaining serum from whole blood must be standardized and followed strictly (see “Serum 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 serum 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 serum 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 Serum, 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 serum samples is recommended. We recommend aliquot volumes of 225 µL serum.

Serum, 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 serum samples for microRNA analysis, which have been stored at -80°C for 15 years. In case the kit is intended to be used for serum 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.

UniSp4 – The synthetic RNA spike-in "UniSp4" is added to the sample during RNA extraction. It is used to monitor RNA extraction efficiency.

Cel-miR-39 – The synthetic RNA Spike-In "cel-miR-39" 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

UniSp3 – The synthetic DNA UniSp3 is present at a fixed position on every osteomiR™ test plate. It 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 osteomiR™ software app. 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.

Hemolysis

Hemolysis can be a major cause of variation in serum/plasma 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 serum or plasma samples. The osteomiR™ 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 serum or plasma 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 serum or plasma samples is not readily detectable by visual or spectrophotometric means.

RNA Yield

Determination of RNA yield from 200 µL serum 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 osteomiR™ kit uses 200 µL serum 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

Table 2
osteomiR™ kit components

Kit Components

The osteomiR™ test kit contains:

1 miRCURY™ RNA Isolation Kit – Biofluids

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

1x	Lysis solution BF	13 mL
1x	Protein precipitation solution BF	5 mL
1x	Wash solution 1 BF	10 mL
1x	Wash solution 2 BF (concentrate) Kit also contains rDNase+buffer	25 mL*
1x	RNase-free water	13 mL
1x	microRNA mini spin columns BF	50
1x	Collection tube 1.5 mL	50
1x	Collection tube 2 mL	50
1x	Collection tubes with lid 2 mL	50

* Add 100 mL 99% Ethanol

2 osteomiR™ Test Kit, 48 reactions

This box contains spike-in controls, all reagents for cDNA synthesis, as well as the osteomiR™ qPCR plates which contain the lyophilized microRNA primers.

8x	96-well osteomiR™ microRNA PCR plates	
1x	Enzyme mix	64 µL
1x	5x Reaction buffer	128 µL
1x	Nuclease-free water	1.25 mL
1x	cel-miR-39-3p	
1x	UniSp4	
1x	Glycogen (5 mg / mL)	110 µL

3 ExiLENT SYBR® Green Master Mix, 2.5mL

This box contains the qPCR master mix.

2x	ExiLENT SYBR® Green master mix	1.25 mL
2x	Nuclease-free water	1.25 mL

Consumables and Instruments Not Supplied with the Kit

- 99 % Ethanol, pro analysis
- Isopropanol, pro analysis
- RNase-free water
- 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 cycler
- Vortexer
- Calibrated pipettes
- Centrifuge for <2 mL tubes and 96-well plates
- qPCR Cycler: Roche LightCycler® 480 (I or II)

Serum Collection

The osteomiR™ Kit requires 200 µL serum.

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

Filled collection tubes should sit upright after the blood is drawn at room temperature for a minimum of 30 to a maximum of 60 minutes for the clot to form. The red top tubes do not have to be full to be used.

Centrifuge the blood sample at 2,500g for 10 minutes at room temperature in a horizontal rotor (swing-out head). If the blood is not centrifuged immediately after the clotting time (30 to 60 minutes at room temperature), the tubes should be refrigerated (4°C) for no longer than 4 hours.

After centrifuging, the clot is located at the bottom of the tube, and the serum is on top of the clot.

Use a clean pipette and nuclease-free filter tips to transfer the serum (recommendation: do not pour!). Pipette serum into the labeled nuclease-free (1.5 ml) tubes, filling the vials in sequential order. Aliquot volume is recommended to be 225 µL for the osteomiR™ kit, so that 200 µL can be safely used for RNA extraction. Close the caps on the vials tightly. This process should be completed within 1 hour of centrifugation.

Note: Be very careful not to pick up red blood cells when aliquoting. This can be done by keeping the pipet above the red blood cell layer, slow pipetting and leaving a small amount of serum in the tube.

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 osteomiR™ kit has been **standardized to a volume of 200 µL serum** as starting material to ensure high RNA yield and prevent inhibition of downstream PCR applications.

If less than 200 µL serum is available we recommend to fill available serum 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

Before getting started ensure that

- **Isopropanol, ethanol and 1.5 mL nuclease-free low bind tubes are available.**
- **Centrifuge is cooled to 4°C for the first 12,000 g for 5 min centrifugation step.**
- **Afterwards, the centrifuge is warmed to run at room temperature.**

This protocol is designed for human serum.

Notes

Step 1 **Preparation of Wash Solution 2 BF** First time use only	Add 100 mL of >99 % ethanol to the supplied Wash Solution 2 BF. **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 (UniSp4 and cel-miR-39)** First time use only	• Spin down vial 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). • Store at -20°C.
Step 3 **Thaw serum sample**	After thawing on ice, 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.
Step 4 **Serum 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 UniSp4 to 60 µL Lysis solution BF and mix it with the 200 µL serum. Vortex for 5 sec and incubate for 3 min at room temperature. For multiple samples prepare a mastermix including 5 % extra volume. **Important note: The UniSp4 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.**

Notes

Step 5 **Protein precipitation**	Add 20 µL of Protein Precipitation Solution BF. Vortex for 5 sec and incubate for 1 min at room temperature. Centrifuge for 3 min at 11,000 g at room temperature.
Step 6 **Transfer supernatant**	Transfer exactly 200 µL of the clear supernatant (aqueous phase) into a new collection tube (2 mL, with lid) and add 2 µL glycogen (5 mg/mL). Vortex and spin down.
Step 7 **Adjust binding conditions**	Add 270 µL Isopropanol. Vortex for 5 sec.
Step 8 **Load column**	Place a microRNA Mini Spin Column BF in a collection tube and load sample onto the column. Incubate for 2 min at room temperature. Centrifuge for 30 sec at 11,000 g at room temperature. Discard flow-through and place column back into the collection tube.
Step 9a **Wash and dry**	Add 100 µL Wash Solution 1 BF to the microRNA spin column BF. Centrifuge for 30 sec at 11,000 g. Discard flow-through and place column back into the collection tube.
Step 9b **Wash and dry**	Add 700 µL Wash Solution 2 BF to the microRNA spin column BF. Centrifuge for 30 sec at 11,000 g at room temperature. Discard flow-through and place column back into the collection tube.
Step 9c **Wash and dry**	Add 250 µL Wash Solution 2 BF to the microRNA spin column BF. Centrifuge for 2 min at 11,000 g at room temperature to dry the membrane completely.
Step 10 **Elute**	Place the microRNA spin column BF in a new low bind tube (1.5 mL). Add 30 µL RNase free water directly onto the membrane of the microRNA spin column BF. Incubate for 1 min at room temperature. Close the lid and centrifuge for 1 min at 11,000 g at room temperature.
Step 11 **Storage**	Store the RNA sample immediately at -80°C.

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 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 5 % extra volume.

Reagent (Volumes in µL)	10 µL Rxn
5x Buffer	2
cel-miR-39-3p Spike-In	0.5
Nuclease-free water	4.5
Enzyme mix	1
Total Volume Mix	8
Total RNA	2

Dispense the cDNA synthesis mastermix into nuclease free tubes and add template RNA in each tube (i.e. for 10 µL cDNA reaction: 8 µL RT mix + 2 µL RNA).

Mix the mastermix and RNA by gentle flicking 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 4°C.

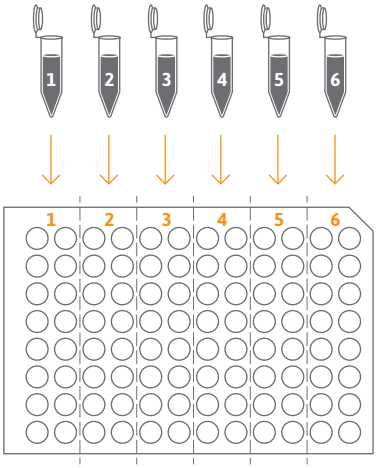
Step 16
Storage

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

qPCR Amplification

The osteomiR™ test plate contains 16 different miRNA or QC primer sets. Six samples can be measured on one 96-well plate. Two columns are used per sample.

Figure 5
PCR amplification
Plate use



Notes

Step 17
Thaw reagents

Thaw cDNA (from step 16) and SYBR® Green 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.

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 SYBR® Green 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 six samples.

Step 19
Prepare osteomiR™ plate

Centrifuge the osteomiR™ 96-well plate at 1,500 g for 90 sec, then remove the seal. Add 10 µL reaction mixture (from step 18) (cDNA, NFW, master mix) to each of the 16 wells. Seal the plate with optical sealing.

Note: The experiment can be paused at this point, the plate can be stored at +4°C protected from light for up to 16 hours.

Notes

Step 20
Perform qPCR

Before running the qPCR, spin plate for 1,500 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 , 10 min, ramp-rate 4.4°C/sec
Amplification – 45 cycles	Analysis mode: Quantification 95°C, 10 sec, ramp 4.4°C/sec 60°C, 60 sec, ramp 1.6°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

Data Analysis

Please refer to the TechNotes on data analysis available at www.tamirna.com/TechNotes.

Troubleshooting

RNA Isolation

Notes

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 serum 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.

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

Problem	Suggestion
No fluorescent signal is detected during the PCR	Confirm that the PCR setup was correct by checking the signal obtained for the qPCR spike-in control „UniSp3 IPC“.
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 SYBR Green or FAM/FITC Check that the optical read is at the correct step of the qPCR cycles.

Notes

Related Products

TAmiRNA offers Demo-Kit versions of all its research-use kits. The Demo-Kit versions enable the analysis of 12 samples and contain two proprietary qPCR detection plates. TAmiRNA offers research-use kits for novel microRNA biomarkers in cardiovascular disease. Read more about these products at www.tamirna.com.

Further Reading

TAmiRNA has published extensively on circulating microRNAs as biomarkers for age-associated diseases. Below you find a list of publications, which describe the identification and utility of osteomiRs.

1. Weilner S, Skalicky S, Salzer B, Keider V, Wagner M, Hildner F, et al. Differentially circulating miRNAs after recent osteoporotic fractures can influence osteogenic differentiation. Bone 2015;79:43–51.

2. Weilner S, Schraml E, Wieser M, Messner P, Schneider K, Wassermann, Klemens Micutkova L, et al. Secreted microvesicular miR-31 inhibits osteogenic differentiation of mesenchymal stem cells. Aging Cell 2016:1–11.

3. Hackl M, Heilmeier U, Weilner S, Grillari J. Circulating microRNAs as novel biomarkers for bone diseases – Complex signatures for multifactorial diseases? Mol Cell Endocrinol 2015 432:83-95.

4. Heilmeier U, Hackl M, Skalicky S, Weilner S, Schroeder F, Vierlinger K, et al. Serum microRNAs Are Indicative of Skeletal Fractures in Postmenopausal Women with and without Type 2 Diabetes and Influence Osteogenic and Adipogenic Differentiation of Adipose-Tissue Derived Mesenchymal Stem Cells In Vitro. J Bone Miner Res 2016.

5. Kocijan R, Muschitz C, Geiger E, Skalicky S, Baierl A, Dormann R, Plachel F, Feichtinger X, Heimel P, Fahrleitner-Pammer A, Grillari J, Redl H, Resch H, Hackl M. Circulating microRNA signatures in patients with idiopathic and postmenopausal osteoporosis and fragility fractures. J Clin Endocrinol Metab. 2016 Aug 2

Further reading on quality controls for circulating microRNA experiments

1. Blondal T, Jensby Nielsen S, Baker A, Andreasen D, Mouritzen P, Wrang Teilum M, et al. Assessing sample and miRNA profile quality in serum and plasma or other biofluids. Methods 2013;59:S1–6.

2. Shah JS, Soon PS, Marsh DJ. Comparison of methodologies to detect low levels of hemolysis in serum for accurate assessment of serum microRNAs. PLoS One 2016;11:1–12.

3. Mestdagh P, Hartmann N, Baeriswyl L, Andreasen D, Bernard N, Chen C, et al. Evaluation of quantitative miRNA expression platforms in the microRNA quality control (miRQC) study. Nat Methods 2014;11.

Notes

Notes

Notes

Notes

Notes

Notes

Notes

Notes

Notes

Notes

Notes

Notes

Notes

Notice to purchaser

Imprint

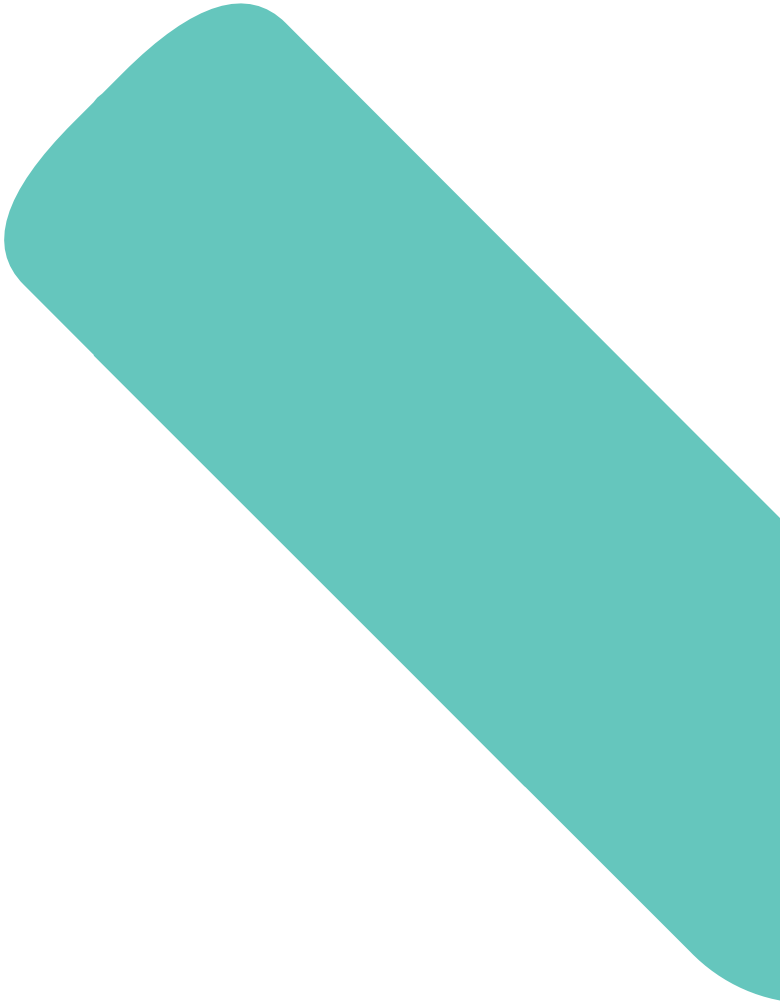
Copyright: 2016 / TAmiRNA GmbH

Text: TAmiRNA GmbH

Concept & Design: www.fuergestaltung.at

Illustrations: www.birgitlohmann.at

Photography: Carina Brunthaler



TAmiRNA GmbH
Muthgasse 18
1190 Vienna, Austria
+43 660 420 58 56
www.tamirna.com