# osteomiR™

microRNA Biomarkers of Bone Quality

osteomiR™ kit

Wet Lab Instruction Manual v2.0

August 2019 for product

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

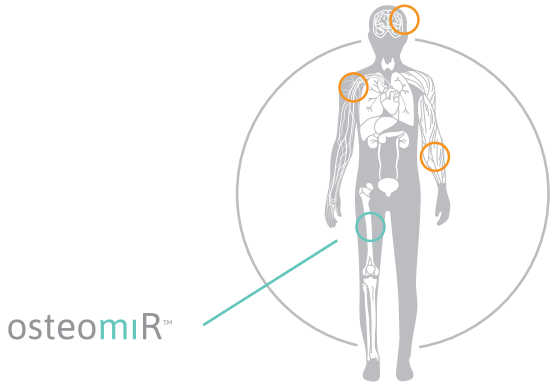
# 1

# Product Summary

# Intended-Use

The osteomiR™ kit has been developed to standardize the quantification of selected microRNA biomarker candidates for fracture-risk in postmenopausal and diabetic osteoporosis. The osteomiR™ kit is intended 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 19 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
included in the osteomiR™ kit

**Summary of evidence for microRNA bone biomarkers included in the osteomiR™ kit**

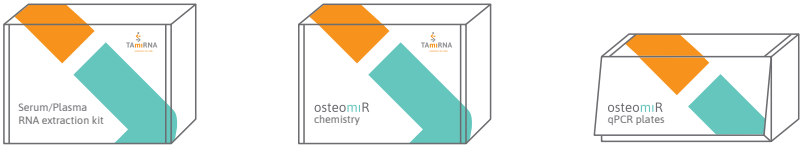
| miRNA ID | Bone Turnover | Microstructure and Histomorphometry | Osteoporosis & other bone diseases | Bone Loss & Treatment Response | Calcification | Therapeutic Activity | Mechanism of action |
|---|---|---|---|---|---|---|---|
| let-7b-5p | • | • | • | | | | VEGF, HMGA2 |
| miR-127-3p | | | • | | | | S1PR3 |
| miR-133b | • | | • | • | | | Runx2/FOXC1 |
| miR-141-3p | | | • | | • | | WNT |
| miR-143-3p | | | • | | | | 17β-estradiol, osterix |
| miR-144-5p | • | | | | | | RANK |
| miR-152-3p | | | • | | | | |
| miR-17-5p | | | | • | • | | Smad5, BMP2 |
| miR-188-5p | | | • | • | | • | PPARγ via HDAC9/ RICTOR |
| miR-19b-3p | • | | • | • | | | |
| miR-203a | | | • | • | • | | Runx2, Dlx5, 17β-estradiol |
| miR-214-3p | | | | | • | • | ATF4 |
| miR-29b-3p | • | • | • | | • | | HDAC4, TGFβ3, CTNNBIP1 |
| miR-31-5p | | | • | • | • | • | WNT via FZD3 |
| miR-320a | | | • | | • | | HOXA10 |
| miR-335-5p | • | • | • | • | • | | WNT via DKK1 |
| miR-375 | | | • | | • | • | WNT via LRP5 and β-catenin |
| miR-550a-3p | • | • | • | | • | | |
| miR-582-5p | | | • | | | | |

# Workflow Components

**The following components are required for the measurement of 19 informative microRNAs and 5 quality controls in human serum samples:**

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

**Figure 1**
Overview of required components




Serum/Plasma RNA extraction kit, RT

osteomiR™ chemistry, including spike-ins, RT chemistry and miGreen Mix, -20°C

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

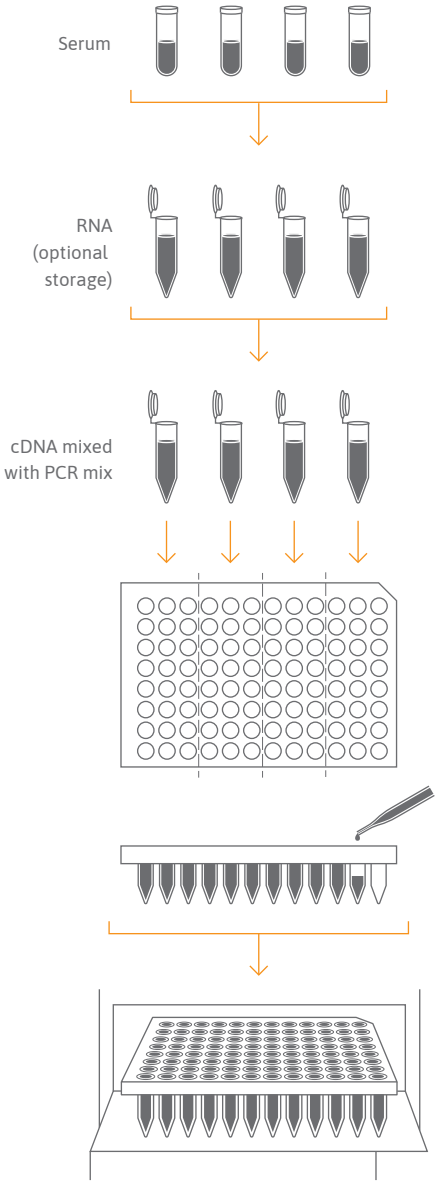
**This combination of kits enables the measurement of microRNAs in serum in a single day.**

**The entire workflow consists of three main steps:**

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

## Workflow summary

**Figure 2**
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 osteomiR™ 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 osteomiR™ 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 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, 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.

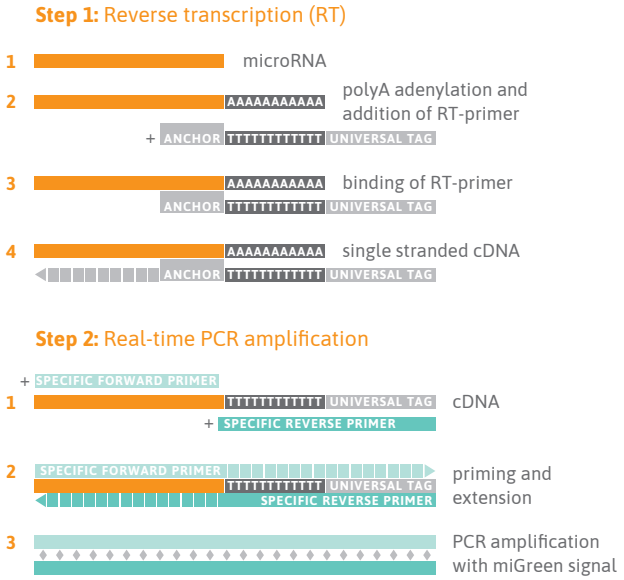
## Reverse Transcription Quantitative PCR Detection

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 miGreen 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 osteomiR™ chemistry will be shipped on dry ice and **must be stored at -20°C.** The osteomiR™ 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.

# 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 20 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.

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 osteomiR™ 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 osteomiR™ 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.

## 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™ analysis toolkit 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

# Essential components

## 1 Serum/Plasma RNA extraction kit

This box contains enough reagents to extract RNA from 50 serum 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 osteomiR™ 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 |
| 4x | RNase-free water | 1.5 mL |
| 6x | 2x miGreen Mix | 1 mL |
| 1x | Glycogen (5 mg/ml) | 0.125 mL |


## 3 Primer coated osteomiR™ qPCR plates

Depending on the qPCR cycler

| | | |
|---|---|---|
| 12x | Primer coated 96 well qPCR plates | |
| 3x | 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 cycler
- 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) |

# Serum Collection

**The osteomiR™ Workflow 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 microRNA 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, 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 RNA extraction 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

### 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!**

This protocol is designed for human serum.

| | | Notes |
|---|---|---|
| **Step 1**<br>**Preparation of Wash Buffer 1 and Wash Buffer 2**<br>First time use only | Add 2 volumes of >99 % ethanol to Wash Buffer 1 and 4 volumes of >99 % ethanol to Wash Buffer 2.<br>**Note: The label on the bottle has a box that should be checked to indicate that the ethanol has been added.** | |
| **Step 2**<br>**Reconstitution of lyophilized spike-in controls (RNA spike-in and cDNA spike-in)**<br>First time use only | • Spin down vials before use by centrifugation at 3,000 g for 30 sec at room temperature.<br>• Resuspend the spike-ins by adding 80 µL nuclease-free water.<br>• Mix by vortexing and spin down. Store on ice for 20 min.<br>• Mix by vortexing again and aliquot in low bind tubes. (20 µL aliquots are recommended)<br>• Store at -20°C. | |

Notes

**Step 3**
**Thaw serum 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**
**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 RNA spike-in to 60 µL Lysis Buffer and mix it with the 200 µL serum. 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. Centrifuge 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.

**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.

| | | Notes |
|---|---|---|
| **Step 9a**<br>**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**<br>**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**<br>**Wash and dry** | Add 500 µL 80% ethanol to the spin column. Centrifuge for 2 min at 8,000 g at room temperature to dry the membrane completely. | |
| **Step 10**<br>**Elute** | Place the spin column in a new low bind collection tube (1.5 mL). Centrifuge with open lid for 5 min at 12,000 g at room temperature. 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**<br>**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**<br>**Thaw total RNA** | Thaw total RNA (from step 11) on ice. | |
| **Step 13**<br>**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. | |

Notes

**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 osteomiR™ test plate contains 19 different microRNA or QC primer sets. Four samples can be measured on one 96-well plate. Three columns are used per sample. 16 samples can be measured on one 384-well plate, using one row per sample.

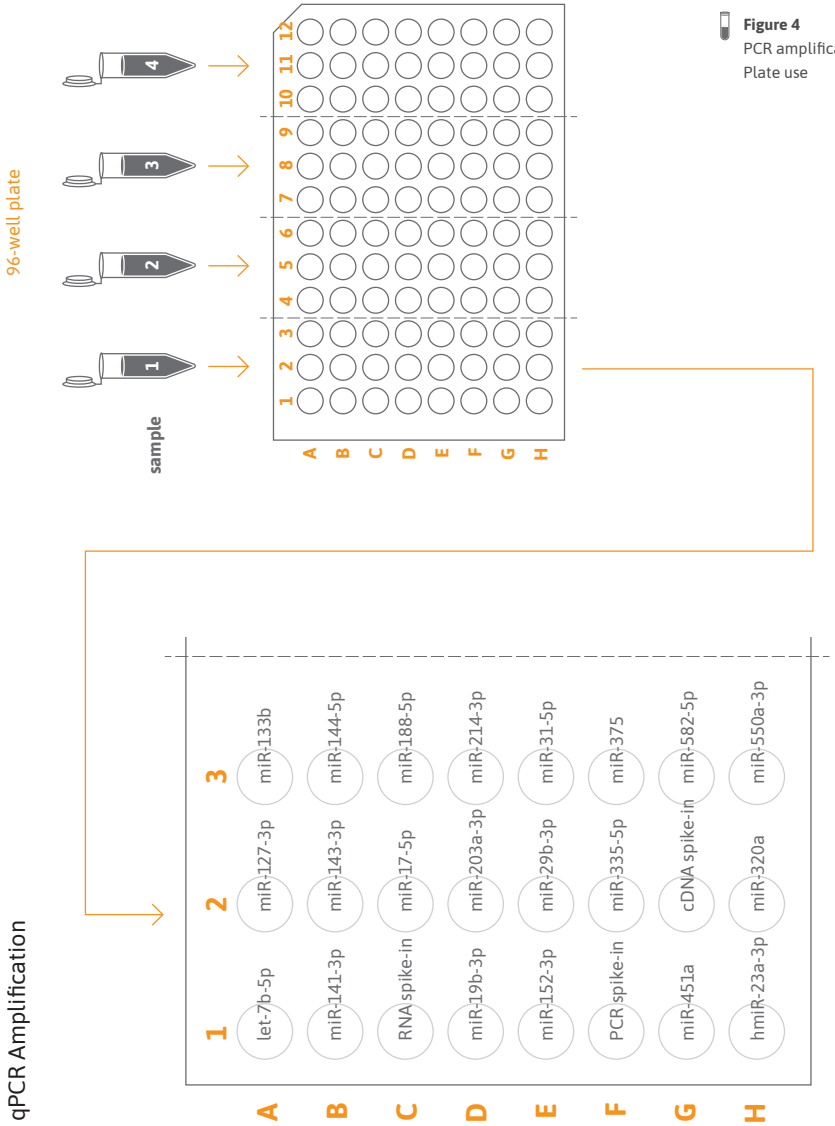
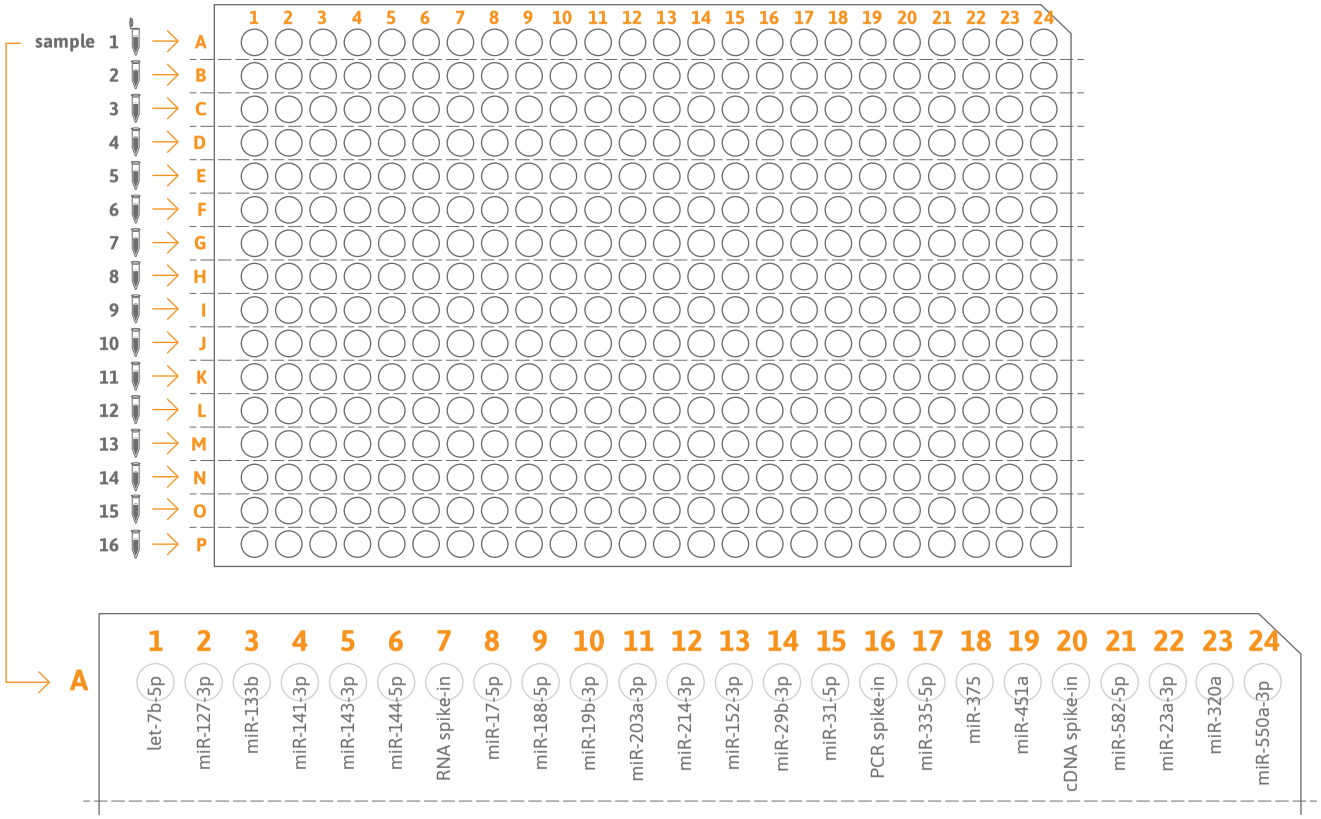



**Figure 4**
PCR amplification
Plate use

## qPCR Amplification

384-well plate



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 2.6 µL cDNA with 127.4 µL nuclease free water, then add 130 µL miGreen Master mix (in total 260 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 osteomiR™ plate**

Add 10 µL reaction mixture (from step 18) (cDNA, NFW, Master mix) to each of the 24 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**

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<br>95°C, 10 sec, ramp 4.4°C/sec<br>60°C, 60 sec, ramp 2.2°C/sec<br>Acquisition mode: single |
| Melting curve | Analysis mode: Melting curve<br>95°C, 10 sec, ramp 4.4°C/sec<br>55°C, 60 sec, ramp 2.2°C/sec<br>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.**
**Mix cDNA with water and qPCR Master mix**

| | Low ROX | High ROX |
|---|---|---|
| miGreen mastermix | 130 | 130 |
| Nuclease Free water | 126.1 | 114.4 |
| ROX | 1.3 | 13 |
| cDNA | 2.6 | 2.6 |

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 osteomiR™ plate**

Add 10 µL reaction mixture (from step 18.1) (cDNA, NFW, ROX, master mix) to each of the 24 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**

Before running the qPCR, spin plate at 1,000 g for 90 sec. Perform qPCR and melting curve analysis as shown below.

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

# Data Analysis

A data analysis application (osteomiR™ 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

| | |
|---|---|
| **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. |

Notes

Notes

| | |
|---|---|
| **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<br>**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 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<br><br>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 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 re cent 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.

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Patents for the in-vitro diagnostic application of microRNAs for diagnosis of osteoporosis and determining the risk of fractures are granted in the EU (EP Pat No 3,155,120) and US (US Pat No. 10,128,398), and pending in Canada, China, Japan, India, and Brazil.

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