Profiling of microRNA in Blood Serum/Plasma

Guidelines for the miRCURY LNA™ Universal RT microRNA PCR System

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Introduction

MicroRNA profiling in serum and plasma samples holds great promise as a non-invasive way to discover important new biomarkers for a wide range of diseases and biological processes. However, getting microRNA profiles from such samples can be challenging.

The miRCURY LNA™ Universal RT microRNA PCR system offers the sensitivity and specificity needed for performing accurate microRNA expression profiling from serum and plasma samples (Figure 1). This document contains a set of guidelines for setting up microRNA profiling experiments from blood serum and plasma and provides important information and tips to ensure successful microRNA quantification using the miRCURY LNA™ Universal RT microRNA PCR system.

Challenges of microRNA profiling from serum and plasma

MicroRNA profiling in serum and plasma samples holds great promise but there are several challenges to overcome in order to successfully perform such experiments.

First, plasma and serum are cell-free samples with very low amounts of RNA. This means that normal RNA quality control using Bioanalyzer or OD measurements is not suitable for such samples.

Second, even the purest RNA prepared from plasma or serum contains inhibitors of the reverse transcriptase and Taq polymerase enzymes used in the qPCR reaction.

Finally, some of the larger RNA species, such as U6 RNA, normally used for qPCR normalizations are present in extremely low concentrations in serum and plasma. This means that great care must be taken when choosing controls for normalization.

In this document, we will present recommendations on ways to overcome these challenges.

How to choose between serum and plasma

Total RNA from both plasma and serum can be accurately profiled using the miRCURY LNA™ Universal RT microRNA PCR system. When choosing between which of the preparations to use, one reason for choosing plasma over serum, is the more controlled sampling method used for plasma preparation. In addition to this, the Cq values from plasma tend to be slightly lower than for serum indicating a higher microRNA content. Typically, plasma samples prepared with anticoagulants such as citrate or EDTA are used as these types of plasma preparations give better results in the subsequent microRNA profiling experiment. In contrast, the use of heparin plasma is not recommended. Heparin is known to inhibit enzyme activity in both the reverse transcription and the PCR. One does not obtain entirely the same microRNA profile in plasma and serum, although changes in the circulating microRNA profile is most likely reflected in both sample types.

Figure 1

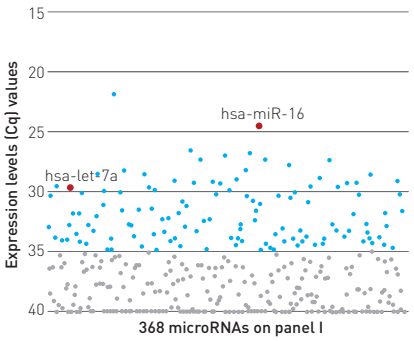


Figure 1. Expression profiling of 368 microRNAs using total RNA from 35µL serum. Real-time PCR was performed using triplicate RT reactions on total RNA purified from serum. Average Cq (quantification cycle) values from the microRNA ready-to-use PCR panel I are shown (368 microRNAs). Over 120 microRNAs showed robust expression with Cq values below 35. hsa-miR-16 (known to be highly expressed in serum/plasma) and hsa-let-7a are indicated in red.

Proper collection and preparation of blood plasma and serum

Collection of whole blood is the first step in preparation of plasma and serum. A major challenge in this process is the instability of the cellular RNAs *in vitro*. Reports from the literature indicate that the copy numbers of individual RNA species (e.g. mRNA) in whole blood can change more than 1000-fold during storage or transport at room temperature[1]. This is caused both by rapid RNA degradation and by induced expression of certain genes after the blood is drawn. Such changes in the RNA expression profile make reliable studies of gene expression in whole blood impossible. Though RNA species present in serum and plasma are relatively unaffected by induced expression of genes, they will be affected by degrading RNases. Therefore, a method that preserves the RNA expression profile during and after collection of whole blood is essential for accurate analysis of gene expression in blood plasma and serum. Generally, it is recommended to process the whole blood right away into either serum or plasma. In all processing steps from whole blood to serum and plasma, measures should be taken to prevent lysis of cells. Failure to do so may lead to contamination of the serum and plasma fractions with RNA from intact cells.

"Immediate separation of whole blood into serum and plasma is essential for the RNA stability"

Figure 2

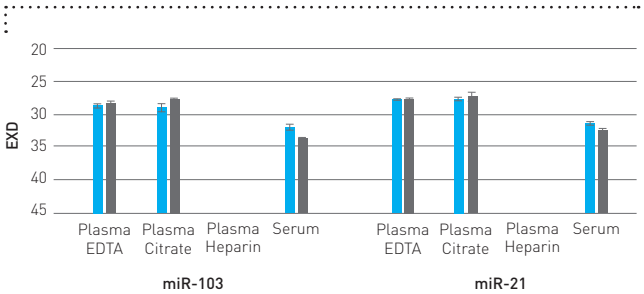


Figure 2. Suitability of different blood preparations for microRNA PCR analysis. Real-time PCR for miR-103 and miR-21 was performed using triplicate RT reactions on total RNA purified from either EDTA-plasma, citrate-plasma, heparin-plasma, or serum. Average Cq values for each triplicate are shown, demonstrating robust amplification from all sources except heparin-plasma.

The RNA from intact cells will mask or prevent the subsequent detection of subtle changes in the microRNA expression profile. In the preparation of samples, it is recommended to carry out the sampling using a well described and fixed procedure to avoid technical variation from this step in the data. In addition, samples should be collected under similar conditions and preferably at the same time. In the preparation of plasma, anticoagulants like EDTA and sometimes citrate are normally used. These anticoagulants are both fine to use, in contrast to heparin which is known to cause inhibition of downstream enzymatic steps such as cDNA synthesis and PCR (Figure 2 and 3). Currently, there is no reliable way of removing heparin from RNA samples or from the original blood serum/plasma samples. Therefore, AVOID HEPARIN in any of the processing steps of whole blood.

Once prepared, serum and plasma may be stored in RNase-free tubes (e.g. cryo-tubes) at -80° C. Alternatively, RNA isolation may be carried out right away. It has been shown that RNA in plasma and serum is generally stable at higher temperatures than -80° C for elongated times. However, it is not known how the microRNAs are preserved.

Optimal Isolation of total RNA

RNA work requires special precautions to prevent RNase contamination of the reagents and degradation of the RNA sample. The tips box below provides simple guidelines for good laboratory practice to ensure optimal performance of PCR experiments:

Tips: General guidelines for handling and storage of RNA

The following precautions should be taken to prevent RNase contamination and degradation of the RNA sample and reagents:

- Always wear disposable gloves, and work in a nuclease-free environment
- Use nuclease-free, low nucleic acid binding plastic ware and filter barrier pipette tips
- Keep tubes capped when possible, always spin tubes before opening
- For storage of RNA, precipitate the RNA with ethanol and store at -20° C.
- For long-time storage, RNA may be stored at -80° C. Avoid repeated freeze-thaw cycles

1. Rainen et al. (2002), Clin Chem, 48, 1883

Figure 3

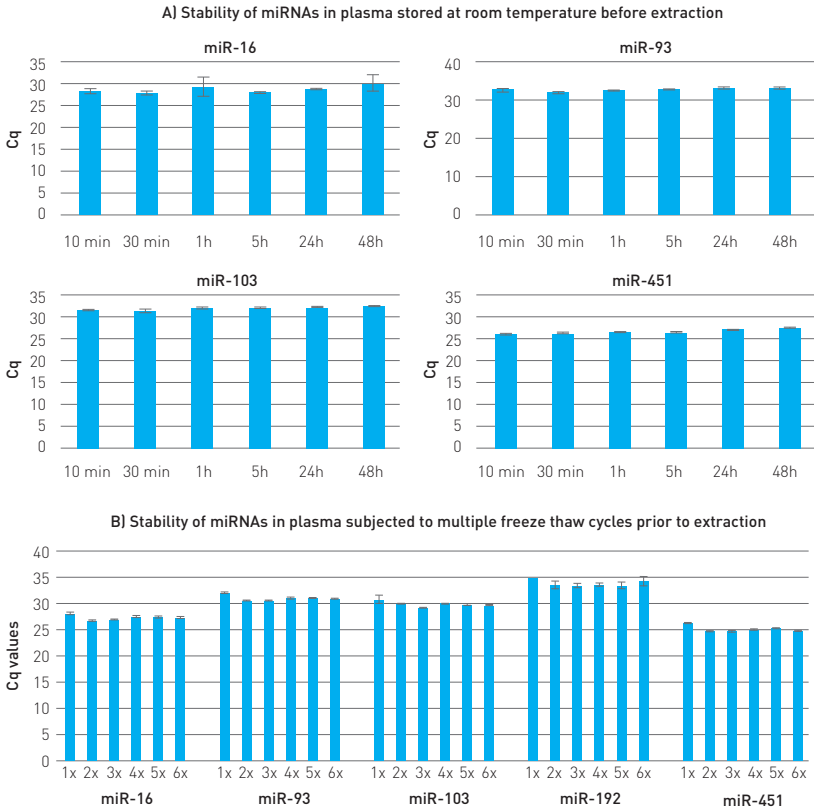


Figure 3. High stability of microRNA in EDTA-plasma samples. A) Real-time PCR for four microRNAs was performed using triplicate RT reactions on total RNA purified from EDTA-plasma. The plasma was stored at room temperature before RNA extraction for the indicated times. No evidence of microRNA degradation in plasma was observed, even after prolonged storage of up to 48 hours at room temperature. **B)** Real-time PCR for six microRNAs was performed using triplicate RT reactions on total RNA purified from EDTA-plasma. The plasma was submitted to 1-6 freeze-thaw cycles as indicated with no effect on miRNA amplification, demonstrating that plasma microRNA is stable towards multiple freeze-thaw cycles.

As mentioned earlier, serum and plasma contain only very minute amounts of RNA which means that there is a high risk of loss of a significant amount of the RNA during extraction. For this reason, we recommend adding carrier RNA during the extraction procedure, and subsequently using RNA amounts based on starting volume rather than RNA quantity in downstream enzymatic steps. The use of a carrier ensures the highest and most consistent yield in the samples. When selecting a carrier, it is important to choose a source which is guaranteed free from microRNAs: Such a source is RNA from the bacteriophage MS2 (available from Roche Applied Science cat. no. 10165948001), which has been used successfully at Exiqon for this purpose.

Exiqon has developed protocols for RNA extraction from human as well as from mouse serum and plasma. In the box below is a brief overview of the protocol. The full protocol is available at www.exiqon.com/serum-plasma-RNA-isolation

Additional tips to ensure a successful RNA extraction and template preparation is provided in the tips box on page 6.

RNA extraction from human or mouse serum and plasma in brief:

- Start with no more than 200 µL human serum (50 µL for mouse)
- Follow the above-mentioned RNA extraction protocol including MS2 carrier RNA
- Full microRNA genome profiling is possible from a total input of as little as 35 µL of serum/plasma per plate
- For high reproducibility in the elution of the minute amount of RNA bound on the column membrane, we recommend using a relatively high elution volume of 50 µL
- For general guidelines on handling and storage of RNA, please view the tips boxes

Total RNA and RNA spike-ins

The efficiency of the RNA extraction step may be monitored by addition of the RNA spike-in (synthetic control template) which is provided with Exiqon's Universal cDNA synthesis kit. The RNA spike-in will be reverse transcribed and the cDNA will be detected with a primer set provided with Exiqon's SYBR® Green master mix, or MicroRNA Ready-to-Use PCR Panels.

In short, add 1 µL of the re-suspended RNA spike-in (10^8 copies/µL) per 200 µL of human serum. Add the spike-in right after addition of QIAzol and MS2 RNA – see step 6 in the RNA extraction protocol. Alternatively, the RNA spike-in can be used to monitor the efficiency of the cDNA synthesis step. This will allow for assessment of residual components present in the RNA preparation which may act as inhibitors of the cDNA synthesis and PCR enzymes. To monitor the efficiency of the cDNA synthesis step, the RNA spike-in should be added to the cDNA synthesis reaction instead – please refer to the miRCURY LNA™ Universal RT microRNA PCR Instruction manual for more information.

Conducting quality control of RNA

Standard methods for measurement of the RNA yield and quality are inappropriate for use with serum and plasma samples. The use of an RNA carrier in these samples makes RNA measurements by OD260 and Bioanalyzer non-informative. The signal from the RNA carrier will simply be too dominant relative to the other RNA species and shield the signal for the RNA of interest. Even if carrier was not included during the isolation, the RNA concentration in the eluate would still be too low for reliable OD260 quantification on a NanoDrop or other spectrophotometers. The quantification itself would also use a significant amount of the RNA sample and since these samples are often limited and precious this loss cannot be afforded. Therefore, an alternative way of normalizing sample input has to be used. Exiqon recommends using RNA amounts based on starting volume rather than RNA quantity (see below under cDNA synthesis).

Tips: Successful RNA extraction and template preparation

Protocols for RNA extraction from human or mouse serum and plasma are available at www.exiqon.com/serum-plasma-RNA-isolation In addition to this protocol, some more general considerations and procedures should be taken into account.

The first critical step for a successful expression profiling analysis of microRNAs involves the purification and preparation of total RNA that includes small RNAs (<200 nt) from a biological sample. Therefore, the method used for RNA sample preparation is critical to the success of the experiment. The following points should be considered before starting the experiment:

- If commercially available purified RNA is used, it is important to make sure that the RNA is guaranteed to contain small RNAs and is representative of the microRNAs in the species and/or tissue from which it was isolated
- The comparison of samples prepared using different RNA isolation methods is not recommended. However, if this is necessary, it is recommended to include the measure of a reference small RNA which has a consistent and unvaried expression level in order to allow for comparison of microRNA levels in the different sample preparations
- The isolation of RNA and the reaction steps preceding real-time PCR should be performed in rooms separate from where real-time PCR experiments are carried out in order to avoid contamination with amplicon
- It is recommended that the integrity of the isolated RNA be assessed before proceeding with quantitative real-time PCR experiments
- If necessary, treat RNA preparations with DNases to remove contaminating DNA that may interfere with the real-time PCR results
- Purified total RNA should be dissolved in nuclease-free water at a stock concentration of at least 1 µg/µL. For further recommendations on RNA storage conditions see tips box on page 4

In the assessment of the quality of total RNA isolated from the cell-free serum and plasma there are mainly three parameters that should be investigated:

1. Presence of typical serum and plasma microRNAs
2. Absence of cell RNA which could distort the plasma and serum microRNA profile
3. Absence of inhibitors of the cDNA synthesis and PCR enzyme

Parameter 1: Check for presence of typical serum and plasma microRNAs

Typical serum and plasma microRNAs can be detected by qPCR using the miRCURY LNA™ Universal RT microRNA PCR system. Detection of such microRNAs is a first indication that RNA isolation was successful.

Here are some of the microRNAs that are typically detected at constant levels in serum and plasma samples:

- hsa-miR-16
- hsa-miR-103
- hsa-miR-192
- hsa-miR-93
- hsa-miR-451

The expression of hsa-miR-451 seems to vary depending on the way the samples have been prepared. However, if the samples were prepared in the same way – which is highly recommended - this assay should also be suitable as control for the quality of the RNA isolation. Often, these microRNA assays may even be good for the normalization of expression data, but the stability of their expression needs to be determined (see below).

The five microRNA assays may be used to identify sample outliers but are not recommended for normalization of the sample input. The five microRNA assays may be used to identify sample outliers but should not be used to equalize RNA input to cDNA synthesis for different samples. As mentioned above, equalizing the RNA input to cDNA synthesis should be based on an equal loading of the sample (serum or plasma) for the RNA isolation. In addition, measures should be taken to treat and process the samples as similarly as possible. This is also the case for other types of samples that include cells (e.g. small LCM samples from FFPE samples and small amounts of FACS sorted cells); here sample input will be normalized by adding an equal amount of cells into the RNA isolation.

Parameter 2: Check for absence of cell RNA which could distort the plasma and serum microRNA profile

Presence of RNA species which are normally only detected in white or red blood cells or other cell types is an indication that cells have lysed during at some point prior to the RNA isolation. The presence of cellular RNA species may obviously disturb the serum /plasma microRNA profiling experiment resulting in a distorted and non-reproducible profile. A few candidate microRNA markers of venous blood are[2]:

- hsa-miR-20a
- hsa-miR-106a
- hsa-miR-185
- hsa-miR-144

Parameter 3: Check for absence of qPCR inhibitors

One of the challenges when working with plasma or serum is the high content of RT and/or PCR inhibitors in these types of samples. The presence of inhibitors means increasing the amount of sample input in order to obtain better sensitivity is not possible. The amount of inhibitors left after extraction may vary greatly between different extraction methods, but also from sample to sample. It is therefore highly recommended to test varying total RNA sample input amounts in the cDNA synthesis reaction (e.g. 0.5 µL, 1.0 µL, 2.0 µL, 4.0 µL, and 10 µL in a 40 µL RT reaction) on a few individual assays in order to get an idea of the microRNA level and possible presence of inhibitors in your particular serum/plasma sample. The different assays mentioned in the section describing typical serum and plasma microRNAs (see Parameter 1 above) are good candidate assays to analyze for the presence of inhibitors. The analysis should be conducted on several samples (>5) as the presence of inhibitors often vary from sample to sample irrespective of the timing of the individual sample preparations or other attempts to harmonize the sample preparation procedures.

"Plasma and serum have high content of RT and PCR inhibitors"

2. Zubakov et al. (2010), Int J Legal Med 124, 217

First strand cDNA synthesis

The quality control of total RNA described above will indicate whether inhibitors in general are present in the RNA samples. To minimize or alleviate the effect of smaller variations in inhibitor content between samples, it is recommended to perform cDNA synthesis in a larger reaction volume than for other samples (see the Instruction Manual for miRCURY LNA™ Universal RT microRNA PCR at www.exiqon.com/mirna-pcr).

In our experience, 8 µL of eluted human plasma/serum RNA or 1 µL of eluted mouse RNA in a 40 µL RT reaction generally give a good signal with more than 100 microRNAs called per sample (Cq's below 35). These values are only valid for RNA purified using the Exiqon protocol. The 40 µL RT reaction will provide sufficient cDNA for one 384-well plate.

The following protocol applies for cDNA synthesis with these modifications using human serum total RNA for each 384-well plate:

Reagent	Volume (µL)	Final conc./
Mix 8 µL 5x reaction buffer	8	1x
Nuclease-free water	19	
RNA spike-in	1	
Enzyme mix	4	1x
Total volume "base mix"	32	

If setting up cDNA synthesis reactions for multiple samples, add 10% excess volume to allow for loss during pipetting when preparing a "base mix" of this reaction mixture.

Final cDNA synthesis mix (per tube):

Reagent	Volume (µL)
"base mix"	32
Plasma or Serum	8
Total volume	40

Keep all reagents on ice while setting up.

For additional protocol details please consult the Instruction Manual for miRCURY™ LNA Universal RT microRNA PCR. The equivalent of 32 µL original serum/plasma is used per plate assuming RNA isolation was conducted on 200 µL serum and eluted from the column in 50 µL (see above).

Real-Time PCR amplification

Dilution of the cDNA synthesis reaction is a standard procedure conducted prior to PCR when using the miRCURY LNA™ Universal RT microRNA PCR system. Due to the larger RT reaction volume used for serum and plasma samples, the dilution factor of the cDNA is reduced relative to normal samples:

- For full 384-well plates, dilute the cDNA 55x (e.g. for screening on panels) by adding 2,160µL nuclease-free water to 40µL cDNA per plate
- For individual assays, dilute the cDNA 40x by combining 2.2 µL undiluted cDNA with 85.8 µL nuclease free water = 88 µL which will be sufficient for ten 20 µL PCR reactions, assuming 10% excess of the diluted cDNA is sufficient to allow for pipetting losses

Figure 4 shows a high reproducibility of RT reactions on total RNA from serum can be achieved using the above reaction volumes. From this point on, all handling and PCR cycling conditions follow procedures described in the Instruction Manual for miRCURY LNA™ Universal RT microRNA PCR.

Figure 4

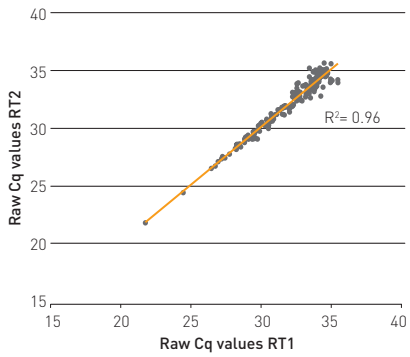


Figure 4. Excellent reproducibility between RT reactions on total RNA from serum. Raw Cq values from two separate RT reactions (RT1 and RT2) on total RNA purified from 65 µL serum are shown. A total of 730 microRNAs were profiled. Only microRNAs with Cq values below 35 have been included (133 data points).

ROX:
The miRCURY LNA™ Universal RT microRNA PCR, SYBR® Green master mix does not include ROX passive reference dye. This may be needed for some instruments.

AB instruments:
Use manual baseline and threshold settings. SDS template files for this and with pre-defined plate layout can be downloaded at www.exiqon.com/sds

Normalization

The purpose of normalization is to remove technical variation in data which is not related to the biological changes under investigation. Proper normalization is critical for the correct analysis and interpretation of results from real-time PCR experiments (Figure 5). The most commonly used methods for normalization are:

1. To identify and use stably expressed reference genes.
2. To use the mean expression value of all expressed microRNAs in a given sample as normalization factor[3].

Exiqon's GenEx software supports both of these approaches and it's recommended to investigate which of these methods will provide the best normalization of the data in question.

Serum and plasma samples typically do not contain larger RNA species, e.g., 5S, U6 and snoRNAs, that are sometimes used for normalization in other samples. Another difference is that the number of called microRNAs often is lower in serum/plasma samples than in other samples. When using mean expression values for normalization, a high number of microRNAs need to be expressed. Therefore, as a general guideline, consider to identify and use stably expressed reference genes for normalization if the number of expressed microRNAs is considerably below 80-100.

3. Mestdagh et al. (2009), Genome Biol, 10, R64

When using stably expressed reference genes for normalization, it is recommended to test several control candidates (reference genes) before setting up the actual microRNA expression analysis. These candidates should be chosen among genes that can be expected to be stably expressed over the whole range of samples being investigated.

When working with serum and plasma the candidates will have to be found amongst stably expressed microRNAs, which are chosen based on literature or pre-existing data (e.g. qPCR panel screening).

Figure 5

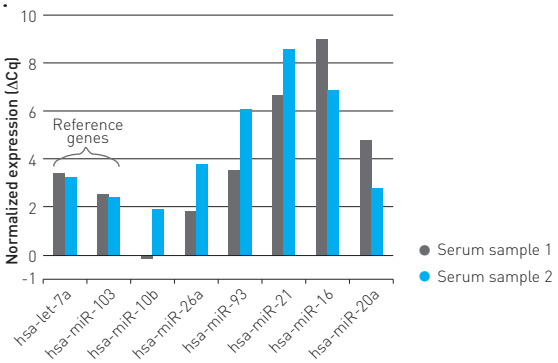


Figure 5. Differences in microRNA expression between serum samples. Normalized expression levels of eight different microRNAs in two serum samples is shown. Total RNA purified from the equivalent of 8 µL serum was used in the RT reaction. Hsa-let-7a and hsa-miR-103 were used as reference genes for normalization.

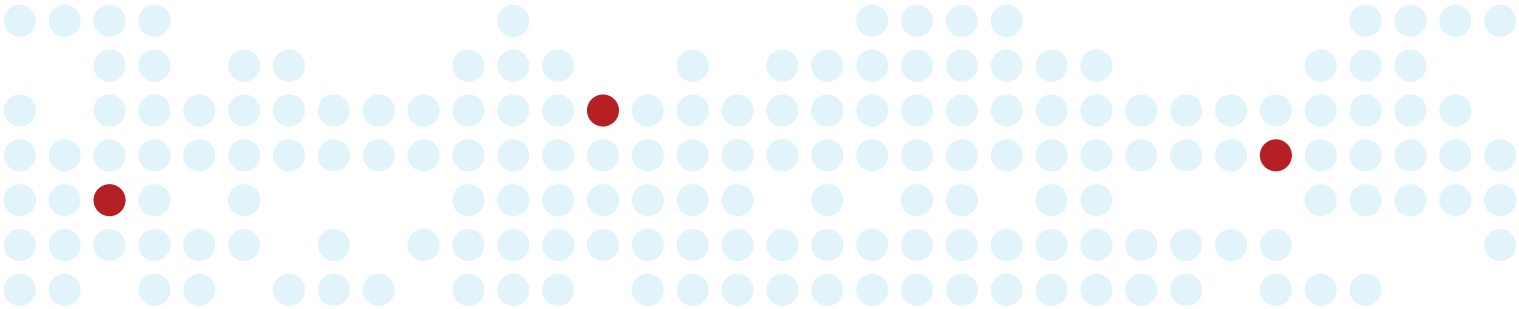
Additional reading and resources

The following publications can be downloaded from **www.exiqon.com/mirna-pcr**

- Instruction Manual for microRNA LNA™ Universal RT microRNA PCR
- RNA Purification from Blood Plasma & Serum – Human
- RNA Purification from Blood Plasma & Serum – Mouse
- SDS template files for AB instrument
- Exiqon GenEx software

Data analysis software and tools are described in more details at **www.exiqon.com/qPCR-resources**

www.exiqon.com/e-talk displays presentations about the miRCURY LNA™ Universal RT microRNA PCR system and the advantages of using the LNA™ technology



Contact information

Outside North America
Exiqon A/S · Skelstedet 16
DK-2950 Vedbaek · Denmark
Phone +45 45 660 888
Fax +45 45 661 888

North America
Exiqon Inc. · 14 F Gill Street
Woburn, MA 01801 · United States
Phone (781) 376 4150
Fax (781) 376 4152

exiqon.com

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