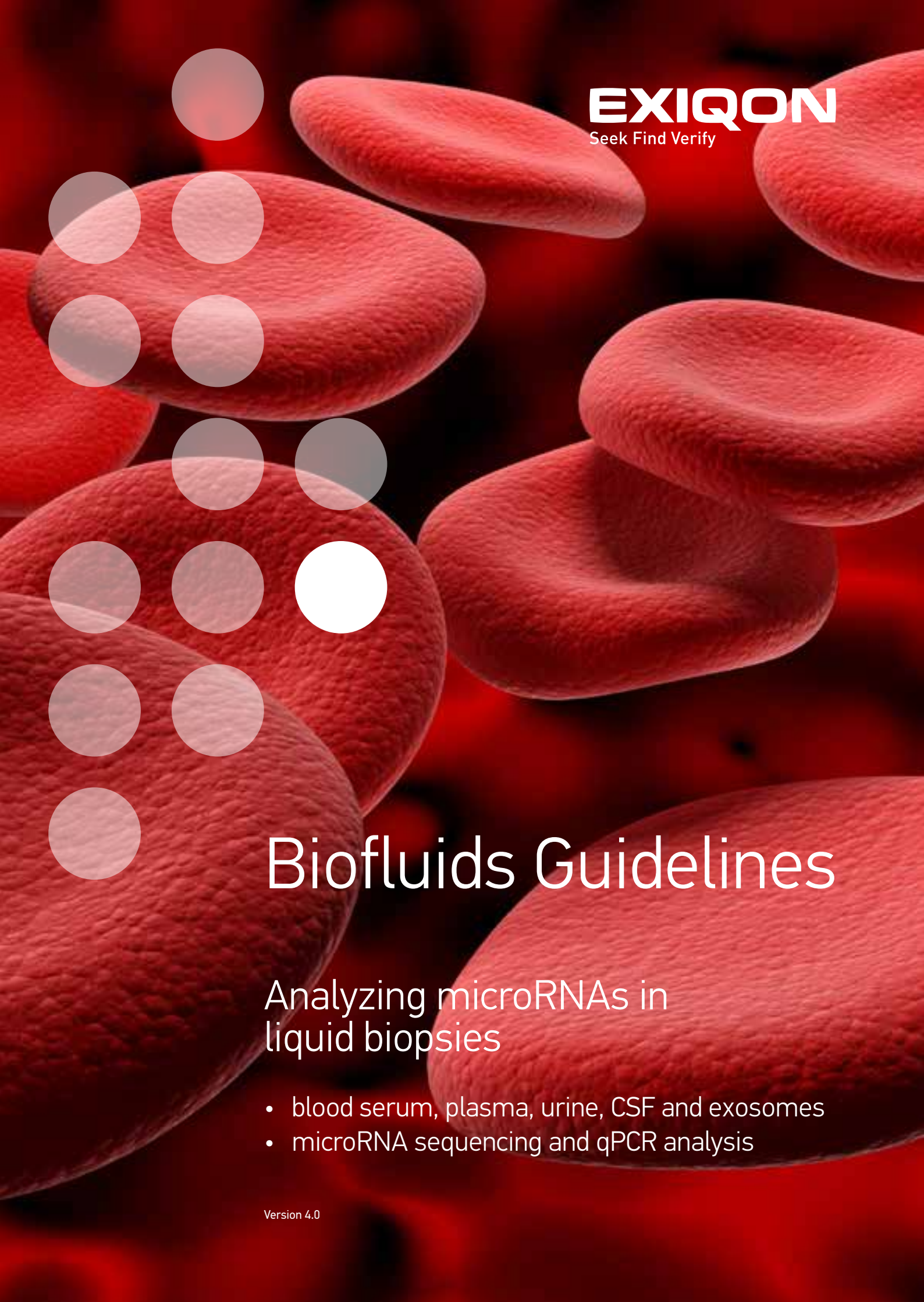


EXIQON
Seek Find Verify

Biofluids Guidelines

Analyzing microRNAs in liquid biopsies

- blood serum, plasma, urine, CSF and exosomes
- microRNA sequencing and qPCR analysis

Version 4.0

The Guidelines

microRNA sequencing using Exiqon's NGS Services and microRNA qPCR analysis using the miRCURY LNA™ Universal RT microRNA PCR System.

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1 Introduction

MicroRNAs in liquid biopsies hold great promise as minimally invasive diagnostic biomarkers for a wide range of diseases and biological processes. These short regulating RNAs have wide-ranging biological potential, are limited in number and are relatively stable in clinical samples such as serum / plasma, urine and other biofluids. However, microRNA profiling in biofluid samples is challenging in many ways. Biofluids contain low levels of RNA, high levels of inhibitors and are susceptible to many pre-analytical variables. To address these challenges, we have focused on developing highly sensitive and accurate microRNA detection methods, combined with optimized protocols for sample handling and preparation, and extensive QC procedures.

Exiqon offers a range of products and services for highly sensitive, specific and robust detection of microRNAs in biofluids. The techniques we have developed are based on many years' experience with both microRNA profiling and biomarker discovery and validation in biofluid samples. Next Generation Sequencing (NGS) is a powerful tool for the discovery of novel microRNAs. We have optimized microRNA sequencing specifically for serum / plasma, to enable reliable results using Exiqon's NGS Services. Our rigorously validated LNA™- enhanced qPCR assays offer sensitive and specific analysis of both known and novel microRNAs.

These guidelines focus on setting up microRNA profiling experiments from blood serum and plasma, and urine, but contain useful information for microRNA experiments using other biofluids as well. The guidelines provide important information and tips to ensure successful microRNA profiling using either Next Generation Sequencing or the miRCURY LNA™ Universal RT microRNA PCR System.



2 How to choose a microRNA profiling platform for biofluids

There are two platforms used for profiling microRNAs in biofluids: NGS or qPCR. The features of these platforms are summarized in Table 1.

NGS is the method of choice if your main goal is the discovery of novel microRNA sequences, or studying isomiRs or microRNA editing. The most abundant isomiR for a particular microRNA may vary between different sample types. One advantage of NGS is that it has the possibility to detect all isomiRs, whereas standard microRNA qPCR assays are designed to detect the specific isomiR listed in miRBase as the major sequence for that microRNA, which may not be the main isomiR present in your particular sample type (Figure 1).

qPCR offers the flexibility to perform either miRNome profiling or validation of a subset of microRNAs, and is well suited for accurate differential expression analysis of a defined set of microRNAs (which can include custom qPCR assays designed to detect novel microRNAs or isomiRs). One advantage of the miRCURY LNA™ Universal RT microRNA PCR System in particular is the high sensitivity and reproducibility of the platform. Using NGS in theory you have the possibility to detect "all microRNAs". However, this is dependent on the sensitivity of the platform.

At Exiqon we have developed optimized procedures for biofluids to ensure highly sensitive and robust microRNA results using NGS or qPCR. Microarrays are not considered to offer the required sensitivity and dynamic range for optimal analysis of microRNAs in biofluids. The choice of platform will depend on many factors including your research goal, species, and amount of sample available. NGS usually requires a larger amount of sample than qPCR (see Table 4). As illustrated in Table 8, the combination of NGS and qPCR offers a powerful experimental design strategy.

Variation in hsa-let-7b-5p (isomiRs)

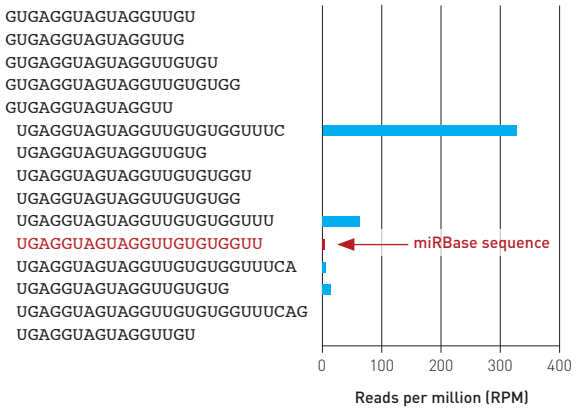
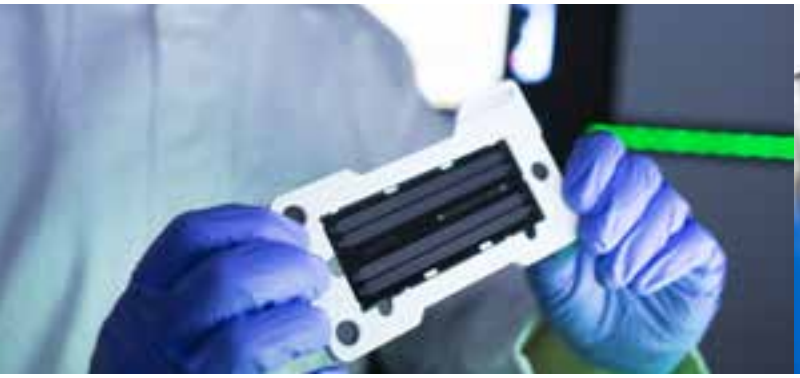


Figure 1: Sequencing RPM data extracted from miRBase.org (not complete list of isomiRs, red sequence shown is the main isomiR listed in miRBase).

Features of NGS and qPCR platforms for microRNA profiling of biofluid samples

NGS	qPCR
Hypothesis free	Hypothesis driven
Discovery of novel microRNAs, isomiRs or microRNA editing	Highly sensitive and reproducible differential expression
Analysis of "all microRNAs"	Profiling or validation of a subset of microRNAs
Larger amount of sample	Smaller amount of sample

Table 1: Features of Next Generation Sequencing and qPCR platforms to consider when choosing a platform for profiling microRNAs from biofluid samples.





3 Challenges of microRNA profiling from biofluids

MicroRNA profiling in biofluid samples holds great promise, but there are several challenges to overcome in order to successfully perform such experiments. First, biofluids contain very low amounts of RNA. This means that normal RNA quality control using Bioanalyzer or OD measurements is not suitable for these type of samples. Second, microRNAs detected in biofluids may have a cellular or an extracellular origin, and both may be relevant in terms of biomarker discovery. Circulating tumor cells (CTCs) may be isolated to examine their cellular microRNA profile. However, if the goal is to analyze the extracellular microRNA profile of biofluids, it is important to avoid cellular contamination and hemolysis (lysis of red blood cells in the case of serum / plasma samples or other biofluids contaminated by blood). Third, biofluids contain inhibitors of the reverse transcriptase and polymerase enzymes that can inhibit the enzymatic reactions in RT-qPCR or library preparation for NGS. Minimizing the carry-over of inhibitors into the RNA sample as well as monitoring sample quality are important obstacles for consideration.

Finally, normalization of qPCR data from biofluid samples can be challenging and great care must be taken when choosing controls for normalization. Some of the larger small RNA species frequently used as reference genes (such as U6 snRNA) originate from the nucleus, and may not be secreted or protected in cell-free biofluids in the same way that microRNAs are. When working in biofluids like urine or cerebrospinal fluid (CSF), another challenge is the large variations in microRNA content between samples. Urine and CSF from healthy individuals typically contain much less microRNA compared to samples from diseased individuals or those exposed to drugs or toxins, which may contain significantly elevated levels of organ-specific microRNAs. In this document, we will present recommendations on ways to overcome these challenges, summarized in Table 2.

Biofluid microRNA profiling: Challenges and Solutions

Challenge	Solutions
Limited amount of RNA	• Exosome isolation to enhance signal (urine and CSF samples) • Optimized RNA isolation protocol • Carrier RNA during RNA isolation (except for NGS) • Quantification of blank purification to assess any background signals • Monitor RNA isolation efficiency (using RNA spike-ins for PCR QC) • Optimized NGS library preparation • Highly sensitive detection method
Undesired components e.g. inhibitors of downstream enzymatic reactions	• Do not use heparin tubes • Use a column or bead based RNA isolation method • Monitor RNases & inhibitors (using RNA spike-ins for PCR QC) • Highly specific detection method (to discriminate tRNA fragments) • Sequencing QC (reproducibility of >50 spike-ins) • When establishing a new protocol, confirm linear relationship between sample input and signal
Cellular contamination & hemolysis	• Ensure consistent and optimal sample collection • Spin samples when fresh to remove cells • Compare data to reference range • Monitor Hemolysis Indicator (PCR QC) or absorbance of oxyhemoglobin (414 nm)
Pre-analytical variables	• Optimal experimental design (biological replicates) • Control sources of technical variation (e.g. collection sites)
Normalization	• Normalize to mean expression value for call rates > 20-50 microRNAs • Alternatively internal normalizers (stable endogenous microRNAs) • For urine from small animal models, consider normalization to urinary volume / creatinine • Consider microRNA ratios to normalize dramatic overall changes in microRNA content (e.g. drug-induced toxicity)

Table 2: Overview of the main challenges and solutions proposed for microRNA profiling of biofluid samples using NGS or qPCR.

4 Blood serum and plasma

Serum or plasma, or exosomes?

RNA from both plasma and serum can be accurately profiled using both Next Generation Sequencing and the miRCURY LNA™ Universal RT microRNA PCR System. Both sample types have been used successfully for biomarker discovery.

In general we observe slightly higher signals from plasma samples, which could be due to varying levels of thrombocyte contamination in the samples. On the other hand, differences in coagulation times and temperature may lead to variation between serum samples.

Comparisons of normal serum and plasma indicate that there is less variation within a properly sampled serum dataset compared to a corresponding plasma dataset. Figure 2 shows a comparison between serum and plasma samples.

Exosomes are of interest due to their proposed role as intercellular hormone-like messengers. MicroRNAs are actively and selectively incorporated into exosomes, hence the exosomal microRNA population represents a subset of the microRNAs found in serum / plasma (Table 3).

MicroRNA profiles from serum and plasma are very similar

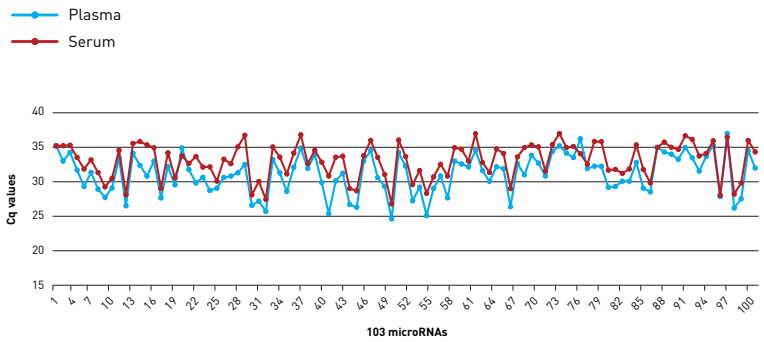


Figure 2: Comparison of Cq values from 103 of the most commonly expressed microRNAs in serum and plasma, profiled using the miRCURY LNA™ Universal RT microRNA PCR System.

Most microRNAs are detected in the whole biofluid, rather than exosomes or exosome-free supernatant derived from serum / plasma

Fraction	Serum	Plasma
Exosome Pellet	127	104
Supernatant	46	74
Whole biofluid	**151**	**142**

Table 3: All of the microRNAs detected in the exosome pellet or supernatant could also be detected in the whole biofluid samples. Exosomes and supernatant were isolated from 200 µl serum or plasma using the miRCURY™ Exosome Isolation Kit. RNA was isolated using the miRCURY™ RNA Isolation kit – Biofluids either directly from 200 µl serum or plasma, or from the exosome pellet and supernatant. Average number of microRNAs detected on Exiqon's miRCURY LNA™ microRNA PCR Serum / Plasma Focus Panel containing 179 microRNAs.

Proper collection and preparation of blood serum and plasma samples

In all steps from collection of whole blood to preparation of serum and plasma, measures should be taken to prevent lysis of cells – this is also important for other biofluid samples. Failure to do so may lead to contamination of the samples with RNA from intact cells. The RNA from intact cells could mask or prevent the subsequent detection of subtle changes in the microRNA expression profile. Hemolysis may affect the microRNA profile.

Minimizing pre-analytical variables

Collection of whole blood is the first step in the preparation of plasma and serum. To avoid hemolysis, which can occur at the time of phlebotomy, it is important to ensure blood is collected by staff highly experienced in venipuncture. A method that preserves the RNA expression profile during and after collection of whole blood is important for accurate analysis of microRNA expression in blood plasma and serum. Use a high quality collection tube, a standardized procedure for sampling, and where possible collect samples at the same time to avoid introducing technical variation at this step.

For optimal results from archived samples, care should be taken to select only samples that have been collected and processed according to the same protocol. For multi-center studies, bear in mind that different hospitals may use different equipment and procedures for sample collection. It is important that the sampling method is consistent throughout the study to minimize any pre-analytical variables.

Collection protocols and tubes

We recommend the NCI's Early Detection Research Network (EDRN) standard operating procedures for the collection and preparation of serum and plasma. At Exiqon we have successfully used BD Vacutainer® tubes, but other tube types may also be applicable. In the preparation of plasma, anticoagulants such as EDTA and sometimes citrate are normally used. These anticoagulants are both fine to use, in contrast to heparin, which is known to inhibit downstream enzymatic steps such as cDNA synthesis and PCR (Figure 3). Currently, there is no reliable way of removing heparin from RNA samples or from the original blood serum / plasma samples. Therefore, **do not use heparin** in any of the processing steps of whole blood.

"Consistent and standardized sample collection and handling is essential"

Heparin tubes are not suitable for microRNA PCR analysis

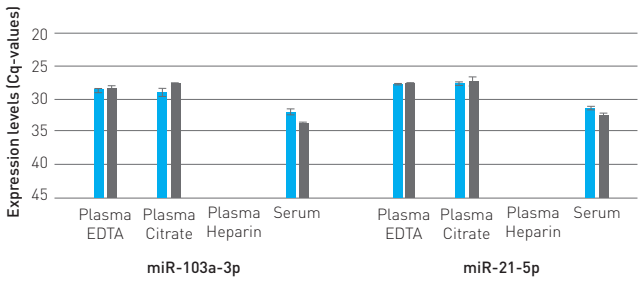


Figure 3: Suitability of different blood preparations for microRNA PCR analysis. Real-time PCR for miR-103a-3p and miR-21-5p was performed using triplicate RT reactions on total RNA purified from either EDTA-plasma, citrate-plasma, heparin-plasma, or serum. Average Cq values for each triplicate are shown, demonstrating robust amplification from all sources except heparin-plasma.

It is recommended to process the whole blood immediately into either serum or plasma. If the whole blood is not centrifuged immediately after collection (or after the clotting time, in the case of serum), the samples should be stored at room temperature for no longer than 4 hours. Storage at 4 °C as stated in the EDRN standard operating procedures may result in lysis of thrombocytes and contamination of the cell-free microRNA profile. As mentioned above, it is important to handle all samples consistently to avoid introducing any technical variation.

Screening for hemolysis

A quick and low cost method to screen serum and plasma samples for hemolysis is to use a spectrophotometer and measure oxyhemoglobin absorbance at 414 nm. Usually an OD scan is performed from around 200 to 700 nm, and distinct absorbance peaks at 414 nm can be used to disqualify hemolysis samples (Blondal *et al.*, 2013). Residual platelets and microparticles can also affect the microRNA profile. Platelets and microparticles can be effectively removed even from archival samples by centrifugation (Cheng *et al.*, 2013).

Storage

Once prepared, serum, plasma and other biofluid samples should be centrifuged for 5 minutes at 3,000 x g to remove cells (prior to freezing), and then stored in RNase- free tubes (e.g. cryo-tubes) at -80 °C. Cells must be removed prior to freezing as the cells will otherwise lyse and release their microRNA content into the cell-free biofluid. Alternatively, RNA isolation may be carried out immediately after removal of cells.

Serum and plasma samples intended for exosome isolation should be centrifuged for 5 minutes at 10,000 x g to remove cell debris (prior to freezing). If cell debris cannot be removed immediately, samples should be stored at 4 °C for maximum 24 hrs until centrifugation. Before centrifugation, plasma samples are treated with thrombin to ensure removal of fibrin which makes the exosome pellet easier to dissolve. The cell-free serum or plasma is then stored at either -20 °C (short term) or -80 °C (long term), or processed immediately to exosomes.

High stability of microRNA in EDTA-plasma samples

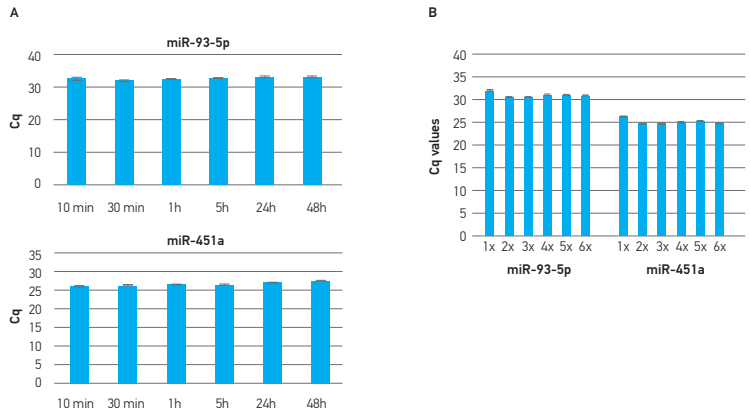


Figure 4: A) Real-time PCR was performed using triplicate RT reactions on total RNA purified from EDTA plasma. The plasma was stored at room temperature before RNA extraction for the indicated times. No evidence of microRNA degradation in plasma was observed, even after prolonged storage of up to 48 hours at room temperature. B) Real-time PCR 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 microRNA amplification, demonstrating that plasma microRNA is stable towards multiple freeze-thaw cycles.

5 Urine

Whole urine, cell-free urine, or exosomes?

Urine microRNAs may be present within exosomes or protein complexes, or within cells that may be shed into the urine. In pre-clinical animal studies, analyses are usually performed on whole urine samples due to limitations in the way the urine can be collected. However, in the case of human studies, whole urine may be separated into the cell pellet and "cell-free" supernatant. If desired, exosomes may be isolated from the cell-free supernatant. MicroRNA profiling may be performed on any of these fractions. The choice of sample type will depend on the research objective and disease context. In some cases, performing separate analysis of the cell pellet and cell-free fraction may be relevant.

The number of different cell types present in urine may vary between individuals or disease states, or following certain medical treatments, and this can affect the microRNA profile of whole urine samples (Figure 5).

The "cell-free" supernatant contains microRNAs released in exosomes and protein complexes. There are usually very few microRNAs detected in the cell-free urine of healthy individuals. The cell-free urine may be useful for toxicology biomarker analysis as it is likely to contain microRNAs released as a consequence of cell toxicity and death. Exosome isolation can enhance the microRNA signals in very dilute samples such as urine and CSF, enabling more microRNAs to be detected (Figure 9). This is because exosome isolation acts as a means to concentrate the sample while minimizing co-purification of inhibitory compounds.

microRNA profile of rat urine before and after treatment with nephrotoxin

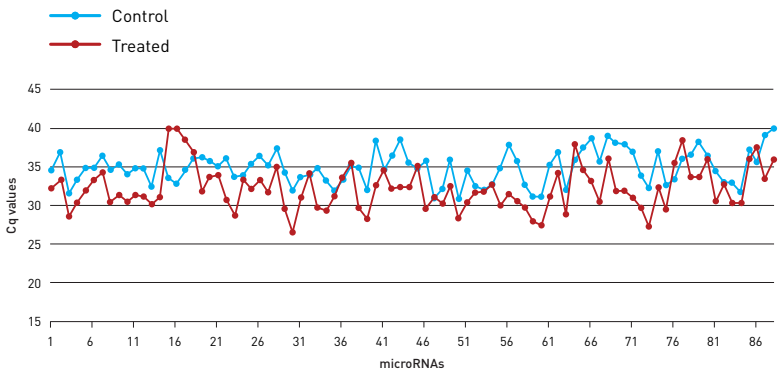


Figure 5: Several microRNAs show elevated expression levels (reduced Cq) in rat whole urine after nephrotoxin treatment. MicroRNA profiling was performed using the miRCURY LNA™ Universal RT microRNA PCR System.



> *"Before freezing, biofluid samples should be centrifuged to remove cell debris"*

High stability of microRNA in human cell-free urine

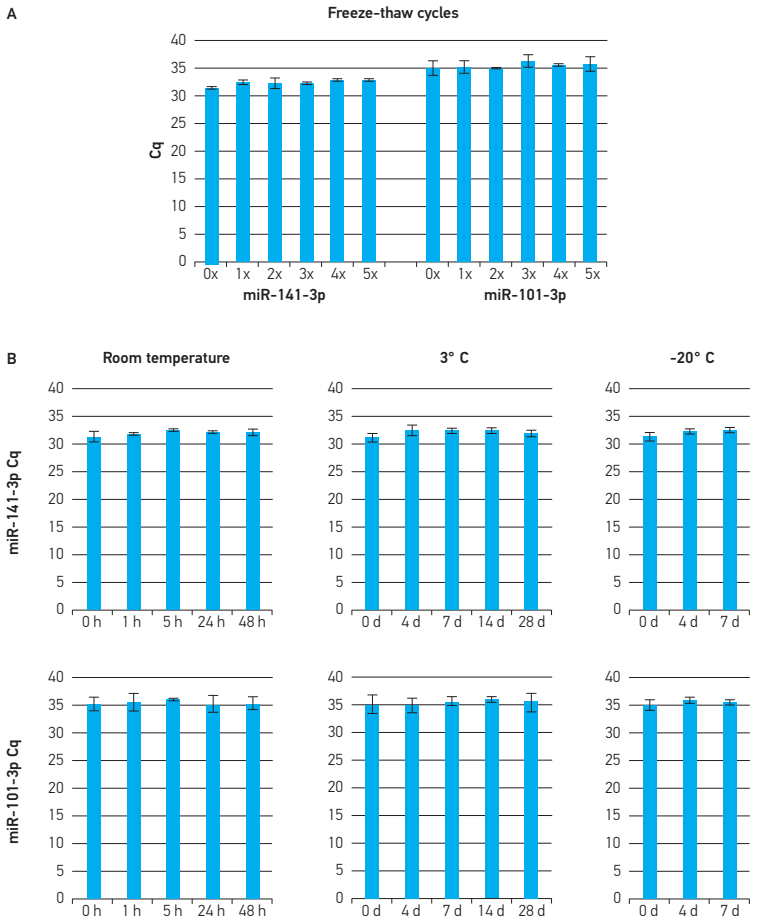


Figure 6: Human urine samples were collected and centrifuged to remove cell debris. Real-time PCR was performed on total RNA purified from urine. A) Cell-free urine samples were subjected to 1-5 freeze-thaw cycles prior to RNA isolation with minimal effect on microRNA amplification. B) Cell-free urine samples were stored at room temperature, 3 °C or -20 °C before RNA extraction for the indicated times. Prolonged storage of up to 48 hours at room temperature, or up to 28 days at 3 °C or -20 °C had minimal effect on microRNA amplification. Although the effects of sample handling and storage resulted in loss of no more than 2 Cq values, we do recommend to standardize sample handling and storage protocols, and to process urine samples immediately (centrifuge to remove cell debris) and store at -80 °C.

Proper collection and preparation of urine samples

MicroRNAs in cell-free urine samples show high stability (Figure 6) but nevertheless we recommend to standardize sample handling and storage protocols. At Exiqon we have successfully analyzed urine samples collected in RNase-free Cryo tubes without stabilizer, however other tube types may also be applicable.

If the goal is to analyze microRNAs in the cell-free portion of the urine, care should be taken in all steps to minimize lysis of cells.

For analysis of cell-free urine, the fresh urine samples should be centrifuged at 3,200 x g for 5 minutes to remove cell debris and stored at -80 °C in RNase-free tubes. If cell debris cannot be removed immediately, samples should be stored at 4 °C for maximum 24 hrs until centrifugation. Centrifugation should be done prior to freezing, as freezing the urine sample before centrifugation will lead to lysis of cells (and release of cellular RNA) upon thawing.

Urine samples intended for exosome isolation should be centrifuged to remove cells then stored at either -20 °C (short term) or -80 °C (long term), or processed immediately to exosomes.

When collecting urine from animals (such as in pre-clinical studies), special consideration should be given to possible sources of contamination and variation due to sample collection procedures, for example the timing of sampling cannot be controlled. We recommend that the urine samples are centrifuged to remove cells and other debris.

> *"Avoid freeze-thaw cycles"*

6 Optimal isolation of exosomes

Why isolate exosomes?

Exosome isolation enables the analysis of those microRNAs which are selectively and actively incorporated into these membrane bound vesicles and secreted by cells into a range of biofluids. In addition, exosome isolation can enhance the signals from very dilute biofluid samples such as urine and CSF, enabling more microRNAs to be detected (Figure 9).

Preparing samples

To ensure optimal exosome isolation, biofluid samples should be centrifuged (prior to freezing) to remove cell debris and stored at either -20 °C (short term) or -80 °C (long term). If cell debris cannot be removed immediately, samples should be stored at 4 °C for maximum 24 hrs until centrifugation. The exosome composition from serum and plasma may differ depending on the treatment of the biofluid prior to RNA isolation. Therefore sample collection and specimen pre-treatment protocols should be standardized and controlled.

The structure of an exosome

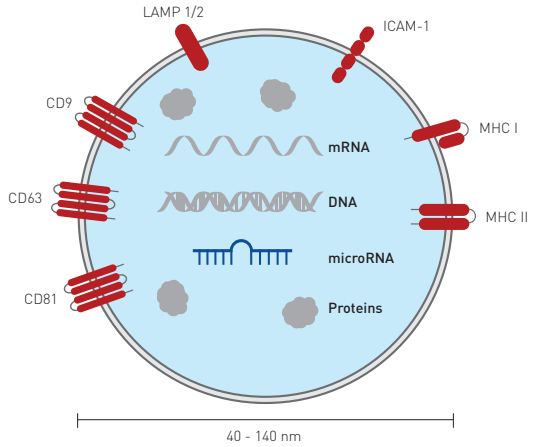


Figure 7: Exosomes are membrane-encapsulated particles ranging from 40 - 140 nm in diameter. They contain proteins, DNA, mRNA and microRNA. A number of surface proteins are found exclusively in exosomes.

Direct comparison of Exiqon miRCURY™ Exosome Isolation Kit and ultracentrifugation

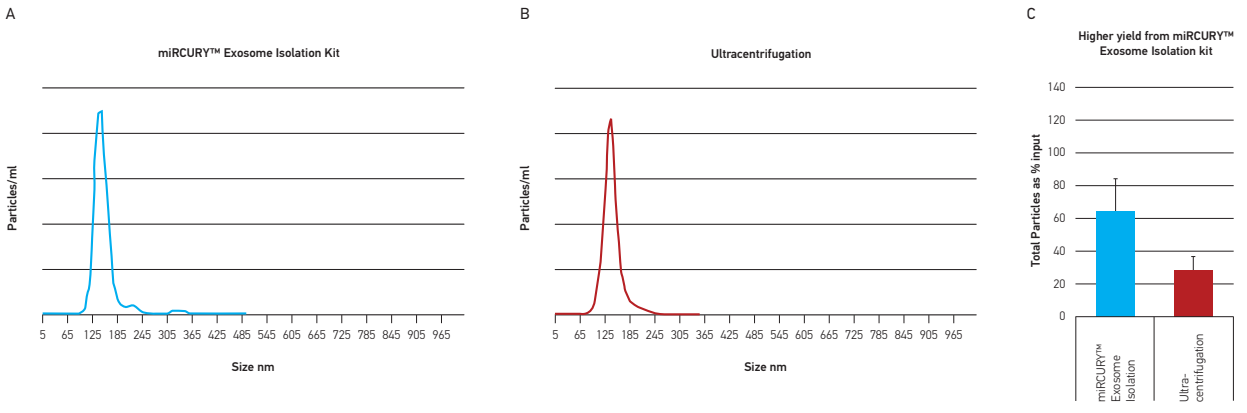


Figure 8: NanoSight measurements showing the similar size distributions of pelleted vesicles obtained using (A) Exiqon's miRCURY™ Exosome Isolation Kit and (B) ultracentrifugation. C) Exiqon's miRCURY™ Exosome Isolation Kit results in higher efficiency of pelleting compared to ultracentrifugation.

Detect more microRNAs in dilute biofluids using exosome isolation kits

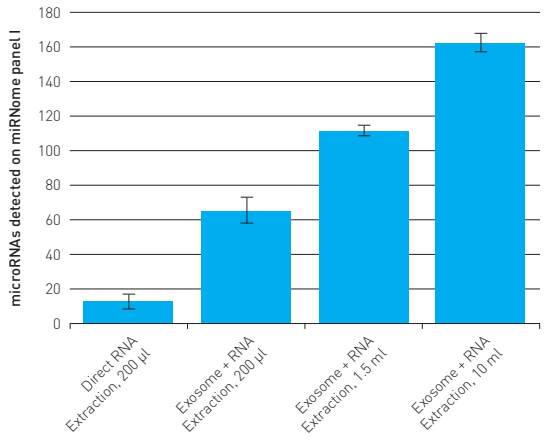


Figure 9: Use of an exosome isolation kit allows greater sample starting volumes from dilute biofluid samples such as urine, resulting in detection of more microRNAs. For direct RNA extraction, 200 µL of urine from a healthy individual was extracted using the miRCURY™ RNA Isolation Kit – Biofluids. In comparison, greater microRNA qPCR call rates were observed when increasing volumes of urine were subjected to exosome enrichment using the miRCURY™ Exosome Isolation Kit – Cells, urine and CSF, prior to RNA extraction by miRCURY™ RNA Isolation Kit – Cell & Plant.

Methods for exosome isolation

Exosomes may be isolated in a number of different ways including precipitation, size exclusion chromatography, differential centrifugation and ultracentrifugation. Different methods can lead to differences in the composition of vesicles isolated, and hence may result in differences in the microRNA profile. Exosome precipitation is a fast and convenient way to isolate exosomes, and results in a similar vesicle size distribution and a higher efficiency of exosome recovery compared to ultracentrifugation (Figure 8).

miRCURY™ Exosome Isolation Kits

For exosome isolation in under 2 hours. Learn more at:

exiqon.com/exosome-isolation-kits



> *"Exosome isolation can enable you to detect more microRNAs"*



7 Optimal isolation of RNA

Working with RNA 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 RNA quality:

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 plasticware and filter barrier pipette tips
- Keep tubes capped when possible, always spin tubes before opening
- For long-term storage, RNA may be stored at -80° C
- Avoid repeated freeze-thaw cycles

Preparation of total RNA that includes small RNAs (<200 nt) from a biological sample is critical for successful microRNA expression profiling. Therefore, the method used for RNA isolation is crucial to the success of the experiment.

Biofluids contain small amounts of RNA

As mentioned earlier, biofluid samples contain only small amounts of RNA which means that there is a high risk that a significant proportion of the RNA is lost during extraction. For this reason, we recommend using low nucleic acid binding tubes, adding carrier RNA during the purification procedure. The use of a carrier ensures the highest and most consistent yield from serum and plasma samples and other low yield biofluids (Figure 10, Andreasen *et al.*, 2010), and may also minimize selective loss of structured low GC content microRNA which can be observed in samples with low RNA content isolated by Trizol (Kim *et al.*, 2012).

Carrier RNA improves RNA isolations from serum / plasma

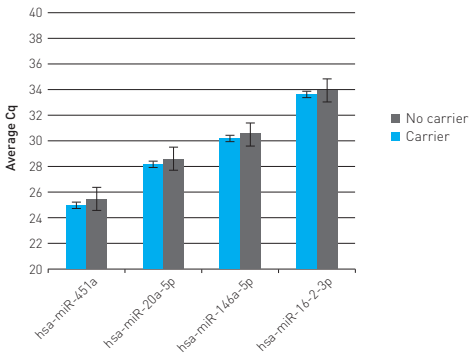


Figure 10: Adding MS2 carrier RNA to the RNA extraction increases reproducibility between isolations and leads to more consistent yields (Andreasen *et al.*, 2010).

When selecting a carrier, it is important to choose a source which is guaranteed to be free from microRNAs. Such a source is RNA from the bacteriophage MS2 (available from Roche Applied Science cat. no. 10165948001), which is routinely used by Exiqon Services. Carrier RNA should not be added to samples intended for NGS. Alternatively glycogen can be used as a carrier, provided it is guaranteed to be microRNA-free. For subsequent enzymatic steps, the input RNA amount is based on starting volume rather than RNA quantity assessed by optical measurements, as OD2600 measurements are not reliable in the case of biofluid samples.

Minimizing inhibitors

In addition to low amounts of RNA, biofluid samples also tend to contain high levels of enzyme inhibitors that can affect the efficiency of the reverse transcription or PCR reactions, or library preparation for NGS. Therefore it is important to choose a purification method, for example a column-based method, which minimizes the carry-over of such inhibitors while maximizing the RNA yield.

Determining background

When profiling very low abundance targets such as microRNAs in biofluids, it is important to ensure the signals you are measuring are reliably above any background signal. A blank purification (water added instead of biofluid sample in the RNA isolation) can be used as a negative control to measure any background signal. As a general rule, the negative control should be at least 5 Cq above the sample Cq. When performing microRNA sequencing, setting a TPM threshold can be a useful way to focus on the most reliable microRNA reads above background (see the section on microRNA sequencing).

Optimized procedures

Exiqon offers a range of kits optimized for isolation of exosomes and/or RNA from serum / plasma, urine, CSF and other biofluids. We also recommend adding certain synthetic RNA spike-ins during the RNA isolation step (included in the RNA spike-in kit), which are used to perform qPCR based quality control of RNA from biofluid samples. To enable robust microRNA sequencing from serum / plasma samples, Exiqon has developed an RNA isolation protocol optimized specifically for this application, which is offered as part of Exiqon's NGS Services. For recommended kits and biofluid starting volumes see Table 4.

> *"Biofluid samples require optimized RNA isolation procedures"*



Recommended biofluid starting volumes and sample preparation for microRNA analysis by qPCR or NGS					
Desired sample type	Recommended exosome isolation kit (starting volume of biofluid)	Recommended RNA isolation kit (starting volume of biofluid)	Recommended microRNA analysis method		
			LNA™ PCR	Exiqon PCR Services	Exiqon NGS Services
Serum / Plasma		Exiqon Services proprietary Protocol (human 500 µl)			✓
Serum / Plasma		miRCURY™ RNA Isolation Kit – Biofluids (human 200 µl, rodent 50 µl*)	✓	✓	
Whole urine**, CSF** or other biofluid		miRCURY™ RNA Isolation Kit – Biofluids (human 200 µl, rodent***)	✓	✓	
Exosomes from serum / plasma	miRCURY™ Exosome Isolation Kit – Serum and Plasma (0.2 – 1.4 ml)	miRCURY™ RNA Isolation Kit – Biofluids	✓	✓	
Exosomes from cell culture media, urine, or CSF	miRCURY™ Exosome Isolation Kit – Cells, urine and CSF (1 – 10 ml)	miRCURY™ RNA Isolation Kit – Cell and Plant	✓	✓	

Table 4: Recommended biofluid starting volumes and sample preparation methods for Exosome and/or RNA isolation prior to microRNA analysis using the miRCURY LNA™ Universal RT microRNA PCR System or Exiqon's PCR or NGS Services. RNA spike-ins (included in the RNA spike-in kit) should be added during the RNA isolation step for quality control purposes.

* Add RNase-free water to rodent serum / plasma to keep final volume at 200 µl.

** For urine and CSF samples, exosome isolation is highly recommended to enable a larger starting volume and thereby increase the number of microRNAs detected.

*** Rodent urine 200 µl, rodent CSF not tested, rodent other biofluids 50 µl (add RNase-free water to keep final volume at 200 µl, and the starting volume of biofluid may need to be adjusted to keep PCR inhibitors at a minimum).

A fraction of the RNA isolated is used as input into Exiqon's Universal cDNA synthesis kit prior to PCR analysis (4 µl for serum, plasma, urine and other biofluid samples, and 8 µl for CSF samples is used per 20 µl RT reaction). Please consult the **PCR Services Guidelines** for volumes of biofluid needed when using Exiqon's Exosome/RNA Isolation and PCR Services.

Tips: Successful RNA extraction and cDNA synthesis

- Include a carrier RNA* like MS2 RNA, in order to ensure the highest and most consistent yield from the samples
- Choose the appropriate RNA isolation kit, depending on whether the RNA will be isolated from the whole biofluid, or from exosomes (see Table 4)
- The comparison of samples prepared using different RNA isolation methods is not recommended
- Don't forget to add RNA spike-ins (included in the RNA spike-in kit) during the RNA isolation and cDNA synthesis steps, which can be later used for assessing the quality of the RNA (see Table 5)
- The isolation of RNA and the reaction steps preceding real-time PCR should be performed in rooms separate from where real-time PCR reactions are carried out in order to avoid contaminating the cDNA with PCR amplicons
- Do not use heparin tubes, as heparin is a strong inhibitor of all enzymatic processes
- Don't increase the volume of RNA in the cDNA synthesis reaction to increase the signal – this can have the opposite effect. Ensure you are working in the linear range of the system (see Figure 12)
- Before isolating RNA from biofluids other than serum / plasma, consider whether to include the cellular fraction

* Important: Carrier RNA should not be added to samples that will be used for microRNA sequencing.

The miRCURY™ RNA Isolation Kit Biofluids – best in class

- Detect more microRNAs
- Quicker and easier to use (45 min protocol)
- Silica carbide matrix - optimized for low RNA content samples, minimizes inhibitors
- No phenol/chloroform – no tricky phase separation

> ***"Our results indicate that the Exiqon miRCURY™ Biofluids Kit out-performs other RNA isolation methods"***

McAlexander *et al.* Comparison of Methods for miRNA Extraction from Plasma and Quantitative Recovery of RNA from Cerebrospinal Fluid. Front Genet. 2013, 4:83. PMID: 23720669.

> ***"Highest amount of isolated miRNA obtained using the miRCURY™ RNA Isolation Kit - Biofluids"***

Sedlackova *et al.* Selection of an optimal method for co-isolation of circulating DNA and miRNA from the plasma of pregnant women. Clin Chem Lab Med. 2014, 52(11):1543-8. PMID: 24815052.

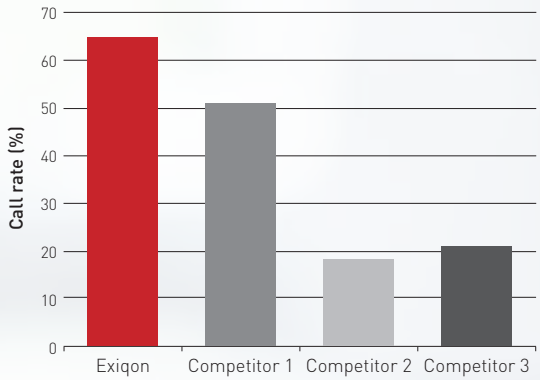
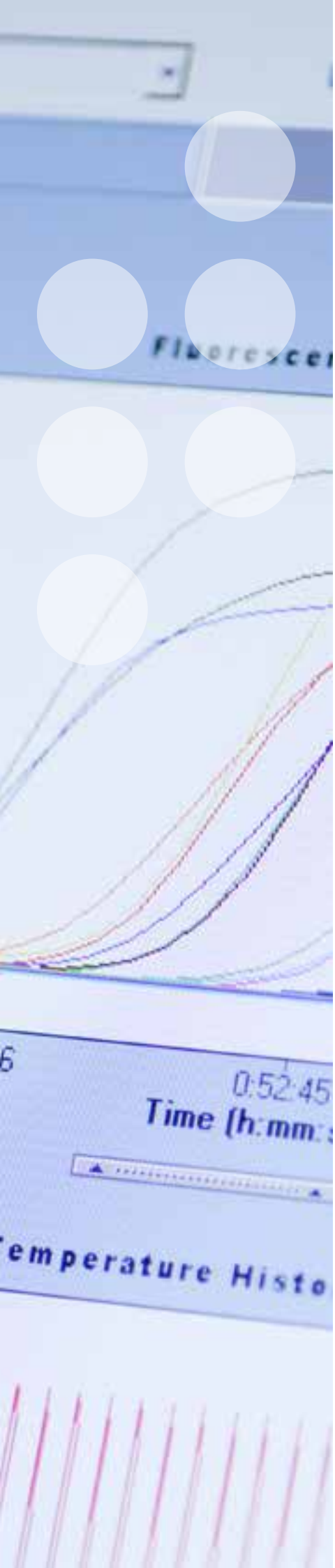


Figure 11: The miRCURY™ RNA Isolation Kit –Biofluids allows detection of more microRNAs compared to three other column based RNA isolation kits.
RNA isolations were performed from the same plasma sample, using 4 different RNA isolation kits. MicroRNA profiling was performed using the Exiqon Serum / Plasma Focus microRNA PCR panel (168 microRNA assays in total). Call rate (percent of microRNAs detected) for each kit is shown. Only microRNAs detected as present in all 5 replicates were calculated as a call.

miRCURY™ RNA Isolation Kit – Biofluids

Superior RNA isolation from biofluids. Learn more at:
exiqon.com/mirna-isolation-biofluids



8 Conducting quality control of RNA

These guidelines focus on the QC of RNA isolated from biofluid samples prior to microRNA qPCR analysis. Exiqon has developed specialized RNA isolation and QC procedures prior to microRNA NGS analysis of biofluid samples, which are performed as part of Exiqon's NGS Services.

> *"qPCR based methods have been developed to reliably assess the quality of RNA from biofluids"*

OD260 quantification is not reliable for biofluid RNA

Standard methods for measurement of the RNA yield and quality are inappropriate for use with biofluid samples. The presence of carrier RNA in these samples makes measuring the low levels of endogenous RNA by OD260 impossible. 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, and presence of contaminants that absorb around 260 nm can confound the readings.

Standardize input amounts based on volume

RNA concentration measurements cannot be used in the case of biofluid samples to monitor yield and ensure consistent RNA input across all samples. For this reason, Exiqon recommends standardizing input amounts based on starting volume rather than RNA quantity (starting with the same volume of biofluid in each RNA isolation, and using the same volume of purified RNA for all samples).

RNA Spike-in Kit

Reliable RNA Quality Control for Biofluids. Learn more at:
exiqon.com/rna-spike-in-kit

Key QC parameters

To assess the quality of RNA isolated from the cell-free biofluids these parameters should be considered:

1. The efficiency of the RNA extraction and the yield
2. The absence of any inhibitors of downstream enzymatic processes e.g. cDNA synthesis, PCR or NGS library preparation
3. The absence of nucleases
4. The presence or absence of RNA resulting from cellular contamination or hemolysis
5. The presence of endogenous microRNAs (expected to be present in the particular type of biofluid being analyzed)

Assess RNA quality by PCR

These parameters can be investigated using a combination of synthetic spike-in RNAs and selected endogenous microRNA assays, enabling inferior or potential outlier samples to be identified prior to further analysis. If working with other biofluid samples, endogenous microRNAs relevant for that type of sample need to be determined, either by literature study or a pilot study using a miRNome panel.

Exiqon has developed a set of synthetic RNA spike-ins and endogenous microRNAs that can be used to perform qPCR based quality control of RNA samples. The RNA spike-ins can be used to monitor the efficiency of RNA isolation, cDNA synthesis and PCR amplification (see Table 3 for details). The use of RNA spike-ins may also reveal potential presence of nucleases.

All spike-ins are available in the RNA spike-in kit and assays for the spike-ins are present on Exiqon's ready-to-use microRNA PCR panels, or available in individual tubes. In addition, a separate microRNA QC PCR panel is also available which contains a combination of 12 qPCR assays for the RNA-spike-ins and endogenous microRNAs.

Overview of Basic and Extended QC for assessment of biofluid sample quality by PCR

Quality Control of biofluid samples by PCR	
Basic QC	• Standard human serum / plasma samples • Use assays listed below • Single RNA input volume for all samples
Extended QC	• Challenging biofluid samples • Use assays listed below • Test a range of RNA input volumes • Proceed with Basic QC when optimal input volume established
Assay name	**Applications for biofluid samples**
UniSp2, UniSp4 and UniSp5	• Three different concentrations • Add to lysis buffer during RNA purification • Use to monitor RNA isolation efficiency
UniSp6 and cel-miR-39-3p	• Different concentrations • Add to cDNA synthesis reaction • Check for RT and PCR inhibitors
UniSp3	• Template and primers present in PCR panels • Independent PCR monitoring • Use as Inter-plate calibrator, IPC
miR-451a and miR-23a-3p	• Unique Hemolysis indicator • Check ΔCq(miR-23a-3p – miR-451a)
miR-30c-5p, miR-103a-3p, miR-124-3p and miR-191-5p	• Biologically relevant, endogenous microRNAs • Should be present in RNA from serum/ plasma, urine or CSF and some other biofluids • Use for general sample quality check

Table 5: Overview of assays in the microRNA QC PCR Panel and how they are used for Basic and Extended QC. The assays are used to monitor the efficiency and yield of the RNA isolation, the performance of the cDNA synthesis and PCR reactions as well as general sample quality. Assays are available on the microRNA QC PCR Panel or as individual assays. For detailed information, please see the manuals for the microRNA QC PCR Panel and the RNA Spike-in kit. UniSp2, UniSp4, UniSp5, and cel-miR-39-3p spike-ins are part of the RNA Spike-in Kit. The UniSp6 spike-in is provided with the Universal cDNA Synthesis Kit II and its corresponding primer set available in Exiqon's ExiLENT SYBR® Green master mix.

Basic QC
– standard human serum / plasma samples

The amount of RNA that can be extracted as well as the amount of inhibitors left after extraction can vary from sample to sample. When performing microRNA profiling from standard human serum / plasma samples it is recommended that all samples are quality controlled in order to monitor the purification yield, absence of PCR inhibitors, and identify any potential problems in the sample set, before proceeding with microRNA profiling by qPCR or NGS. For studies involving a very large number of samples, at least a subset of the samples should be quality controlled.

Basic QC is easily done using the microRNA QC PCR panel, using a single RNA input amount (the same volume of RNA for all samples). Samples with low RNA yield, signs of inhibition or hemolysis, can be excluded from further studies based on the results of the quality control by qPCR. For a list of assays included in the Basic QC, please see Table 3. Please refer to the miRCURY LNA™ Universal RT microRNA PCR, RNA spike-in kit and microRNA QC PCR panel manuals for details of how to interpret the QC results.

Assessing cellular contamination

The presence of cellular RNA species may disturb the "cell-free" biofluid microRNA profiling experiment resulting in a distorted and non-reproducible profile. In the case of serum or plasma, the circulating, or extracellular microRNAs (found within exosomes, microparticles, protein complexes, or complexed with HDL/LDL), are often considered the most interesting targets for microRNA profiling. An overrepresentation of RNA species from white or red blood cells in the profile may be an indication that cells have lysed at some point prior to the RNA isolation. This may be due to incomplete removal of white blood cells or platelets from the sample, and/or due to hemolysis.

Monitoring hemolysis in serum and plasma samples (or other biofluid samples that may be contaminated with blood) can be done by various spectrophotometric methods that measure free hemoglobin levels. However, if the RNA has already been extracted or the original sample is no longer available, an alternative strategy would be to compare the level of a microRNA highly expressed in red blood cells (hsa-miR-451a), with a microRNA unaffected by hemolysis (hsa-miR-23a-3p) (Blondal *et al.*, 2013). Blondal *et al.* found that delta Cq (miR-23a-3p - miR- 451a) is a good measure of the degree of hemolysis where values of more than five is an indicator of possible erythrocyte microRNA contamination, and a delta Cq of 7–8 or more indicates a high risk of hemolysis affecting the data obtained in human samples (the values are different in mouse and rat samples). A similar approach can be taken to identify contamination from cellular components in other biofluid samples, by identifying and monitoring the levels of cellular-derived endogenous microRNAs. For example, miR-142 is considered a marker for thrombocyte / cellular contamination.

It is important to note that many microRNAs in serum and plasma are not affected by hemolysis and therefore it may still be possible to detect disease associated microRNA biomarkers even from samples affected by hemolysis. However it is crucial to be aware of the possible effects on the microRNA profile when performing normalization and data analysis so that any systematic bias can be eliminated.

miRCURY™ microRNA QC PCR Panel

Rapid Quality Control for Biofluids.
Learn more at:
exiqon.com/qc-panel



Tips: How to avoid hemolysis

- Use good and consistent sample collection device throughout study (e.g. BD Vacutainer)
- Follow manufacturer's instructions!
- Avoid drawing blood from a hematoma
- Avoid frothing of the sample
- Make sure the venipuncture site is dry
- Avoid a probing, traumatic venipuncture
- Avoid prolonged tourniquet application or fist clenching
- Use correct size needle (~22 gauge)
- Vacuum tubes should be filled completely



> *Extended QC is recommended for challenging biofluids"*

Extended QC – challenging biofluid samples

When establishing a new protocol or working with a new or challenging type of biofluid e.g. urine or CSF, we recommend testing different RNA sample input amounts in the cDNA synthesis reaction (e.g. 1.0 µL, 2.0 µL, 4.0 µL and 8.0 µL in a 20 µL RT reaction). This dilution series is performed using assays for the RNA-spike-ins and a few microRNA assays (see Table 5) in order to confirm there is a linear relationship between sample input and signal, and identify a suitable RNA input amount for further studies. Samples containing PCR inhibitors will show dilution curves without the expected linear relationship between sample input and signal (Figure 12).

Performing an RNA dilution series

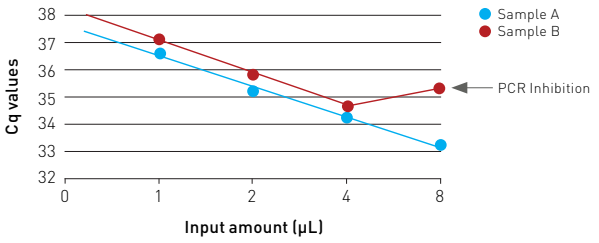


Figure 12: Performing an RNA dilution series can be used to determine that input amounts are within the linear range of the system, and to ensure a good level of signal, with no sign of inhibition (e.g. Sample A at 8 µL). Increasing the RNA volume to increase signal levels can have the opposite effect and result in less signal due to carry-over of more inhibitory compounds into the RT-PCR reaction (e.g. Sample B at 8 µL). Input amount refers to µL RNA in a 20 µL RT reaction.

The results of the dilution curve analysis should be used to assess the quality of the obtained RNA and the potential for inhibition of the RT or PCR reactions. If you wish to adjust the sample input volume in the RT reaction based on these results, this should be done with care, and for all samples in the study. As mentioned above, we recommend including the same volume of purified RNA in the RT for all samples. A dilution series also confirms that there is a linear relationship between the amount of RNA target and the signal obtained, which is essential for accurate microRNA quantification.

Good endogenous microRNAs for this Extended QC include the following microRNAs which are typically detected at medium to high levels and are listed in Table 6.

microRNAs detected or stably expressed in human biofluid samples

microRNA	Serum / plasma	Urine	CSF (exosomes)
hsa-let-7i-5p	*		
hsa-miR-23a-3p	✓		✓
hsa-miR-23b-3p		✓	✓
hsa-miR-30c-5p		✓	✓
hsa-miR-30e-5p	*		
hsa-miR-93-5p	✓		✓
hsa-miR-103a-3p	✓	✓	✓
hsa-miR-106b-5p	✓		
hsa-miR-107		✓	✓
hsa-miR-141-3p		✓	
hsa-miR-148b-3p	*		
hsa-miR-191-5p	✓		✓
hsa-miR-222-3p	*		
hsa-miR-423-5p/3p	✓		
hsa-miR-425-5p	*		✓
hsa-miR-484	*		

Table 6: Endogenous microRNAs typically detected or stably expressed in human biofluid samples. Often, one or more of these microRNA assays may be good for the normalization of expression data, but the stability of their expression needs to be determined in each sample set (see section on Normalization). MicroRNAs identified by Exiqon as stably expressed in human serum / plasma samples, based profiling thousands of serum / plasma samples from individuals with a range of different diseases as well as healthy individuals are indicated by asterisks.
* very often stably expressed in serum / plasma
✓ typically detected but not necessarily stably expressed

Once the optimal sample input has been established using the Extended QC, samples should be routinely quality controlled with a single sample input volume prior to microRNA profiling using the Basic QC (see the section Basic QC - standard human serum / plasma samples).



miRCURY LNA™ Universal RT microRNA PCR System

Highest sensitivity and linearity for Biofluids. Learn more at:

exiqon.com/mirna-pcr

9 microRNA qPCR analysis

Protocols optimized for biofluids

The quality control of RNA described above will indicate whether inhibitors in general are present in the RNA samples. Every step of the procedure has been optimized to maximize signal and minimize the effects of inhibitors (see Table 7 and the Instruction Manual for miRCURY LNA™ Universal RT microRNA PCR for serum / plasma samples at www.exiqon.com/mirna-pcr). It is recommended to perform cDNA synthesis in a larger reaction volume than for other samples, in order to minimize or alleviate the effect of smaller variations in inhibitor content between samples. If the amount of RNA input exceeds 20% v/v in the RT reaction, it is very important to monitor the samples for any signs of inhibition as described in the section Basic QC – standard human serum / plasma samples.

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 biofluid samples, the dilution factor of the cDNA is reduced relative to normal samples (see Table 7). Figure 13 shows the high reproducibility between different reverse transcription reactions on RNA from serum that 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 for biofluid samples.

Experimental set up using the miRCURY LNA™ Universal RT microRNA PCR System

Sample Type	Serum, plasma or other biofluids				
Panels or primer sets	miRNome panel I	miRNome panels I + II	Pick-&-Mix/Focus panels		Individual assays (<96)
			1-96 assays	97-192 assays	
Universal cDNA reactions per kit	16	8	64	32	64
Universal cDNA reaction volume (µl)	40	80	10	20	10
Dilution of cDNA in ExiLENT Master Mix	50x				40x
ExiLENT Master Mix consumption (µl)	2000	4000	5/assay		

Table 7: The miRCURY LNA™ Universal RT microRNA PCR System is optimized to maximize signal and minimize the effects of inhibitors present in biofluid samples. The experimental set up can be scaled to fit all project sizes, from full miRNome profiling to validation.

Excellent reproducibility between technical replicates from serum

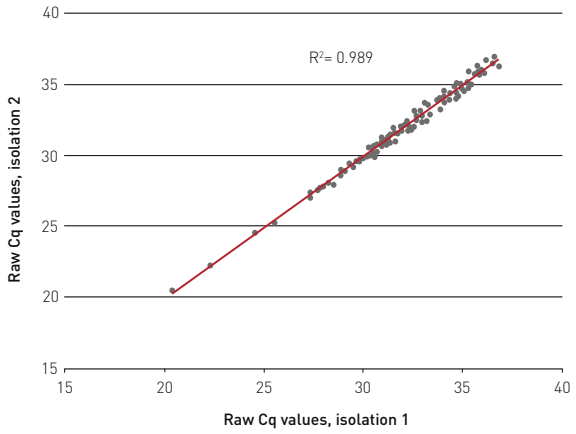


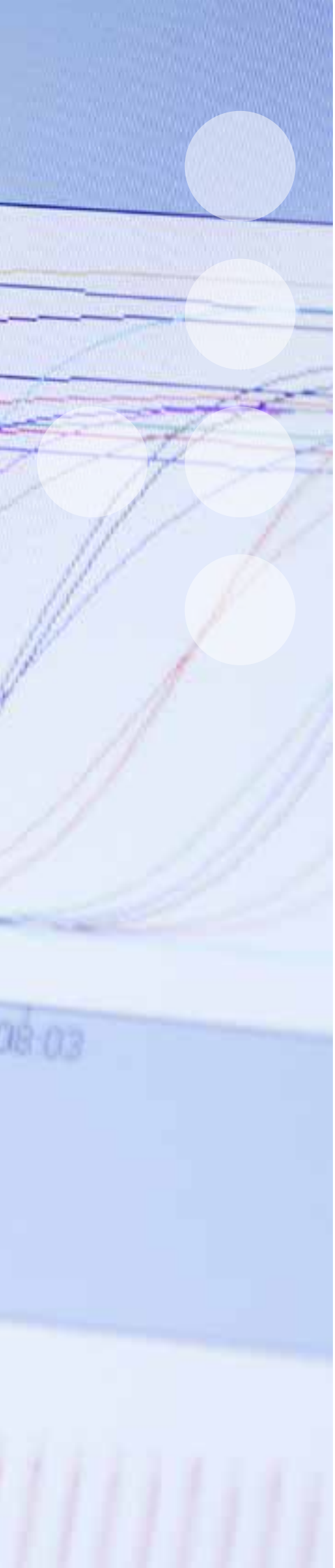
Figure 13: Two independent RNA isolations were performed from the same serum sample and a total of 742 microRNAs were profiled using the miRCURY LNA™ Universal RT microRNA PCR System. Raw Cq values from two separate RNA isolations are shown. The equivalent of 64 µL serum was used per RT reaction. Only microRNAs with Cq values below 35 have been included.

For a general overview of which PCR panels and services Exiqon offer for microRNA profiling in biofluids, please see Table 8.

Exiqon's products and services for microRNA profiling in biofluids

	Initial screening	Profiling	Validation
	Limited sample size:  **Identify subset of relevant microRNAs**	Medium sample size:  **Profile microRNAs & reference genes**	Large sample size:  **Validate differentially expressed microRNAs**
Serum / Plasma	**NGS Services, microRNA PCR Panels:** miRNome, Serum / Plasma Focus	**microRNA PCR Panels:** Serum / Plasma Focus, *Custom Pick-&-Mix	**microRNA PCR Panels:** *Custom Pick-&-Mix ***Individual assays**
Urine	**microRNA PCR Panels:** miRNome, Urine Exosome Focus, Toxicology Focus	**microRNA PCR Panels:** Urine Exosome Focus, Toxicology Focus, *Custom Pick-&-Mix	**microRNA PCR Panels:** *Custom Pick-&-Mix ***Individual assays**
CSF	**microRNA PCR Panels:** miRNome, CSF Exosome Focus	**microRNA PCR Panels:** CSF Exosome Focus, *Custom Pick-&-Mix	**microRNA PCR Panels:** *Custom Pick-&-Mix ***Individual assays**

Table 8: Overview of Exiqon's products and services for microRNA profiling in biofluids by qPCR and NGS. All PCR panels are available both as products and also through Exiqon's PCR Services. Pick-&-Mix PCR panels can be fully customized to include any number of selected relevant microRNAs. Assays to detect novel microRNAs* for validation of NGS data are available on Pick-&-Mix PCR panels, or as individual assays.



Sensitivity is defined by the limit of detection

Biofluids contain very low amounts of RNA, so it is essential to use a highly sensitive detection system. It is a common misconception that lower Cq values from qPCR assays mean higher sensitivity. A Cq value has to be compared either to another Cq value in another sample (leading to a calculation of relative expression) or to a standard curve where known amounts of the target has been analyzed (leading to absolute quantification). In addition, a Cq value has to be compared to the Cq value obtained when the target is not present (i.e. the background signal), usually in RT and PCR control reactions.

In order to find out what the true sensitivity of a qPCR assay is, it is necessary to run a dilution series of known input amounts including a negative control sample where the template is not present (e.g. a blank purification where water is included instead of biofluid). The limit of detection is usually defined as the last point on that curve where the curve is still linear (any point that does not lie on the linear regression curve must be excluded). An example of a dilution series used to compare the sensitivity of different qPCR assays is shown in Figure 14.

Validated PCR assays

Over 1,400 of the primer sets available in Exiqon's miRCURY LNA™ Universal RT microRNA PCR portfolio have been wet lab validated (Figure 15) to ensure that they meet strict performance criteria, and over 95% are sensitive down to less than 10 RNA copies in the PCR reaction Below 10 copies, variation in Cq values can be affected simply by stochastic variance in the number of template molecules pipetted into the qPCR reaction.

Performing an RNA dilution series

A dilution series is also used for efficiency calculation (an alternative is LinRegPCR), or for absolute microRNA quantification. When performing a dilution series we recommend an RNA dilution series (rather than a cDNA dilution series) as an RNA dilution series will also take into account any factors limiting the RT reaction, which can often be the limiting step for sensitivity, rather than the subsequent qPCR. We recommend diluting the RNA sample into carrier RNA e.g. bacteriophage MS2 total RNA to avoid loss of RNA due to plastic adhesion.

The best combination of sensitivity and specificity

Biofluid samples may also contain fragments or degradation products of other RNA species, so it is important to use a detection method which is highly specific, and able to discriminate the microRNAs from other RNA fragments, as well as other closely related microRNA family members. The miRCURY™ LNA Universal RT microRNA PCR System is the only platform that offers both high sensitivity and high specificity. Read more about miRQC, the largest study comparing microRNA profiling platforms, published in Nature Methods (Mestdagh et al.,2014).

Sensitivity is defined by the limit of detection, not the Cq value

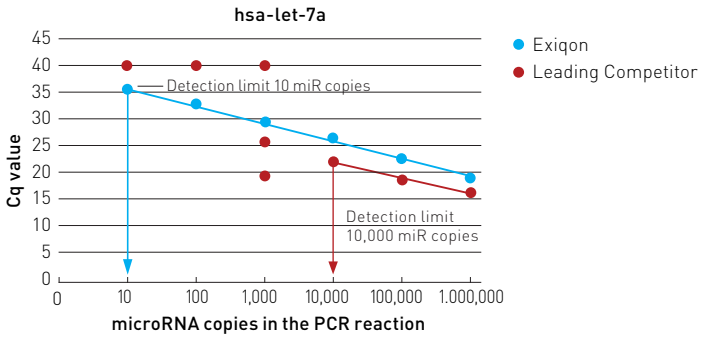


Figure 14: The sensitivity of microRNA PCR assays are compared using a dilution series of synthetic microRNA template in order to identify the limit of detection (the last point where the curve is still linear). The figure shows a dilution series of hsa-let-7a, an example where Cq values from one assay (competitor) are lower than those of another assay (Exiqon). Although the Cq values of the competitor assay are lower, the competitor assay is not as sensitive as the Exiqon assay, as the linear regression curve from the dilution series can only be extended to 10,000 copies and not 10 copies.

Excellent sensitivity and linearity of the miRCURY LNA™ microRNA PCR assays

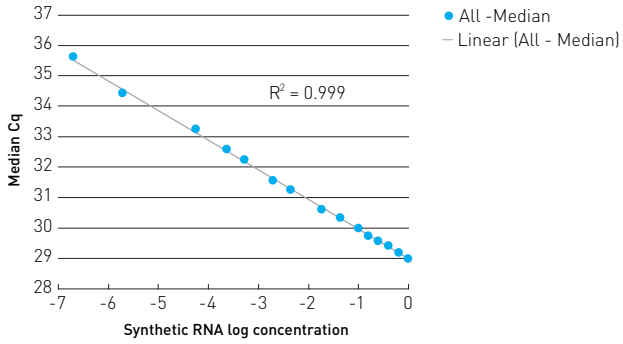


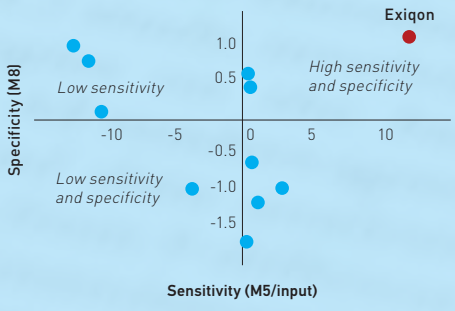
Figure 15: miRCURY LNA™ Universal RT microRNA PCR assays demonstrate excellent sensitivity and linearity over a wide range of template concentrations, including low microRNA concentrations found in biofluids. A linear relationship between signal and template concentration is essential for accurate microRNA quantification.
Study details: A pool of synthetic templates for 647 microRNAs was subjected to serial dilution (15 – 1,500 copies of each template RNA in the PCR reaction) and then assayed by RT-qPCR.
The median Cq value for all assays was then plotted against template concentration, demonstrating linearity of the platform over a wide range of template concentrations. The dilution series was performed in a background of MS2 bacteriophage total RNA.

Exiqon performs best

miRQC – the largest microRNA benchmarking study to date

In a new large study published in Nature Methods, Pieter Mestdagh *et al.* compare the performance of commercially available of microRNA profiling platforms in key areas. Exiqon is the only platform to be a top performer in all areas, combining both high sensitivity and specificity.

To find out more information, please go to:
exiqon.com/mirqc



Data QC

In challenging samples such as biofluids, characterized by low levels of microRNAs combined with the presence of PCR inhibitors, the Cq values obtained will often be at the high end (in the range 30 – 35). This is acceptable as long as the signal can be easily discerned from the background. Some assays may be more sensitive to inhibition than others, so it is a good idea to monitor efficiency on the actual study data. To ensure reliable data points, it is recommended to check the following for quality control purposes:

- Dissociation curve with a single clean peak indicating a unique amplification product
- Amplicon melting temperature is consistent for the same assay between samples
- Amplification efficiency (using LinRegPCR or similar algorithm, Ruijter et al., 2013) shows no signs of PCR inhibition
- Sample Cq is clearly discerned from any background (e.g. at least 5 Cq below negative control / blank purification)

> *“SYBR Green based qPCR systems enable important quality control of the data by melting curve analysis”*

It is worthwhile examining the microRNA profiles to look for any signs of variation in the dataset that might result from technical differences in sample handling or processing. In our experience the microRNA profile may also reveal information about physiological state, for example high levels of miR-122 can often be found in the serum / plasma of individuals subject to liver stress as a result of certain treatments.



10 Normalization

Methods for normalization

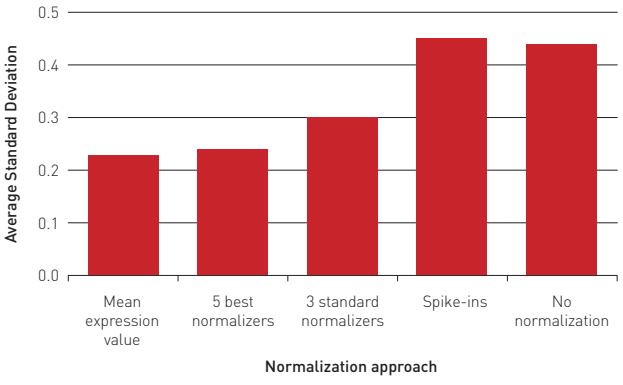
The purpose of normalization is to remove technical variation in data which is not related to the biological changes under investigation. Proper normalization is critical for the correct analysis and interpretation of results. The most commonly used methods for normalization are:

1. To use the mean expression value of all commonly expressed microRNAs in a given sample as normalization factor (Mestdagh et al., 2009). This is usually the best approach when analysing PCR panels containing a larger number of microRNA assays.
2. To identify and use stably expressed reference genes.

> *"Stably expressed endogenous microRNAs are recommended for normalization"*

Exiqon's GenEx software supports both of these approaches and it is recommended to investigate which of these methods will provide the best normalization of the data in question. Normalization using spike-in RNAs is not recommended as this approach does not correct for many aspects of technical variation e.g. variation in endogenous RNA content (Figure 16).

Normalization to the mean expression value is the best approach for normalization of microRNA qPCR data from biofluids



Normalization approach	Normalization to the average of	Conclusion
Mean expression value	90 microRNAs detected in all samples	Superior method for normalization
5 best normalizers	the 5 most stably expressed microRNAs in this study: hsa-let-7i-5p hsa-miR-222-3p hsa-miR-425-5p hsa-miR-93-5p hsa-miR-152	Works well if good normalizers can be selected
3 standard normalizers	3 microRNAs selected without prior knowledge: hsa-miR-423-3p hsa-miR-103a-3p hsa-miR-191-5p	Acceptable, if information on good normalizers is lacking
Spike-ins	UniSp2, UniSp4 and UniSp5	Does not perform well if RNA isolation performed in multiple batches

Figure 16: Normalization was performed using four different approaches. Normalization to the mean expression value was most successful in minimizing standard deviation across samples. RNA isolation was performed from 120 plasma samples in duplicate, adding RNA Spike-ins to the lysis buffer. MicroRNA profiling was performed on all RNA samples using miRCURY LNA™ Universal RT microRNA PCR Human Panels I + II (742 microRNAs).

Normalize to endogenous microRNAs

We recommend to use stably expressed endogenous microRNAs as reference genes, rather than larger small RNA species, e.g., 5S, U6 and snoRNAs, that are sometimes used for normalization in other sample types. The main reason for this is that the larger RNAs have a different biogenesis pathway and may not be secreted or protected in biofluids in the same way as microRNAs are. Larger RNAs may also behave differently during RNA purification.

Another difference is that often the number of microRNAs detected in biofluid samples is rather low. 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 detected microRNAs is below 20-50.

Cellular contamination can affect normalization

When using mean expression values for normalization, be aware that cellular contamination or hemolysis can result in release of cellular RNA which may affect the overall microRNA content thus impacting the mean expression value in affected samples. Hence, it is crucial to monitor RNA samples for signs of cellular contamination or hemolysis (see the section Basic QC – standard human serum / plasma samples) and ensure that the microRNAs selected for use as normalizers are not affected by hemolysis (Pritchard et al., 2012).

Selecting reference genes

When using stably expressed reference genes for normalization, it is recommended to test 5-6 candidates (reference genes) before setting up the actual microRNA expression analysis, in order to identify 2-3 stably expressed reference genes for normalization. These candidates should be chosen from a selection of genes that are expected to be stably expressed over the entire range of samples being investigated.

When working with serum / plasma, urine or CSF, the candidates are typically chosen based on the literature or pre-existing data (e.g. qPCR panel screening). Several microRNAs are available on Exiqon's PCR Panels as candidate reference genes. These are typically medium to highly expressed and may be stably expressed, but their use as reference genes needs to be evaluated on a study-to-study basis. Exiqon have identified certain microRNAs which are often stably expressed in serum / plasma (Table 6).

Recommended microRNAs for normalization in serum / plasma

See Table 6, page 25

Challenges particular to biofluids

Certain treatments can result in dramatic changes in the overall microRNA content of biofluid samples, e.g. drug-induced toxicity. In these situations it may be very challenging to identify stably expressed microRNAs, and the mean expression value of all microRNAs may not be stable either, so alternative strategies such as use of microRNA ratios may need to be considered.

Urine samples can pose additional challenges for normalization. There can sometimes be large variation in RNA concentration between urine samples (depending on the time of day, state of hydration as well as disease state), so care should be taken when selecting stable reference genes, and it may also be necessary to normalize to urinary volume or creatinine.

For other biofluid samples we recommend to empirically determine the best reference microRNAs by doing an initial pilot study and use geNorm or NormFinder from the GenEx software to identify the best candidates. For further details regarding experimental set up and data analysis of microRNA qPCR experiments we refer to our microRNA qPCR guidelines available here: http://www.exiqon.com/ls/Documents/Scientific/miRNA-qPCR-guidelines.pdf

GenEx Software

For fast and easy data analysis.
Learn more at:
exiqon.com/qpcr-software

11 microRNA sequencing





Exiqon NGS Services – microRNA Biofluids

Reliable microRNA sequencing from Biofluids. Learn more at:

exiqon.com/ngs-services

Ensuring reliable biofluids NGS data

Obtaining sensitive and reliable microRNA NGS data from biofluid samples is challenging, so it is essential to use protocols for RNA isolation, QC, library preparation and data analysis which are optimized specifically for biofluid samples with low RNA content. As with all microRNA sequencing projects, optimized library size selection is crucial to maximize the number of microRNA reads. All of these protocols form an integral part of Exiqon's microRNA biofluids NGS Service. It is important to perform a thorough QC of the sequencing data. Exiqon's NGS Service for microRNA biofluids includes analysis of >50 RNA spike-ins added during RNA isolation to monitor the reproducibility and linearity of the sequencing (Figure 17).

The number of reads measured by Next Generation Sequencing for a particular sequence is not directly related to its abundance, i.e. NGS is not used for absolute quantification. Therefore NGS data, like microarray and qPCR data, should be normalized and analyzed to identify relative differential expression between samples. In addition, measurement of a particular microRNA by NGS is not independent of other microRNAs, so if a particular microRNA is highly abundant, this can reduce the number of reads available for other microRNAs. Exiqon Services take care to ensure that sequencing depth is sufficient to allow accurate analysis of even lowly expressed microRNAs found in biofluids.

Setting a TPM threshold

Ideally, the number of microRNA reads across the different samples in the study should be similar in order for the samples to be comparable. The normalization procedure used should account for any differences in read number between samples. Calculating the TPM (Tags per Million mapped reads) is a means of normalizing against the total number of mapped reads. Reads detected at less than 1 TPM could represent lowly expressed microRNA or artifacts. In our experience, reads below 1 TPM are usually difficult to validate by other techniques e.g. qPCR, and their detection is normally not improved by increasing the sequencing depth. Setting a TPM threshold is a useful way to focus on the most reliable microRNA reads.

Excellent reproducibility of microRNA Sequencing in Biofluids

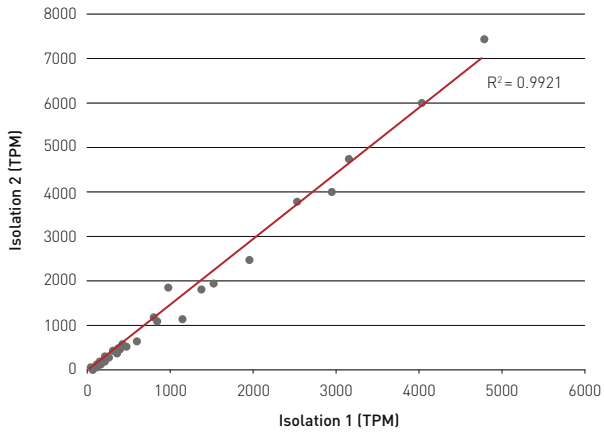


Figure 17: Excellent reproducibility of Exiqon Services' microRNA Biofluids NGS workflow from RNA isolation to sequencing. Two independent RNA isolations were performed using the same pool of serum / plasma samples, followed by two independent library preparations and sequencing runs. This graph shows the excellent reproducibility as reported by the correlation between > 50 RNA spike-ins, added during the RNA isolation. TPM = Tags per Million mapped reads.

12 Additional reading and resources

Find more information in our biofluids reading room: exiqon.com/biofluids

Read more about liquid biopsies and circulating microRNA biomarkers in our biomarker reading room: exiqon.com/mirna-biomarkers

The following publications can be downloaded from www.exiqon.com/rna-isolation
- Instruction Manual for miRCURY™ RNA Isolation Kit – Biofluids
- Instruction Manual for miRCURY™ Exosome Isolation Kit – Serum & Plasma
- Instruction Manual for miRCURY™ Exosome Isolation Kit – Cells, Urine & CSF

The following publications can be downloaded from exiqon.com/mirna-pcr
- Instruction Manual for microRNA LNA™ Universal RT microRNA PCR serum and plasma
- Instruction Manual for miRCURY™ RNA Spike-in kit
- Instruction manual for miRCURY™ microRNA QC PCR Panel
- Pitfalls and recommendations for microRNA expression analysis using qPCR – guidelines to setting up microRNA qPCR
- Urine microRNA profiling to discover biomarkers for nephrotoxicity
- microRNA biomarker discovery for asthma in a mouse model
- SDS template files for ABI instruments
- Exiqon GenEx software

Data analysis software and tools are described in more details at **exiqon.com/mirna-pcr-analysis**

The following publications can be downloaded from exiqon.com/services
- Example Final Reports for Exiqon's PCR and NGS Services
- Guidelines for Exiqon's PCR and NGS Services
- Sample Submission Forms

Challenges and Solutions for microRNA profiling in biofluids:
http://www.exiqon.com/ls/Pages/news/biofluid-presentation.aspx

EDRN Standard Operating Procedures:
Plasma:
http://edrn.nci.nih.gov/resources/standard-operating-procedures/standard-operating-procedures/plasma-sop.pdf
Serum:
http://edrn.nci.nih.gov/resources/standard-operating-procedures/standard-operating-procedures/serum-sop.pdf

exiqon.com/e-talk displays presentations about profiling microRNAs in biofluid samples using the miRCURY LNA™ Universal RT microRNA PCR System and Next Generation Sequencing.

MIQE & qPCR iBook (May 2015) available free from iTunes

13 References

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14 Exiqon Products for Biofluid microRNA Analysis

miRCURY™ Exosome and RNA isolation kits

exiqon.com/rna-isolation

Sample type	Recommended exosome isolation kit	Recommended RNA isolation kit
Serum / Plasma	miRCURY™ Exosome Isolation Kit – Serum and plasma	miRCURY™ RNA Isolation Kit – Biofluids (10 or 50 rxns)
Cell culture media, urine, CSF or other biofluids	miRCURY™ Exosome Isolation Kit – Cells, urine and CSF	miRCURY™ RNA Isolation Kit – Cell and Plant

RNA Quality Control

RNA Spike-In Kit | **exiqon.com/rna-spike-in-kit**
miRCURY™ microRNA QC PCR Panel | **exiqon.com/qc-panel**

LNA™ PCR Primer sets to detect the RNA spike-ins and endogenous microRNAs are also available as individual assays in tubes.

miRCURY LNA™ microRNA PCR reagents

Universal cDNA Synthesis Kit II, 8 – 64 rxns | **exiqon.com/mirna-pcr-kits**
ExiLENT SYBR® Green Mastermix, 2.5 or 20 ml | **exiqon.com/mirna-pcr-kits**
microRNA PCR Starter Kit | **exiqon.com/mirna-pcr-starter-kit**

miRCURY LNA™ microRNA PCR Individual Assays

miRCURY LNA™ microRNA PCR primer sets | **exiqon.com/mirna-pcr-primer**
Custom miRCURY LNA™ microRNA PCR primer sets | **exiqon.com/custom-PCR**
Reference Gene miRCURY LNA™ PCR primer sets | **exiqon.com/mirna-pcr-controls**

miRCURY LNA™ microRNA PCR panels, Ready-to-Use (1 rxn per well)

miRNome panels (human, mouse&rat) (752) | **exiqon.com/mirna-pcr-panels**
Serum / Plasma Focus panels (179) | **exiqon.com/serum-plasma-miRNA-qPCR**
Urine Exosome Focus panels (87) | **exiqon.com/exosome-mirna-pcr**
CSF Exosome Focus panels (87) | **exiqon.com/exosome-mirna-pcr**
Toxicology Focus panels (87) | **exiqon.com/toxicology-mirna-pcr**
Custom Pick-&-Mix panels (any) | **exiqon.com/pick-and-mix**

The numbers in brackets indicate number of human microRNA assays present on each panel. Number of assays for other species may vary. Each panel is available in a range of different types of plates for compatibility with all major real-time PCR instruments.

miRCURY LNA™ microRNA PCR Bulk Plates (200 rxns per well)

microRNA qPCR Custom Plate | **exiqon.com/mirna-pcr-primer**

qPCR Data Analysis

Exiqon GenEx qPCR analysis software | **exiqon.com/qpcr-software**



15 Exiqon Services for Biofluid microRNA Analysis

Exosome and RNA Isolation Services

High quality isolation of exosomes and/or RNA from a blood serum / plasma, urine, CSF and other biofluids upon request | **exiqon.com/rna-isolation-services**

microRNA Biofluids Sequencing Services

Expert microRNA sequencing services developed for biofluid samples | **exiqon.com/ngs-services**

microRNA qPCR Services

A complete microRNA qPCR profiling service tailored to biofluid samples using Exiqon's LNA™-enhanced PCR system | **exiqon.com/microRNA-pcr-services**

Workflow for Exiqon's microRNA Sequencing Service for Biofluids

1. Consultation and experimental design
Tailored experimental design to suit your research needs and budget

2. Sample submission and RNA Isolation
500 µl serum or plasma
Exiqon Services proprietary RNA isolation protocol (including >50 RNA Spike-ins)

3. RNA sample quality control (QC)
PCR QC to monitor RNA isolation efficiency, inhibitors, hemolysis and detect outliers

4. Library preparation
Library generation, quality control and quantification (Bioanalyzer and qPCR)
Size selection of the library (for microRNA and small RNA)

5. Sequencing
Sequencing using the Illumina technology (MiSeq, NextSeq or HiSeq)

6. Data analysis and interpretation
QC of sequencing data (using >50 Spike-ins)
Filtering, mapping, normalization and differential expression analysis
GO Enrichment analysis and Biological Interpretation

7. Report and final consultation
An extensive report is accompanied by follow-up scientific discussion

Workflow for Exiqon's microRNA PCR Services for Biofluids

1. Consultation and experimental design
Tailored experimental design to suit your research needs and budget

2. RNA sample submission or RNA isolation
Submit isolated RNA or 250 µl* biofluid
Exiqon can isolate your RNA for qPCR analysis

3. RNA sample quality control (QC)
PCR QC to monitor RNA isolation efficiency, inhibitors, hemolysis and detect outliers

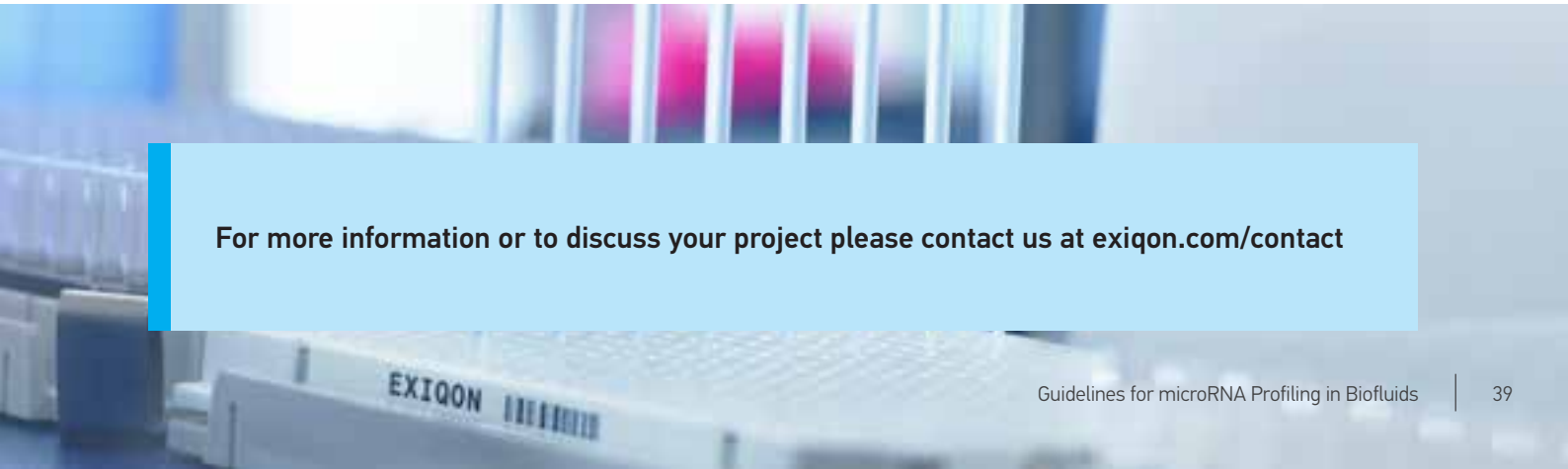
4. RNA qPCR
The qPCR system combines a Universal RT reaction with LNA™-enhanced PCR primers, which results in unmatched sensitivity and specificity
Choose from Exiqon's range of microRNA PCR panels, or a custom selection of microRNAs

5. PCR quality assessment
Rigorous quality assessments of the PCR data are performed before proceeding with the data analysis

6. Normalization and data analysis
Normalization along with a thorough data analysis appropriate for the experiment at hand is performed

7. Report and final consultation
An extensive report is accompanied by follow-up scientific discussion

* Only 75 µl is required for rodent serum / plasma.



For more information or to discuss your project please contact us at exiqon.com/contact

For more information go to: exiqon.com/biofluids

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