Guidelines for Profiling Biofluid miRNAs

Maximize your miRNA sequencing and qPCR success from liquid biopsy samples, including blood, serum/plasma, urine, CSF and exosomes

Sample to Insight

QIAGEN

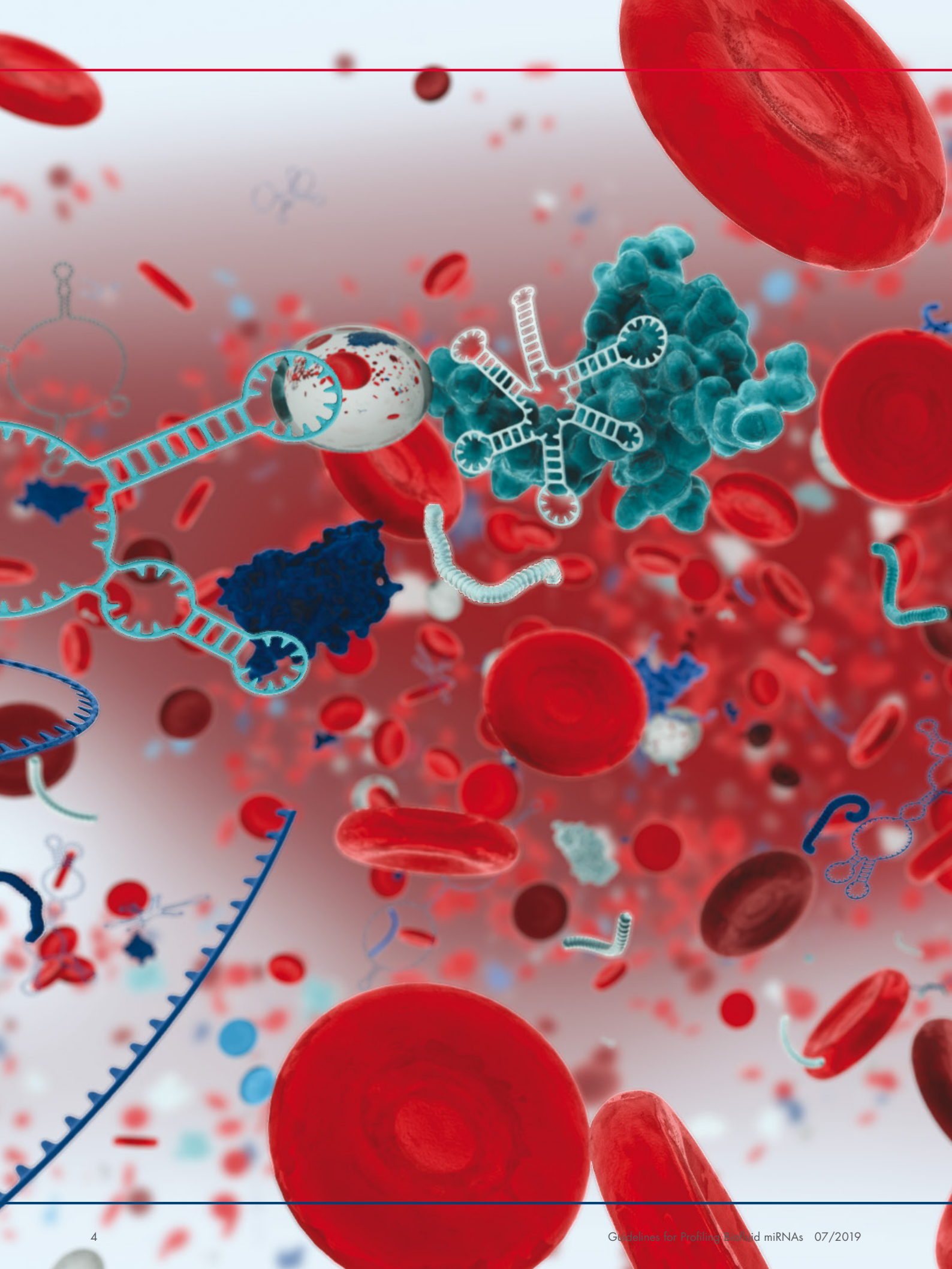
Introduction

MicroRNAs (miRNAs) in liquid biopsies hold great promise as minimally invasive biomarkers for a multitude of diseases and biological processes. These short, regulating RNAs have wide-ranging biological potential, are limited in number and are relatively stable in liquid biopsy samples such as serum/plasma, urine and other biofluids. However, miRNA profiling in biofluid samples is challenging because biofluids contain low levels of RNA, high levels of inhibitors and are susceptible to many preanalytical variables.

To address these challenges, we have optimized sample handling, preparation and quality control methods and developed highly sensitive and accurate miRNA detection techniques, based on many years' experience in miRNA profiling and biomarker discovery and validation in biofluid samples. These guidelines provide important information and tips to ensure successful miRNA profiling using next-generation sequencing or PCR-based techniques. Although the guidelines focus on miRNA profiling in blood, serum/plasma, urine and exosomes, they also contain useful information for working with other biofluids.

Challenge	Solutions
Limited amount of RNA	• Isolate exosomes or extracellular vesicles to enhance signal (urine and CSF samples) • Optimize RNA isolation protocol • Quantify blank purification to assess any background signals • Monitor RNA isolation efficiency (using RNA spike-ins for PCR QC) • Optimize NGS library preparation • Use a highly sensitive detection method
Undesired components (e.g., inhibitors of downstream enzymatic reactions)	• Avoid using heparin tubes • Use a column- or bead-based RNA isolation method • Monitor RNases and inhibitors (using RNA spike-ins for PCR QC) • Use a highly specific detection method (to discriminate tRNA fragments) • Perform sequencing QC (reproducibility of spike-ins) • When establishing a new protocol, confirm linear relationship between sample input and signal • Use an NGS library preparation method that blocks hY RNA
Cellular contamination & hemolysis	• Ensure consistent and optimal sample collection • Spin or pre-filter samples to remove any remaining cells, ideally before freezing • Compare data to reference range • Monitor hemolysis indicator (PCR QC) or absorbance of oxyhemoglobin (414 nm)
Pre-analytical variables	• Optimize experimental design and include biological replicates • Control sources of technical variation (e.g., collection sites)
Normalization	• Normalize to mean expression value for call rates >20–50 miRNAs, or alternatively, to internal normalizers (stable endogenous miRNAs) • For urine from small animal models, consider normalization to urinary volume/creatinine • Consider miRNA ratios to normalize dramatic overall changes in miRNA content (e.g., drug-induced toxicity)

Table 1. Overview of the main challenges and solutions for miRNA profiling of biofluid samples using NGS or qPCR.



Sample Collection and Stabilization

General biofluid recommendations

Profiling of miRNAs in biofluid samples holds great promise due to their potential use as minimally invasive biomarkers for various diseases. Still, a number of challenges are associated with miRNA profiling. Compared to tissues, cells or whole blood, the concentration of miRNAs in cell-free biofluids is very low, making their detection and quantification difficult. Several important pre-analytical factors should be considered: sample collection, storage, hemolysis and platelet content. In particular, the type of blood collection tube used, the time interval between blood draw and preparation of plasma or serum, and procedures such as pre-centrifugation or filtration to remove residual cells and cell fragments can greatly impact the results.

Other RNA isolation-related factors to consider include:

- Compartmentalization: while most of the miRNA in blood is found in platelets, red and white cells, cell-free miRNA is usually present within exosomes and other extracellular vesicles (EVs), or outside of EVs in ribonucleoprotein complexes containing Argonaute (e.g., Ago2 in human samples) or other proteins that protect the miRNA from endogenous RNases.
- Plasma/serum starting volume: we recommend using 200 µl, as larger volumes potentially lead to higher risk of inhibition.
- RNA integrity: while RNA inside EVs is well-protected from endogenous RNases, it is crucial to immediately inactivate RNases while lysing EVs during the RNA isolation process to maintain the integrity of the RNA.

Removal of residual cells & cell fragments & storage conditions

Once collected, serum, plasma, urine and other biofluid samples should be centrifuged for 5–15 minutes at 3000 *x g* or prefilter, e.g., using a 0.8 µm filter to remove residual cells and debris. If debris cannot be removed immediately, samples may be stored at 2–8°C for a maximum of 24 hours. Cells must be removed prior to freezing, as the cells will otherwise lyse and release their miRNA content into the cell-free biofluid. Isolation of RNA or exosomes may be carried out immediately after cell removal, or alternatively, the cell-free samples can be stored at –20°C (for short-term storage) or –80°C (for long-term storage).

»Before freezing, biofluid samples should be centrifuged to remove cell debris«

Blood serum & plasma

RNA isolated from both plasma and serum has been successfully used for biomarker discovery, but there are some subtle differences in interpretation. Typically, slightly higher signals are obtained from plasma, as shown in Figure 1. Our extended comparisons of normal serum and plasma samples indicate that the variation within a properly sampled serum dataset is less than within a corresponding plasma dataset. However, preparation of plasma samples involves fewer procedural variations, such as differences in coagulation times and temperature associated with serum preparation. Thrombocyte contamination and release of vesicles from platelets during coagulation can also increase unwanted background in serum.

Minimizing pre-analytical variables

Collection of whole blood is the first step in preparing both plasma and serum, and certain precautions must be taken to ensure successful analysis. To avoid hemolysis during phlebotomy, it is important to ensure that the blood is drawn by experienced personnel. Preserving the RNA profile both during and after blood collection is crucial for accurate miRNA analysis in plasma and serum samples. To avoid technical variation, we recommend using the same type of high-quality collection tubes for all samples within a study and standardizing the sample handling workflow, especially with respect to time between blood draw and preparation of serum or plasma.

PreAnalytiX provides high quality blood collection tubes, which minimize technical variations by design. For isolation of cellular miRNA from whole blood, the PAXgene® Blood RNA Tubes protect RNA molecules from degradation and completely lyse all cells in the sample to minimize ex vivo changes in gene expression due to gene induction and downregulation. Circulating cell-free miRNA can be isolated from plasma generated from blood collected in PAXgene Blood ccfDNA Tubes, which efficiently stabilize red blood cells and prevent apoptosis of leucocytes. (1)

To obtain optimal results from archived samples, care should be taken to select only those samples that have been collected and processed according to the same protocol. For multi-center studies, it should be considered that different sites may use different equipment and procedures for sample collection

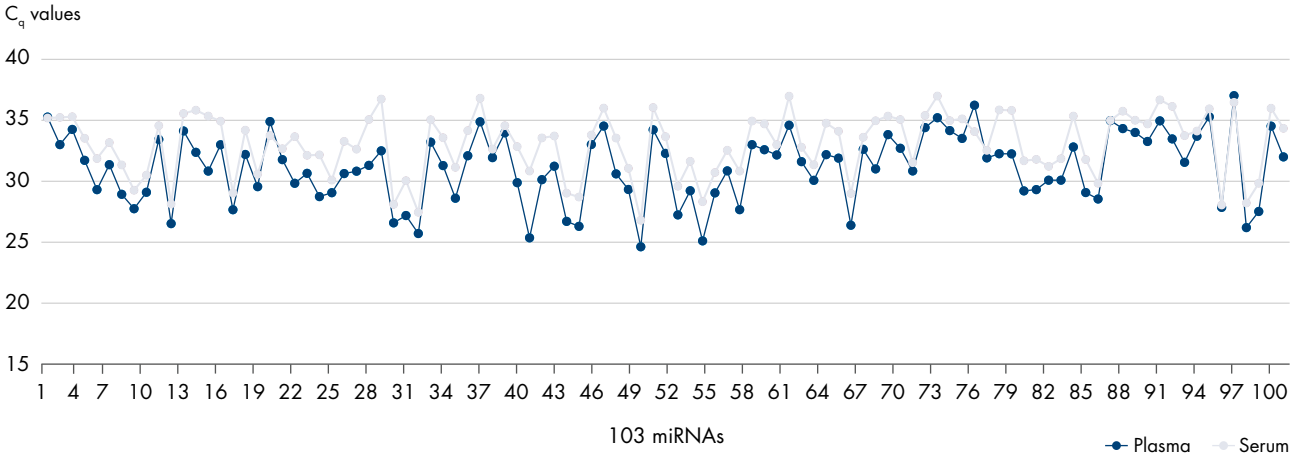


Figure 1. miRNA profiles from serum and plasma are very similar. Comparison of C_q values obtained for 103 of the most commonly expressed miRNAs in serum and plasma, profiled using the miRCURY® LNA® miRNA PCR System.

Tips for avoiding hemolysis

- Use a recommended sample collection device consistently throughout the study (e.g., PAXgene Blood ccfDNA Tubes)
- Always follow the manufacturers' 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)
- Fill vacuum tubes completely

Avoiding cellular RNA contamination

During all steps from blood collection to preparation of serum and plasma, care should be taken to prevent cell lysis to avoid contamination with RNA from intact cells, for example by hemolysis. This may interfere with or inhibit detection of subtle changes in the non-cellular miRNA profile.

Sample handling & pre-treatment

For guidelines on collecting and preparing serum and plasma, we recommend following the Early Detection Research Network's (EDRN) standard operating procedures, as published by the National Cancer Institute (NCI) (2) and the technical specifications CEN/TS 16835-1 & 3:2015 (3) published by the European committee for standardization.

»Consistent and standardized sample collection and handling are essential«

Blood collection tubes

Both PAXgene Blood ccfDNA Tubes as well as BD Vacutainer® tubes have been successfully used in our laboratories, but other tube types may also be used. We recommend only using blood collection tubes containing cell stabilization reagents that do not chemically modify biomolecules. In a recent review article, Groelz et al. (4) summarized the properties of all widely available stabilization tubes used in the field. If no dedicated blood stabilization can be used, EDTA and citrate are recommended for use as anticoagulants, whereas heparin is known to inhibit downstream enzymatic steps such as cDNA synthesis and PCR and should therefore be avoided (Figure 2 and Figure 3). Heparin should be avoided in all processing steps; however, if this is not possible, QIAGEN has developed a protocol to remove heparin from blood, serum or plasma samples or from isolated RNA preparations. Contact QIAGEN technical support for more details.

Even collection tubes and anticoagulants recommended for use with RNA isolation may influence the representation of individual RNA transcripts (Figure 3), so it is strongly recommended to use the same collection tubes throughout a study.

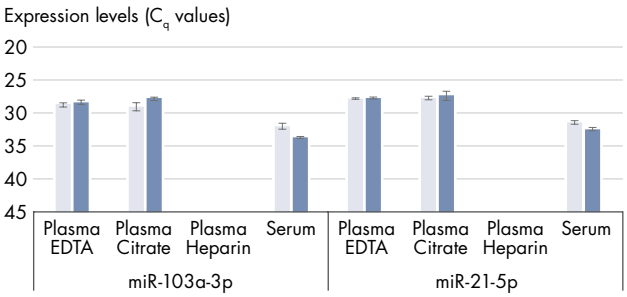


Figure 2. Heparin tubes are not suitable for miRNA analysis. Suitability of different blood preparations for miRNA PCR. Real-time PCR for miR-103a-3p and miR-21-5p was performed using triplicate RT reactions on total RNA purified from either EDTA-plasma, citrate-plasma, heparin-plasma, or serum. Average C_q values for each triplicate are shown, demonstrating robust amplification from all sources except heparin-plasma.

Figure 3. While a wide variety of blood collection tubes is compatible with RNA isolation procedures, representation of individual transcripts may differ. Use of heparin tubes is not recommended. RNA was isolated using the exoRNeasy Maxi Kit.

Processing time from blood collection to plasma/ serum preparation

Blood samples should be processed directly after collection if no cell stabilization tube is used. However, if they cannot be centrifuged immediately after collection (or, in case of serum preparation, after the clotting time), blood samples should be stored at room temperature for a maximum of 4 hours. Storage at 4°C as stated in the EDRN standard operating procedures or in the CEN/TS may result in thrombocyte lysis, leading to contamination of the cell-free miRNA profile. As mentioned previously, it is important to process all samples in the same way to avoid introducing any technical variation.

affected by hemolysis. (5) Residual platelets and micro-particles can also affect the miRNA profile. They can be effectively removed by centrifugation even from archived samples. (6)

Influence of pre-handling steps

Centrifugation or filtration steps are essential to remove cell fragments from plasma prior to isolation of extracellular vesicles or vesicular RNA (refer to section "Removal of residual cells & cell fragments & storage conditions" on page 5 for more details). However, these pre-handling steps may also affect the representation of RNA from different classes of vesicles. The effect on vesicle concentration and

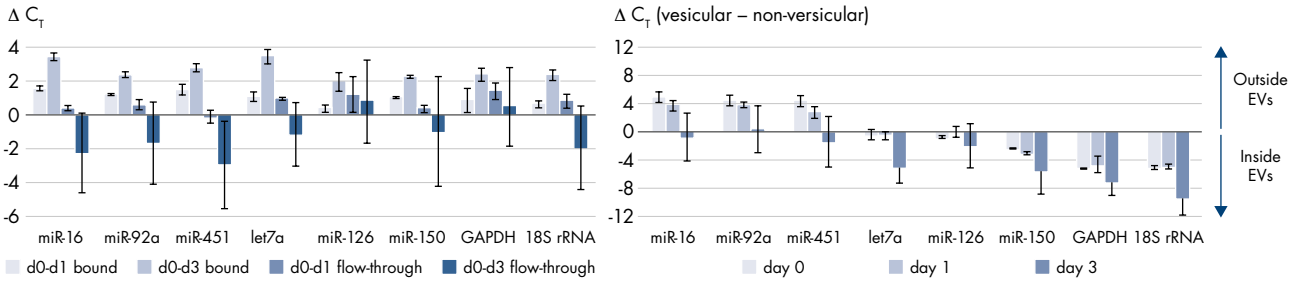


Figure 4. Effect of blood storage time prior to plasma preparation on abundance of selected miRNAs. Blood was either processed to plasma within <2 h after collection or kept at room temperature for 1 or 3 days. RNA from within EVs was isolated using the exoRNeasy Midi Kit, non-vesicular RNA was isolated from the flow-through of the EV-binding exoEasy columns using the miRNeasy® Serum/Plasma Kit, and samples were subsequently quantified by qPCR. RNA outside EVs increases within the first day after blood draw and decreases thereafter. RNA inside EVs increases over time, presumably due to continued release of EVs by blood cells, increasing unwanted background.

Storage of blood prior to plasma preparation can also lead to the release of additional vesicular RNA from blood cells, potentially increasing background signals. In contrast, cell-free, non-vesicular RNA decreases after prolonged blood storage (unless stabilization tubes are used), resulting in a major shift in the ratio of vesicular versus non-vesicular RNA over time (Figure 4).

Spectrophotometric measurement provides a quick and low-cost method to screen for hemolysis in serum and plasma samples. Oxyhemoglobin displays a distinct absorbance peak at 414 nm, which can be used to identify samples

size distribution is not proportional to the effect on RNA representation, indicating that RNA is not evenly distributed within vesicles (Figure 5).

More than 90% of plasma RNA content can be removed by filtration through a 0.8 µm filter, indicating that this RNA originates from cells and cell fragments that remain after conventional plasma generation (centrifugation at 1900 *x g*). The choice of centrifugation speed and filter pore size also affect the representation of RNA transcripts, even though particle concentration and size distribution are not significantly affected, except when the smallest filter pore

size (0.1 µm) is used (Figure 5 and Table 2). We generally recommend centrifugation at 3000 *x g* or filtration through a 0.8 µm filter, unless the goal is to deplete the samples of microvesicles (refer to section "Exosomes & other extracellular vesicles" on page 12).

	Particle size mean (nm)	Particle size D50 (nm)	Particle concentration (per ml plasma)
3000 *x g*	143.3	133.7	5.34e10
16,000 *x g*	162.2	161.3	4.32e10
3000 *x g*, 0.8 µm	159.2	155.6	4.80e10
3000 *x g*, 0.2 µm	165.1	161.9	5.10e10
3000 *x g*, 0.1 µm	123.1	110.9	2.34e10
0.8 µm	153.8	143.2	4.54e10

Table 2. Effect of centrifugation and filtration steps for removal of residual cells and cell fragments from plasma on particle concentration and size distribution, measured by nanoparticle tracking analysis (NTA).

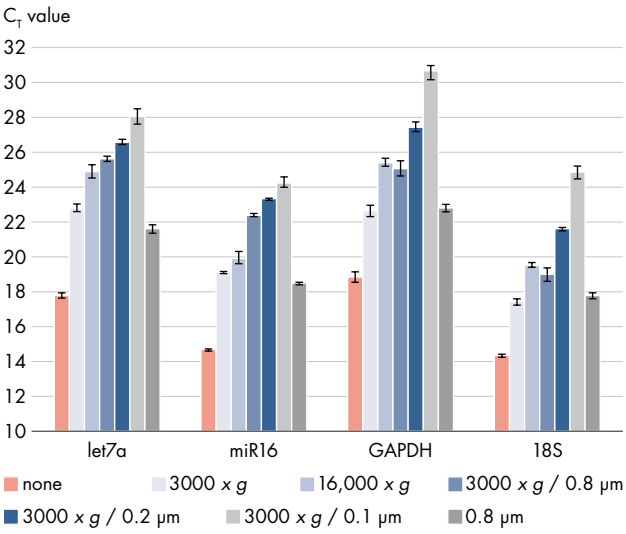


Figure 5. Effect of centrifugation and filtration steps for removal of residual cells and cell fragments from plasma on RNA abundance. Plasma samples were subjected to an additional centrifugation at 3000 or 16,000 *x g* and/or filtration using membrane with 0.8, 0.2 or 0.1 µm pore width, as indicated, followed by RNA isolation using the exoRNeasy Maxi Kit. Without any additional processing, the vast majority of isolated RNA originates from residual cells and cell fragments (>0.8 µm). Centrifugation at 3000 *x g* and 0.8 µm are very similar in efficiency of RNA removal. Higher g-force or filters with smaller pore sizes result in removal of larger EVs, and as a result, less RNA is isolated.

Urine

Whole urine, cell-free urine or exosomes?

In urine samples, miRNAs may be present within exosomes or protein complexes, or in cells that have shed into the urine. In pre-clinical animal studies, analyses are usually performed using whole urine samples due to limitations in the way the urine can be collected. However, in human studies, whole urine may be separated into a cell pellet and a cell-free supernatant. Exosomes can be isolated from the cell-free supernatant. miRNA profiling may be performed on any or several of these fractions, depending on the research objective and disease context, but since RNA levels from residual cells are several orders of magnitude higher than cell-free RNA levels, residual cells must be removed before RNA isolation to allow analysis of cell-free RNA – just like for plasma or serum.

Urine collected in the early morning is generally more concentrated than urine collected later in the day. Urine may contain various metabolites that can interfere with RNA analysis. To reduce the effect of potential inhibitors, the volume of urine used for isolating short RNAs should be limited to a maximum of 2 ml (morning urine) or, alternatively, eluates should be diluted accordingly before analysis.

Collection & preparation of urine samples

Even though miRNAs have been shown to be very stable in cell-free urine samples (Figure 6), we recommend using standardized sample handling and storage protocols. We have successfully analyzed urine samples collected in tubes without stabilizer, but other sample tubes may also be acceptable.

When collecting urine from animals (such as in pre-clinical studies), care should be taken to avoid contamination and variation due to sample collection procedures, for example the time points for sampling. We recommend centrifuging the urine samples to remove cells and other debris. For preparation and storage of cell-free urine, refer to section "Removal of residual cells & cell fragments & storage conditions" on page 5.

The number of different cell types present in urine may vary between individuals or disease states, or as a result of certain medical treatments, affecting the miRNA profile in whole urine samples (Figure 7). The cell-free supernatant contains miRNAs contained in exosomes and protein complexes. In healthy individuals, the level of miRNAs detected in this sample fraction is usually very low. Cell-free urine may be useful for toxicology biomarker analyses as it is likely to contain miRNAs released as a result of toxicity or cell death. When analyzing miRNAs in cell-free urine supernatant, care should be taken at all steps to minimize cell lysis.

»Avoid multiple freeze-thaw cycles«

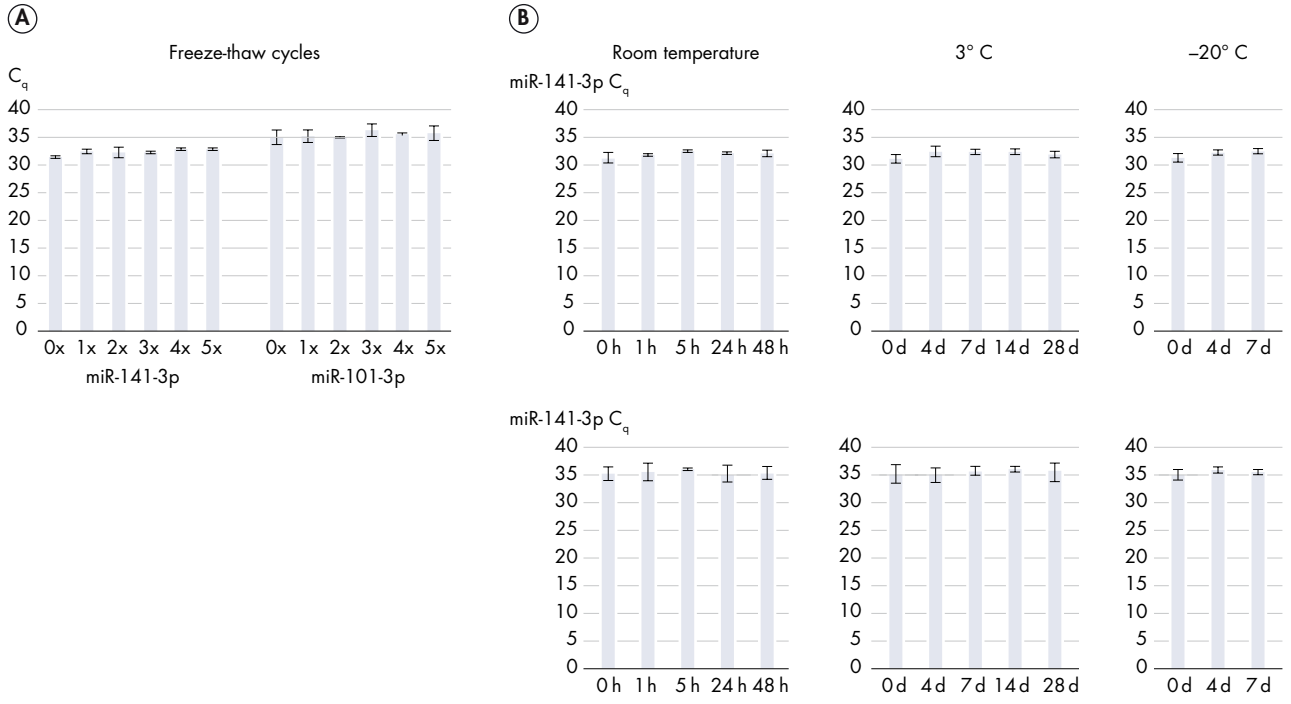


Figure 6. Stability of miRNAs in human cell-free urine. A. Cell-free urine samples were subjected to 1–5 freeze-thaw cycles prior to RNA isolation, leading to minimal effect on miRNA amplification. **B.** Cell-free urine samples were stored at room temperature, 3°C or –20°C before RNA extractions for 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 miRNA amplification. Although the effects of sample handling and storage resulted in loss of no more than 2 C_q values, we do recommend standardizing sample handling and storage protocols, process urine samples immediately by centrifuging to remove cell debris and storing at –80°C.

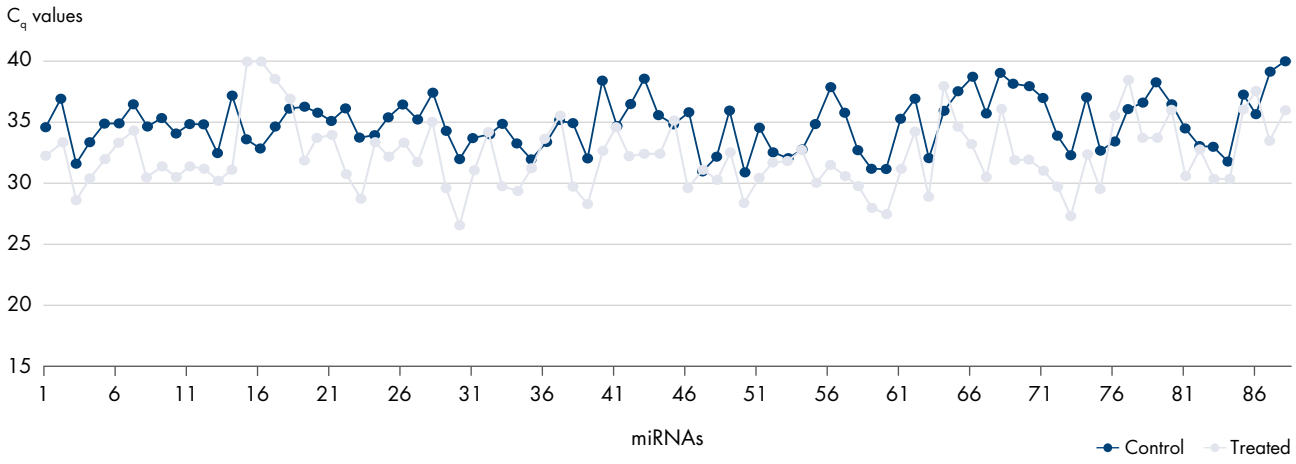


Figure 7. Changes in the miRNA profile detected in rat whole urine as a result of nephrotoxin treatment. Several miRNAs show elevated expression levels (reduced C_q) in rat whole urine after nephrotoxin treatment. miRNA profiling was performed using the miRCURY LNA miRNA PCR System.

Exosomes & other extracellular vesicles

Recent years have seen an increased interest in the significance of RNA and other molecules carried by exosomes and other extracellular vesicles (collectively, EVs). Exosomes are cell-derived membranous particles ranging in size from 20 to 120 nm (Figure 8), approximately the same size as viruses but considerably smaller than microvesicles.

The importance of these vesicles for normal cell function and disease progression has made them interesting possible targets for use as non-invasive, circulating biomarkers.

EVs are excreted from cells into the surrounding media and can be found in many if not all body fluids. They are believed to function as intercellular hormone-like messengers, transferring biomolecules such as proteins and

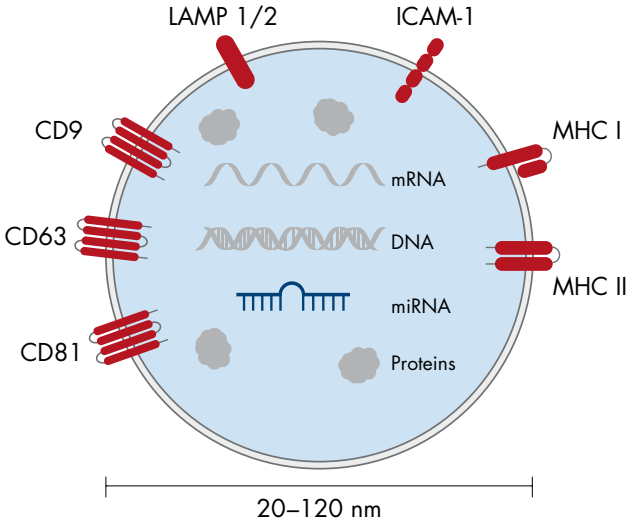


Figure 8. Structure of an exosome. Exosomes are membrane-encapsulated extracellular vesicles typically ranging from 20 to 120 nm in size and contain multiple macromolecules, including proteins, mRNA and miRNA. They have also recently been reported to contain DNA. A number of surface proteins are found exclusively in exosomes, but not in other microvesicles.

RNA between cells for a variety of important cell functions, including:

- Immune regulation
- Blood coagulation
- Cell migration
- Cell differentiation
- Cell-to-cell communication

miRNAs are actively and selectively incorporated into EVs, so the vesicular miRNA population represents a subset of the miRNAs found in serum and plasma (Table 3) or other biofluids. Compared to cells, extracellular vesicles typically contain high amounts of small RNA species and almost no intact ribosomal RNAs, so electropherograms will look different from those obtained with intact RNA isolated from cells or tissue.

Why isolate EVs?

Isolating EVs enables the analysis of miRNAs that are selectively and actively incorporated into these membrane-bound vesicles and secreted by cells into a range of biofluids. In addition, EV isolation increases the concentration of miRNAs in the sample while minimizing co-purification of inhibitory compounds and can therefore enhance miRNA signals in very dilute samples such as urine and cerebrospinal fluid (CSF), enabling detection of a larger number of miRNAs.

Methods for EV isolation

EVs may be isolated in various ways, including affinity-based methods, precipitation, size exclusion chromatography, differential centrifugation or ultracentrifugation. Depending on the preparation method and treatment of the biofluid prior to RNA isolation, the composition of isolated vesicles and co-purification of non-vesicular, cell-free miRNA may vary, resulting in inconsistent miRNA profiles. Therefore, sample collection and specimen pre-treatment protocols should be standardized and controlled.

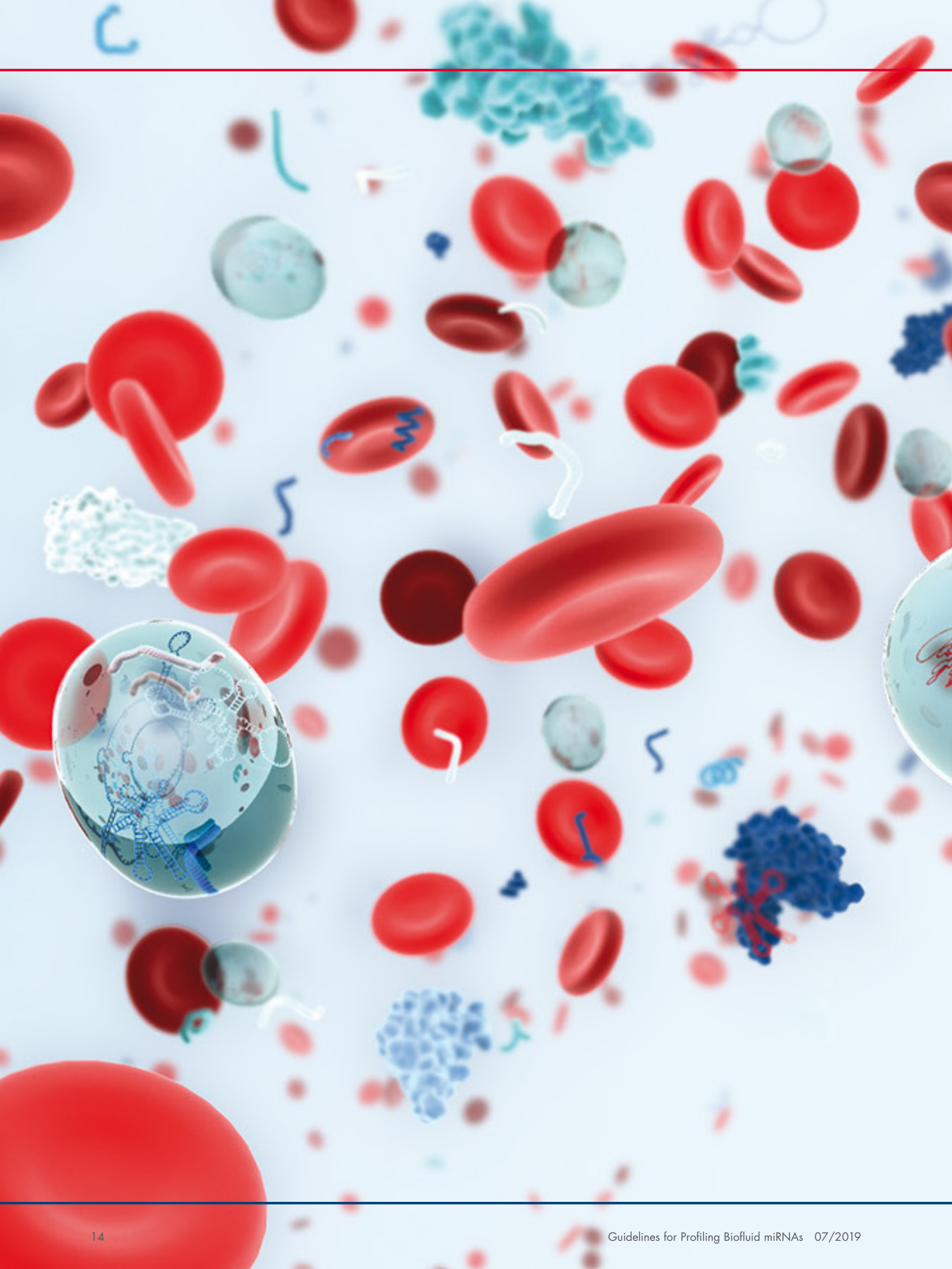
Fraction	miRNAs detected in serum	miRNAs detected in plasma
EV pellet	127	104
Supernatant	46	74
Whole biofluid	151	142

Table 3. Most miRNAs are detected in the whole biofluid, rather than in EVs or EV-free supernatants derived from serum/plasma. All of the miRNAs detected in the EV pellet or supernatant could also be detected in the whole biofluid samples. EVs and supernatant were isolated from 200 µl serum or plasma using the miRCURY Exosome Serum/Plasma Kit (cat. no. 76603). RNA was isolated either directly from 200 µl serum or plasma, or from the EV pellet and supernatant. Average number of miRNAs detected on the miRCURY LNA miRNA PCR Serum/Plasma Focus Panel containing 179 miRNAs.

EV isolation procedures are available for most biofluids including serum, plasma, CSF and urine. Because of the higher abundance of RNA in cells (several orders of magnitude), even small amounts of cellular debris can have a significant effect on RNA profiling of cell-free fluids. Therefore, cellular materials should be removed as soon as possible after blood collection to reduce the risk of additional background from blood cell-derived vesicles generated *in vitro*. For more details, refer to section "Removal of residual cells" or "Blood collection tubes" on page 7.

»Isolation of exosomes and extracellular vesicles can enable detection of more miRNAs«

To isolate vesicular RNA from serum and plasma, we recommend performing an initial low g-force centrifugation step to separate cells from plasma or serum, followed by a higher g-force centrifugation or filtration step to remove all remaining cellular debris. The latter centrifugation/filtration step significantly reduces the amount of cellular or genomic DNA and RNA in the sample (see Figure 5). The use of gel barrier tubes also results in fewer residual cells after the initial low g-force centrifugation.



Isolation of miRNA

Interest in smaller RNA species, such as miRNA, has increased over the past years as researchers better understand the regulatory role of small non-coding RNAs. In general, working with RNA requires special precautions to prevent RNase contamination of the reagents, leading to RNA degradation. Preparation of total RNA that includes small RNAs (<200 nucleotides) from a biological sample is critical for successful miRNA expression profiling.

Special considerations for miRNA analysis

Origin of miRNA

The vast majority of miRNA in whole blood is found inside of cells, but the cell-free miRNA population is particularly interesting in biomarker studies. This very small proportion of miRNA is released by various cell types, including blood cells but also endothelial and other peripheral cells from basically any organ in the body.

Cell-free miRNAs in biofluids originate through different biological processes, including the active secretion of extracellular vesicles or passive release after cell death, and both may be relevant in terms of biomarker discovery. These small RNAs are protected from degradation by several mechanisms: miRNAs in human plasma are thought to exist either as "free" miRNAs bound to protein complexes (e.g., Ago2) or contained within extracellular vesicles. The ability to distinguish the two populations may aid in creating meaningful biomarker profiles.

miRNA content

Working with cell-free biofluids can be challenging because of large variations in miRNA content between samples. Samples from healthy individuals typically contain only low levels of miRNA, whereas samples from sick individuals or those exposed to drugs or toxins may contain significantly elevated levels of organ-specific miRNAs.

Determining background

When profiling very low-abundance targets such as cell-free biofluid miRNAs, it is important to ensure the signals being measured are reliably above any background signal. A blank purification (e.g., water added instead of biofluid sample in the RNA isolation) can be used as a negative control to measure any background signal.

»Biofluids require optimized RNA isolation procedures«

Undesired components

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 carry-over of inhibitors into the RNA sample and monitoring sample quality are important factors for consideration.

Limited amount of RNA

Cell-free biofluids in particular contain very low amounts of RNA. Therefore, normal RNA quality control using capillary OD measurements is not suitable for these types of samples. Refer to "Quality Control" on page 17 for more details on implementing appropriate quality control measures.

RNA handling & storage guidelines

The following precautions help 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, and always briefly centrifuge tubes before opening.
- Avoid repeated freeze-thaw cycles.
- Store RNA at the appropriate temperature (refer to "Storage of purified RNA" on page 17).

Spike-in controls

Reproducible RNA isolation may be difficult from some sample types, and some RNA samples may contain inhibitors of cDNA synthesis or PCR, even though they were isolated using the best standard procedures. This can lead to different efficiencies of the reverse transcription or PCR amplification between compared samples. One way to check for differences in efficiencies in isolation, cDNA synthesis and PCR amplification is by adding known amounts of RNA spike-ins to the sample prior to RNA isolation and cDNA synthesis, respectively. Use of RNA spike-ins may also reveal potential presence of nucleases.

The RNA Spike-in Kit, for RT provides several RNA spike-ins. For control of RNA isolation, UniSp2, UniSp4 and UniSp5 are provided pre-mixed in one vial, each at a different concentration in 100-fold increments. For control of cDNA synthesis, a synthetic version of a *C. elegans* miRNA, cel-miR-39-3p,is used in combination with the UniSp6 RNA spike-in template. Following PCR, wells detecting the RNA spike-ins are compared, and outlier samples may be identified and considered for exclusion from further analysis.

RNA spike-ins are also useful for monitoring miRNA sequencing experiments. The QIAseq® miRNA Library QC PCR Panel and Assays contains 52 spike-ins that enable evaluation of RNA sample quality prior to miRNA/small RNA NGS library preparation and assessment of NGS performance post-sequencing (see section "Spike-in controls for NGS" on page 23 for more details).

There is currently no clear consensus in the research community on what should be used as a normalization control for miRNA expression profiling in biofluid samples. Synthetic RNA spike-ins should never be used for normalization, as they do not reveal the RNA content and quality in the biological sample. Normalization should instead be performed using stably expressed endogenous reference genes or, when applicable, the global mean of all expressed miRNAs. Refer to section "Normalization" on page 35 for more details.

Carrier RNA

Use of carrier RNA is optional with many QIAGEN and PreAnalytiX RNA isolation kits. There is no major benefit, but in some applications, users have reported higher reproducibility when carrier RNA is added. For example, RNA from the bacteriophage MS2 (Roche Applied Science, cat. no. 10165948001) can be used. Carrier RNA should not be added to samples intended for NGS applications.

Sample type	Total cell-free miRNA	Exosomal RNA including miRNA	Exosomes (intact vesicles)	Cellular and cell-free miRNA*
Serum/plasma	miRNeasy Serum/Plasma Advanced Kit (automatable on QIAcube®) miRNeasy 96 Advanced QIAcube HT Kit	exoRNeasy Kits (automatable on QIAcube)	exoEasy Maxi Kit miRCURY Exosome Serum/Plasma Kit	
Urine	miRNeasy 96 Advanced QIAcube HT Kit	exoRNeasy Kits (automatable on QIAcube)	exoEasy Maxi Kit miRCURY Exosome Cell/Urine/CSF Kit	
CSF, cell culture supernatant		exoRNeasy Kits (automatable on QIAcube)	exoEasy Maxi Kit miRCURY Exosome Cell/Urine/CSF Kit	
Whole blood				PAXgene Blood miRNA Kit (in combination with PAXgene Blood RNA Tube) QIAsymphony® PAXgene Blood RNA Kit (in combination with PAXgene Blood RNA Tube)

Table 4. Recommended kits for isolation of RNA from biofluids. *Total cellular and cell-free miRNA are isolated, although the large excess of cellular miRNA prohibits analysis of the cell-free miRNA.

miRNA isolation methods

Minimizing inhibitors

QIAGEN and PreAnalytiX offer a range of kits optimized for isolation of miRNAs from exosomes and biofluids such as serum, plasma, urine or CSF. 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 that minimizes the carry-over of such inhibitors while maximizing the RNA yield. The following purification kits have been shown to be suitable for this purpose (Table 4).

Quality Control

Storage of purified RNA

Store purified RNA at –15°C to –30°C or –65°C to –90°C in RNase-free water. Under these conditions, no degradation of RNA is detectable after 1 year.

Integrity of RNA

Integrity of RNA isolated from whole blood can be analyzed with standard gel or capillary electrophoresis. Compared to RNA of from cell cultures, integrity values like RIN or RQI of blood eluates are often lower due to the relative high portion of small RNA species that are mistakenly counted as degradation products by the algorithms behind these values. (7)

Cell-free RNA from serum or plasma mostly contains small RNAs or fragmented RNA <100 nucleotides in size. Integrity of cell-free RNAs cannot be analyzed by standard methods like denaturing agarose gel electrophoresis and ethidium bromide staining or with QIAxcel® Advanced System or Agilent® 2100 Bioanalyzer. Analysis using the Small RNA Chip does not distinguish miRNAs, and since ribosomal bands are not visible for cell-free miRNAs, RIN values are not valid. Appearance of ribosomal RNA bands in these samples usually indicates contamination by cells or cell debris.

Quantification of RNA from biofluids

Standard methods for measuring RNA yield and quality are only appropriate for use with whole blood samples. For cell-free biofluid samples, the RNA concentration in the eluate is too low for reliable OD260 quantification on a NanoDrop® or other spectrophotometer. If carrier RNA is used, it makes measuring the low levels of endogenous RNA by OD260 impossible. In addition, presence of contaminants that absorb around 260 nm can further confound the readings. Fluorometric quantification (e.g., using Qubit) is also often unreliable for short nucleic acid fragments such as miRNA from cell-free samples. If an exact quantification is needed, we recommend a qPCR-based method targeting a selection of the (mi)RNAs of interest.

Standardizing input amounts based on volume

Since spectrophotometric and fluorometric RNA concentration measurements are not sufficient for ensuring consistent RNA input in downstream analyses, we recommend standardizing input amounts based on starting volume rather than RNA quantity. Start with the same volume of biofluid in each RNA isolation, and use the same volume of purified RNA for all samples in downstream analysis.

DNA contamination

No currently available purification method can guarantee that RNA is completely free of DNA, even when it is not visible by agarose gel or capillary electrophoresis. While miRNeasy and PAXgene miRNA kits remove the vast majority of cellular DNA, trace amounts may remain, depending on the amount and nature of the sample. In contrast to whole blood, serum, plasma and other cell-free body fluids contain very little DNA. Furthermore, our PCR assays that detect mature miRNAs do not amplify DNA.

Assessing RNA quality by PCR

RNA can be investigated using a combination of synthetic RNA spike-ins and selected endogenous miRNA assays, enabling identification of inferior or potential outlier samples prior to further analysis. If working with other biofluid samples, endogenous miRNAs relevant for that type of sample need to be determined, either by comparison to literature values or by conducting a pilot study using a miRNome panel.

QIAGEN has developed a set of synthetic RNA spike-ins and endogenous miRNAs for performing qPCR-based quality control of RNA samples. The RNA Spike-In Kit, for

Key QC parameters

Consider the following parameters when assessing the quality of RNA isolated from cell-free biofluids:

- RNA extraction efficiency and yield
- Absence of inhibitors of downstream enzymatic processes, such as cDNA synthesis, PCR or NGS library prep
- Absence of nucleases
- Presence or absence of RNA resulting from cellular contamination or hemolysis, if cell-free biofluids are used
- Presence of endogenous miRNAs (based on the particular type of biofluid being analyzed)

RT includes spike-ins that enable monitoring of the RNA isolation efficiency, cDNA synthesis and PCR amplification; the miRCURY LNA RT Kit includes a spike-in for monitoring cDNA synthesis (see Table 5 for details). The use of RNA spike-ins may also reveal potential presence of nucleases.

The assays to detect the synthetic spike-ins are present in the ready-to-use miRCURY LNA miRNA PCR Focus and miRNome PCR Panels. They can also be added as controls to custom panels or ordered separately. A separate miRNA QC PCR panel, which contains a combination of 12 qPCR assays for the RNA spike-ins and endogenous miRNAs, is also available. The assay for detecting the UniSp3 spike-in is included in the PCR panels and is used as an inter-plate calibrator to monitor the PCR.

»Biofluid RNA quality can be reliably assessed by qPCR«

For NGS applications, the QIAseq miRNA Library QC PCR Array Kit enables rigorous sample QC before NGS library preparation as well as NGS performance post-sequencing (Figure 9).

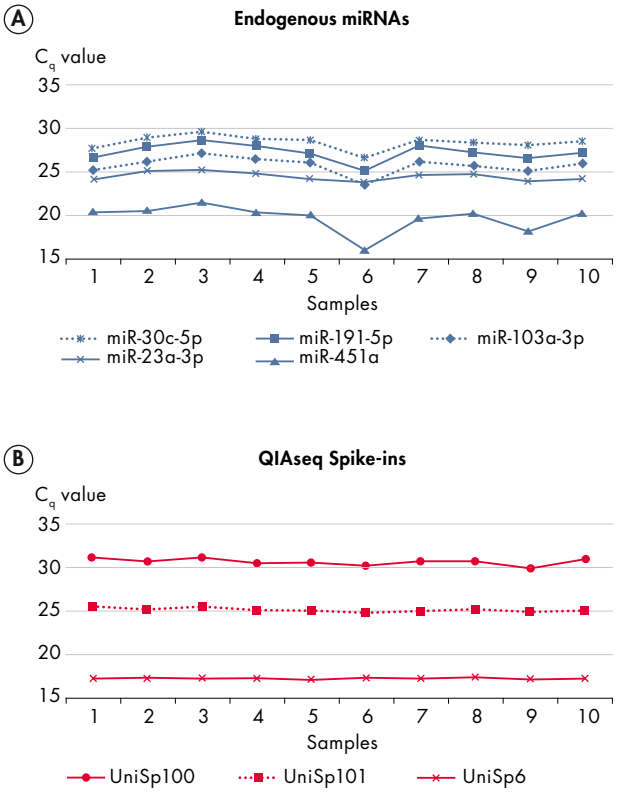


Figure 9. The QIAseq miRNA Library QC PCR Array detects endogenous miRNAs and synthetic RNA spike-ins by qPCR for rigorous, up-front sample QC. A. Outliers and samples affected by hemolysis (samples 6 and 9) can be readily identified. **B.** QIAseq Spike-ins UniSp100 and UniSp101 assess RNA isolation efficiency, and UniSp6 monitors for presence of inhibitors. Collectively, all samples shown pass QC.

Quality control of biofluid samples by PCR	
Basic QC	• Standard human serum/plasma samples • Use assays listed on the right • Single RNA input volume for all samples
Extended QC	• Challenging biofluid samples • Use assays listed on the right • Test a range of RNA input volumes • Proceed with Basic QC when optimal input volume established

Table 5. Overview of assays in the miRCURY LNA miRNA 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 QC PCR Panel or as individual assays. UniSp2, UniSp4, UniSp5 and cel-miR-39-3p spike-ins are part of the RNA Spike-In Kit. The UniSp6 is contained in the miRCURY LNA RT Kit. The UniSp3 is only available on panels. For more details, refer to the corresponding kit handbooks.

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 ΔC_q (miR-23a-3p – miR-451a)
miR-30c-5p, miR-103a-3p, miR-124-3p and miR-191-5p	• Biologically relevant, endogenous miRNAs • Should be present in RNA from serum/plasma, urine or CSF and some other biofluids • Use for general sample quality check

Basic QC – standard human serum & plasma samples

The amount of RNA that can be extracted as well as the amount of inhibitors remaining after extraction can vary from sample to sample. When performing miRNA profiling in standard human whole blood, serum or plasma samples, it is recommended that all samples are quality-controlled to monitor purification yield and check for absence of PCR inhibitors. Any potential issues within the sample set should be identified before proceeding with qPCR or NGS. For studies entailing a large number of samples, at least a subset of the samples should be quality-controlled.

Basic QC can be performed easily with the miRCURY LNA miRNA QC PCR Panel, using a single RNA input amount (the same volume of RNA for all samples). Based on the results of this qPCR quality control step, samples showing low RNA yield or indications of inhibition or hemolysis can be excluded from further studies.

Assessing cellular contamination

The presence of cellular RNA species may disturb cell-free biofluid miRNA profiling experiments and result in a distorted and non-reproducible profile. The presence of RNA species specific for 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 longer, sub-optimal storage of blood in a collection tube without cell stabilization prior to cell removal, incomplete removal of white blood cells or platelets from the sample and/or hemolysis. For more details on preventing hemolysis, refer to "Tips for avoiding hemolysis" on page 7.

Monitoring hemolysis in serum and plasma samples or other biofluid samples that may be contaminated with blood can be performed using various spectrophotometric methods that measure free hemoglobin levels (see "Processing time from blood collection to plasma/serum preparation" on page 9). However, if the RNA has already been extracted or the original sample is no longer available, an alternative strategy is to compare the level of a miRNA highly expressed in red blood cells (hsa-miR-451a) with that of a miRNA unaffected by hemolysis (hsa-miR-23a-3p). As shown in (5), the ΔC_q (miR-23a-3p – miR-451a) is a good measure of the degree of hemolysis. Values higher than five indicate a possible erythrocyte miRNA contamination, and a ΔC_q of 7–8 or more indicates a high risk of hemolysis affecting the data obtained in human samples. Note that these values are different for mouse and rat samples.

A similar approach can identify contamination from cellular components in other biofluid samples, by identifying and monitoring the levels of cellular-derived endogenous miRNAs. For example, miR-142 is considered a marker for thrombocyte or cellular contamination. Many miRNAs in serum and plasma are not affected by hemolysis, so it may still be possible to detect disease-associated miRNA biomarkers even from samples affected by hemolysis. However, it is important to be aware of the possible effects on the miRNA profile when performing normalization and data analysis so that any systematic bias can be eliminated.

Extended QC – challenging biofluid samples

When establishing a new protocol or working with a new or challenging type of biofluid, such as urine or CSF, we recommend testing different RNA sample input amounts in the cDNA synthesis reaction (e.g., 1, 2, 4 and 8 µl in a 20 µl RT reaction). This dilution series is performed using assays for the RNA spike-ins and a few miRNA assays (see Table 5) to confirm 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 a linear relationship between sample input and signal (Figure 10).

»Extended QC is recommended for challenging biofluids«

A dilution series is also used for efficiency calculation or for absolute miRNA quantification. When performing a dilution series, we recommend an RNA dilution series rather than a cDNA dilution series. 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 a solution containing carrier RNA (e.g., bacteriophage MS2 total RNA) to avoid loss of RNA due to plastic adhesion. For dilution of cDNA prior to qPCR, follow the guidance in section "Protocols optimized for biofluids" on page 31 and in the corresponding kit handbook.

Suitable endogenous miRNAs for this extended QC typically detected at medium to high levels are listed in Table 6. Once the optimal sample input has been established using the extended QC procedure, samples should be quality-controlled on a routine basis with a single sample input volume prior to miRNA profiling using the basic QC (see section "Basic QC – standard human serum & plasma samples" on page 20).

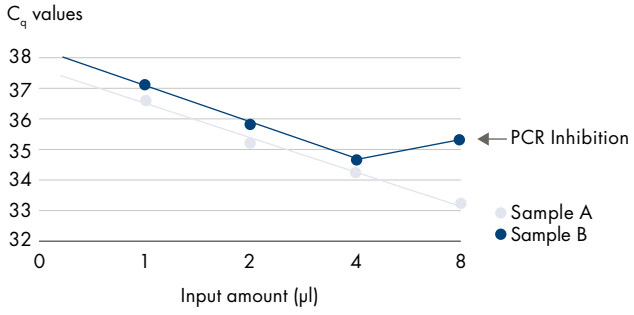
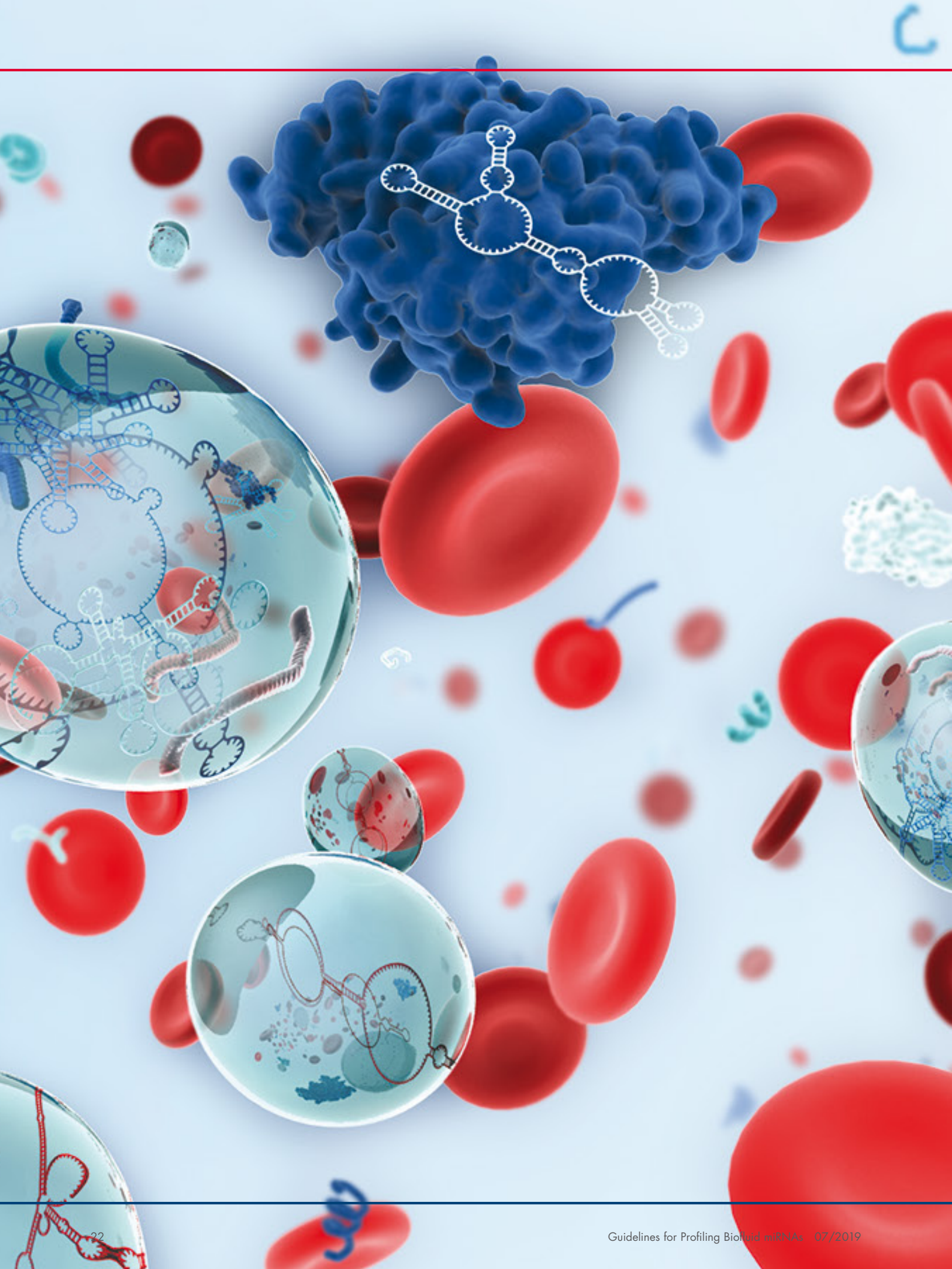


Figure 10. Determining suitable input amounts to achieve a linear signal range. An RNA dilution series can be used to determine that input amounts are within the linear range of the system and to ensure a good signal level, with no sign of inhibition (e.g., Sample A at 8 µl). Increasing the RNA volume to increase signal can have the opposite effect and result in less signal due to carry-over of inhibitors into the RT-PCR (e.g., Sample B at 8 µl). Input amount refers to µl RNA in a 20 µl RT reaction.

miRNA	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 miRNAs typically detected or stably expressed in human biofluids. Often, one or more of these miRNAs may be good for normalization of expression data, but their expression stability needs to be determined in each sample set. miRNAs we have identified as stably expressed in human serum/plasma samples are indicated by an asterisk (*). miRNAs that are typically detected but not necessarily stably expressed are indicated by a circle (•).



miRNA Next-Generation Sequencing

Why choose NGS over other analysis methods?

The choice of analysis platform(s) is an important factor a researcher must consider. Microarrays require an RNA input amount that is prohibitively large for biofluid samples, and the sensitivity/dynamic range is lacking. qPCR is a popular discovery tool because of its high sensitivity and specificity, but the interrogation targets must be known ahead of time. NGS does not require any prior knowledge of the distinct miRNAs present in the samples of interest, as these approaches capture all sequences of a desired size and characteristic. NGS offers a cost-effective approach to profile the entire miRNome and enables the detection or novel miRNAs as well as other types of small RNAs that are potential biomarkers, such as piRNA. This makes NGS the discovery technology of choice for screening applications. New miRNA NGS library preparation methods using unique molecular indices or identifiers (UMIs) overcome bias in quantification.

Ensuring reliable NGS data from biofluids

Spike-in controls for NGS

Obtaining sensitive and reliable miRNA NGS data from biofluid samples is challenging, so it is essential to use protocols for RNA isolation and quality control that are optimized for biofluid samples with low RNA content. Refer to sections "Isolation of miRNA" on page 15 and "Quality Control" on page 17 for more details. Adding synthetic spike-in miRNA during the RNA extraction procedure improves yield and reproducibility. (8) However, care should be taken to optimize the amount of spike-in RNA so it does not consume too many sequencing reads. To facilitate data analysis, the spike-in RNA should be of a species with sufficient evolutionary distance from the species of interest.

The QIAseq miRNA Library QC Spike-Ins, a set of 52 Arabidopsis miRNA sequences, meets these requirements for NGS analysis of human and other mammalian miRNA samples. The success and uniformity of the extractions can be assessed by qPCR detection of the spike-ins. Additionally, any enzymatic inhibitor carried over from the sample can be assessed with spike-ins at the reverse transcription step. The impact on library construction and downstream sequencing can therefore be anticipated, and any negative impact can also be confirmed by inspecting the sequencing data from the 52 spike-ins (Figure 11).

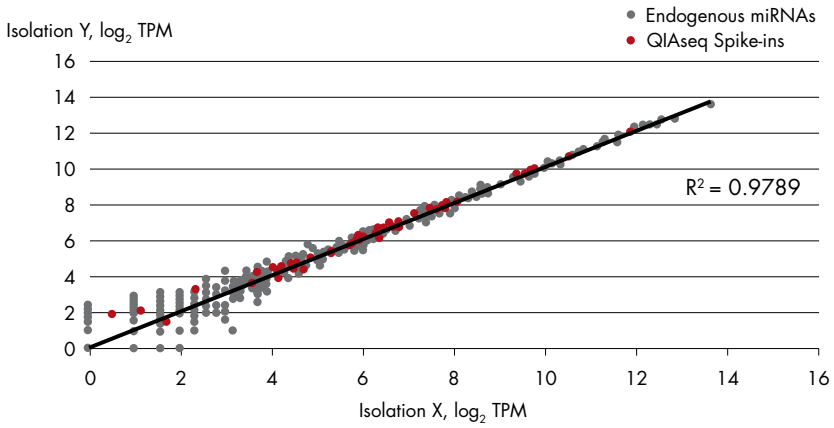


Figure 11. Reproducibility and linearity assessment of miRNA and small RNA for the biofluid NGS workflow from RNA isolation to sequencing. Two independent RNA isolations were performed using the same pool of serum/plasma samples, followed by two independent library preparations and sequencing runs. This graph shows the excellent reproducibility as reported by the correlation between 52 RNA spike-ins, added during the RNA isolation procedure. TPM = tags per million mapped reads.

NGS library preparation

Bead cleanups instead of gel excision

Thus far, miRNA sequencing has been fraught with challenges. Library preparation has often relied on tedious gel purification to eliminate adapter–dimers and contaminating RNA. Prepping, running and then excising the correct band from the gel takes time and can add at least a day to time-critical experiments. Furthermore, band cutting can be quite variable from sample to sample and run to run, and the resulting increase in variability will be reflected in the data quality. For a more reproducible cleanup and adapter–dimer removal, we recommend bead-based cleanup using the QIAseq miRNA Library Kit, which uses proprietary chemistry to eliminate adapter–dimers and contaminating RNAs.

The standard QIAseq miRNA procedure (Figure 12) does not require any gel purification, excision or elution, reducing the required hands-on time and streamlining the entire workflow. In addition, the technology used in the QIAseq miRNA Library Kit blocks human Y4 small RNA products abundant in serum and plasma (10) from being included in the sequencing library, thereby reducing the percentage of wasted reads.

Avoiding sequence bias using UMIs

A significant contributor of sequence bias in the small RNA library construction process is the downstream PCR, as amplification efficiency may vary between templates. The library preparation workflow using the QIAseq miRNA Library Kit avoids PCR bias by introducing UMIs. During library preparation, the RT primers carry and incorporate UMIs of 12 nucleotides in length. Given that there are four nucleotide options at each of the 12 positions, nearly 17 million different combinations are possible, so each individual RNA can be tagged with a UMI (Figure 12).

QIAseq miRNA Library Kit evaluated as leading solution for NGS-based miRNA biomarker detection

Features	NEXTflex® (Bioo Scientific)	SMARTer® (Clontech/Takara)	NEBNext® (New England Biolabs)	QIAseq (QIAGEN)
Alleviating sequencing bias	✓	✓	✘	✓
Inhibition of adaper-dimer formation	✓	✓	✓	✓
% miRNA reads	✓	✘	✓	✓
hY RNA inhibition	✘	✘	✘	✓
Workflow convenience	✓	✓*	✓*	✓

Table 7. Comparison of sequencing efficiency and overall performance obtained with QIAGEN and three other leading library preparation kit providers. To assess sequencing efficiency, the percent of reads passing the filter during adapter trimming and mapping were calculated for each of the four library preparation methodologies. Green ticks indicate a satisfying and yellow ticks an average performance in the respective category. Red crosses either signify a performance below average or in the case of YRNA inhibition, the option is not available. *Requires gel separation. (9)

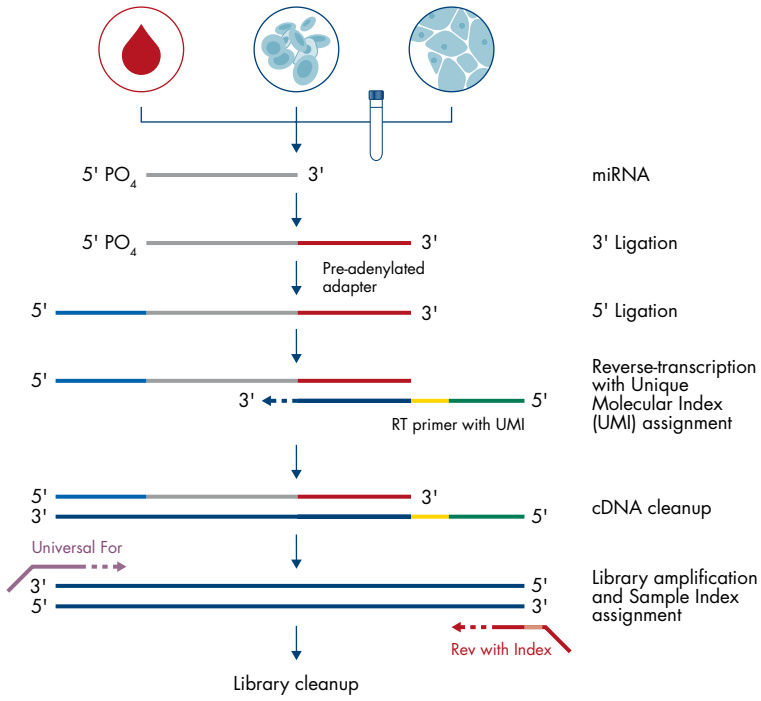


Figure 12. RNA-seq library prep workflow of the QIAseq miRNA Library Kit. TThe enhanced library prep strategy uses UMIs to reflect original RNA molecule quantities and remove PCR bias. The bead-based cleanup enables automating the workflow on a robotic platform.

Sequencing of traditional miRNA libraries produces raw read counts that often reflect PCR bias, and the number of reads does not accurately represent the number of copies of the original RNA. This phenomenon is illustrated in Figure 13, in which the results indicate 6 reads for sample 1 and 3 reads for sample 2, or a 6:3 (2:1) ratio instead of the true 3:1 ratio of the transcripts in the sample.

Sequencing read quantification based on UMIs removes this bias, allowing the data to reflect the original quantities of ligated RNA molecules. By counting all copies of a single UMI as one original RNA, the sequencing data are reduced or "collapsed" back to the original ratios. The use of UMIs allows more accurate interpretation of the quantities and changes in gene expression, and identification of miRNA species of interest can therefore be pursued for further study.

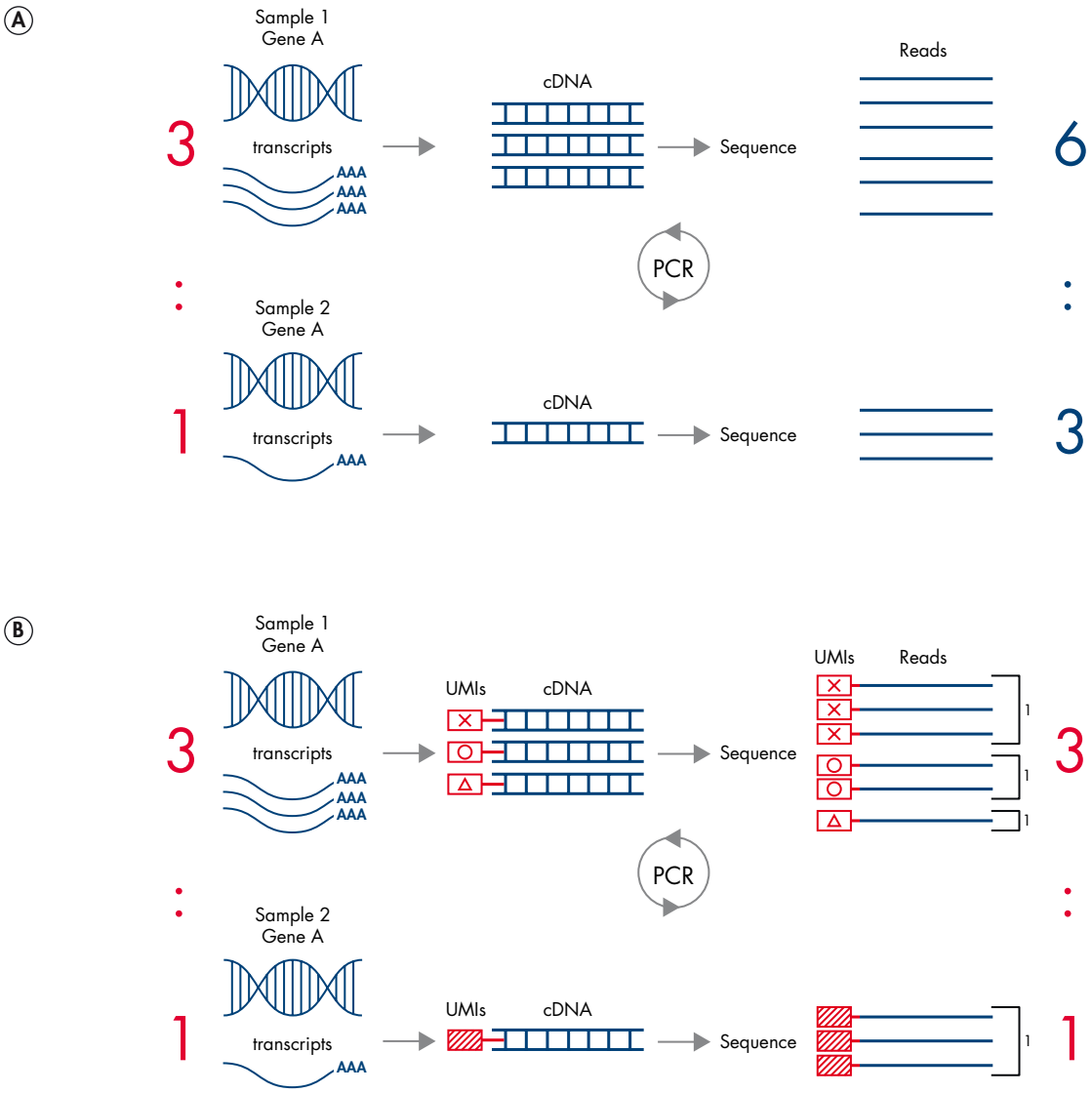


Figure 13. PCR bias correction using UMIs. A. Without UMIs, the number of reads does not accurately represent the number of copies of the original RNA. **B.** With UMIs, the data accurately reflect the original transcript abundance.

Sequencing depth considerations for biofluid miRNA

The number of reads measured by NGS for a particular miRNA sequence is not directly related to its abundance (11), and as a result, NGS is not suitable for absolute quantification. NGS data, like microarray and qPCR data, therefore should be normalized and analyzed to identify relative differential expression between samples. In addition, measurement of a particular miRNA by NGS is not independent of other miRNAs. Thus, if a particular miRNA occurs in high abundance, this may reduce the number of reads available for detection of other miRNAs. Care should be taken to ensure that sequencing depth is sufficient to allow accurate analysis of the low-expressed miRNAs typically found in biofluids.

miRNA library read depth is one of the most crucial factors with regards to both differential expression analysis and discovery of novel miRNAs. Metpally (12) analyzed NGS miRNA data from gastrocnemius tissue (calf muscle) in mice and corresponding plasma samples using different read numbers from 0.5–15 million raw unmapped reads (Figure 14). Deeper sequencing allows the identification of novel putative miRNAs. Based on the experience of our Genomic Services team, 12 million reads have been determined as an ideal number for the capture of miRNAs from biofluid sources.

»Ensure an adequate sequencing depth for analyzing low-expressed biofluid miRNAs«

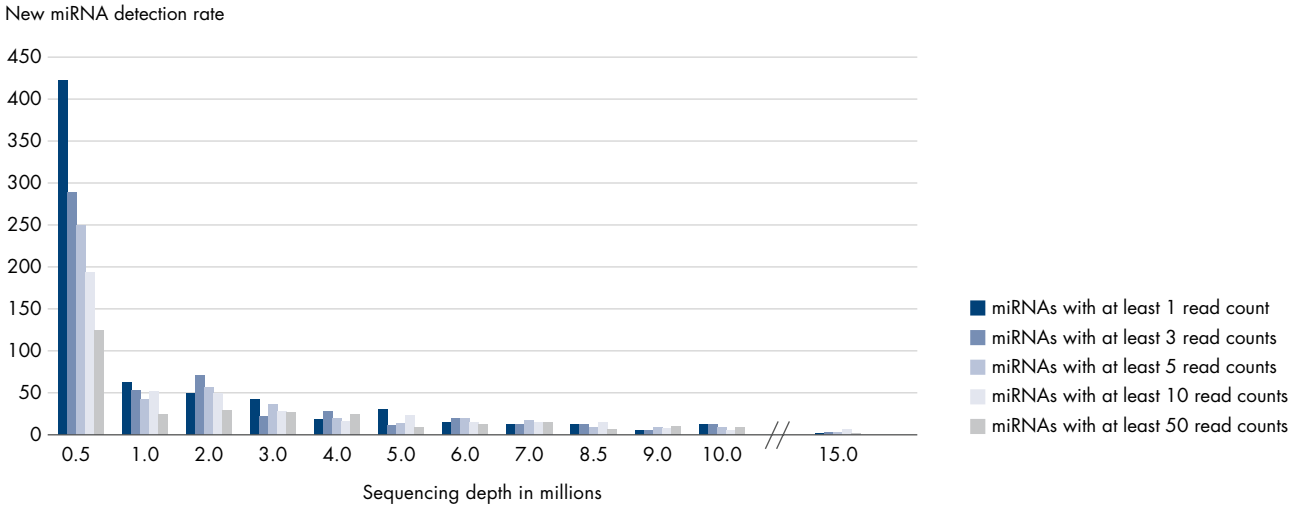


Figure 14. Effect of read depth on discover rate of rare or novel miRNAs. As 1 million reads (filtered but unmapped) at a time are added to the sequencing depth, the number of additional novel miRNAs that are detected is reduced. Each bar on the chart shows the numbers of newly detected miRNAs with at least 1, 3, 5, 10 or 50 reads, respectively, at different sequencing depth. (12)

Setting a TPM threshold

Ideally, the number of miRNA reads across different samples in a study should be similar to allow for sample comparison. The normalization procedure used should account for any differences in read number between samples, regardless of the sequencing depth (Figure 15). Calculating the tags per million (TPM) mapped reads is a means of normalizing against the total number of mapped reads. Reads detected at less than one TPM could represent miRNAs expressed at low levels or artifacts, while reads below five TPM can be difficult to validate by other techniques (e.g., qPCR), and detection cannot usually be improved by increasing the sequencing depth. Setting a TPM threshold is a useful way to focus on the most reliable miRNA reads.

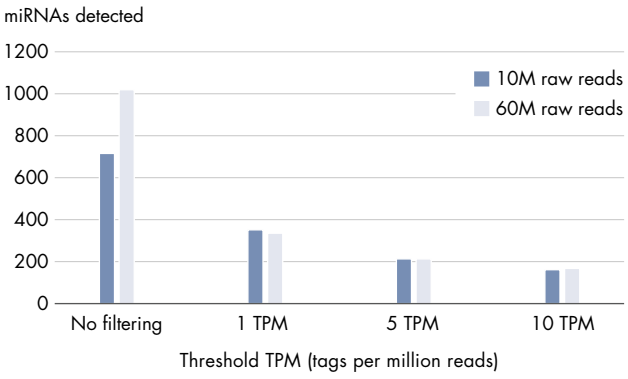


Figure 15. Calculating TPM mapped reads for normalization correction of different sequencing depths. Pooled serum and plasma were sequenced with either 10M or 60M raw reads per sample. After normalization, the number of miRNAs detected is the same. Applying a threshold enables focusing on the most reliable signals.

Normalization for differential expression analysis

While TPM-normalized values can provide an estimate of the RNA amount contained in a sample, comparing between samples or groups of samples requires a different normalization approach that compensates for differences in sequencing depth as well as over- and under-sampling effects. While there are many methods to normalize RNA sequencing data for differential gene expression analysis, these approaches often assume that the majority of genes does not change between sample groups. In a comparison of normalization methods by Seyednasrollah (13), none of the results using these methods diverged significantly from the results of trimmed mean of M values (TMM) normalization.

TMM calculates effective libraries sizes, which are then used as part of the per-sample normalization. TMM normalization adjusts library sizes based on the assumption that most genes are not differentially expressed. The TMM factors are used to obtain normalized read counts. (14) The CLC Genomics Workbench miRNA analysis pipeline (part of the Biomedical Genomics Analysis plugin) makes use of TMM normalization. After differential expression analysis, results tables can be visualized as a Venn diagram and heatmaps. From the CLC Genomics Workbench, the comparison of miRNA seed expressions can be uploaded directly to Ingenuity® Pathway Analysis (IPA®).

The optimized workflow and protocols described form an integral part of our miRNA biofluids NGS services. This workflow produces optimal results and represents the best recommendation for working with miRNA, and in particular, with miRNA from biofluid samples.

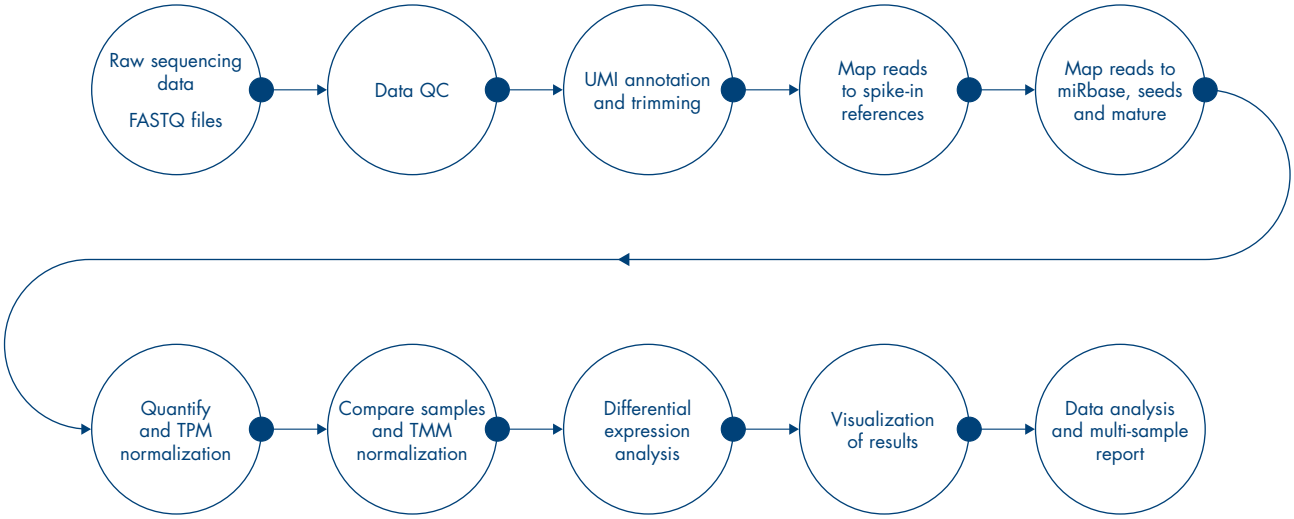


Figure 16. Schematic of the full miRNA NGS data analysis pipeline available through the CLC Genomics Workbench with the Biomedical Genomics Analysis plugin.

Understanding complex 'omics data with IPA

Taking the resulting, high-quality and high-confidence data to the next level of biological interpretation requires advanced bioinformatics tools, such as QIAGEN's Ingenuity Pathway Analysis. IPA software is a powerful analysis and search tool that uncovers the significance of 'omics data and identifies new targets or candidate biomarkers within the context of biological systems. (15) IPA has been widely adopted by the life science research community and is cited in thousands of articles for the analysis, integration and interpretation of data derived from 'omics experiments, such as RNA-seq, small RNA-seq, microarrays including miRNA and SNP, metabolomics and proteomics experiments.

miRNA PCR Analysis with SYBR® Green

Why choose qPCR over other analysis methods?

qPCR has become a widely used tool in miRNA studies. Unlike NGS, it can only be used for studying known targets. However, qPCR is a fast, extremely sensitive and inexpensive method that offers linear detection over a broad order of magnitude. Plus, setup efforts and data analysis are relatively simple for most labs. It also requires low input RNA, a key advantage for biofluid samples, which are often limited. qPCR can be scaled to various research needs, from screening and profiling, to validation of NGS studies and verification of results from functional studies.

Protocols optimized for biofluids

The landscape of miRNA analysis from biofluids is changing rapidly. We offer a number of new technologies for complete RT-qPCR and NGS workflow analyses, including locked nucleic acid (LNA) technology that provides increased sensitivity and specificity to meet the challenging and ever-changing needs of miRNA studies, especially in biofluids.

In the miRCURY LNA miRNA PCR System, every step of the procedure for processing biofluid samples has been optimized to maximize the signal and minimize the effects of inhibitors. For more details, refer to Table 8 and the *miRCURY LNA miRNA PCR – Exosomes, Serum/Plasma and Other Biofluid Samples Handbook*, which is available at www.qiagen.com.

Tips for successful cDNA synthesis

- We do not recommend comparing samples prepared using different RNA isolation methods
- Remember to add RNA spike-ins (included in the RNA Spike-In Kit) during the RNA isolation and cDNA synthesis steps; these will help with assessment of RNA quality later
- Perform the RNA isolation and the reaction steps preceding real-time PCR in rooms separate from where real-time PCR reactions are carried out to avoid contaminating the cDNA with PCR amplicons
- Do not use heparin blood draw tubes, as heparin is a strong inhibitor of all enzymatic processes

To minimize or alleviate the effect of smaller variations in inhibitor content between samples, we recommend performing reverse transcription in a larger reaction volume than for standard samples. If the RNA solution input exceeds 20% (v/v) in the RT reaction, it is important to monitor the samples for any signs of inhibition as described in section "Basic QC – standard human serum & plasma samples" on page 20.

Dilution of the RT reaction prior to PCR is a standard procedure in the miRCURY LNA miRNA PCR protocol. Due to the larger RT reaction volume used for biofluid samples, the dilution factor of the cDNA is reduced relative to standard samples (see Table 8). Figure 17 shows the high reproducibility that can be achieved between RT reactions using serum RNA and the volumes detailed in Table 8. All subsequent handling and PCR cycling conditions follow procedures as described in the *miRCURY LNA miRNA PCR – Exosomes, Serum/Plasma and Other Biofluid Samples Handbook*.

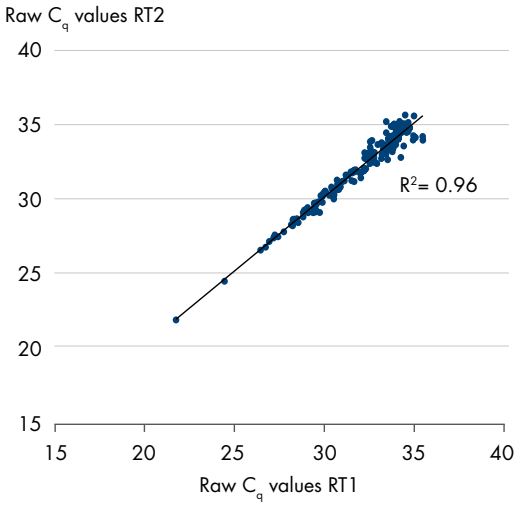


Figure 17. Excellent reproducibility between technical serum replicates using the miRCURY LNA miRNA PCR System. Raw C_q values from two separate RT reactions (RT1 and RT2) on total RNA purified from 65 µl serum are shown. A total of 730 miRNAs were profiled. Only miRNAs with C_q values below 35 have been included (133 data points).

		Serum/Plasma Focus PCR Panel	miRNome PCR Panels: Human and Mouse/Rat	
Assays or panels	**Single assays**	192 assays on 96- or 384-well plate	Panel I (one 384-well plate)	Panel I+II (two 384-well plates)
Number of reactions possible with the miRCURY LNA RT Kit	64	32	16	8
RT reaction volume	10 µl	20 µl	40 µl	80 µl
Dilution of cDNA for qPCR		1:100		

Table 8. Experimental setup for serum/plasma samples using the miRCURY LNA miRNA PCR System.

For a general overview on which miRCURY LNA miRNA PCR panels are available for miRNA profiling in biofluids, refer to Table 9. Initial screenings can be performed with miRNome PCR panels, which contain 752 well-characterized miRNAs. Full or complete miRNome screenings are often done by NGS, followed by PCR validation using Custom PCR Panels and Assays. Focused profiling of miRNAs from biofluids or exosomes isolated from CSF or urine can be directly performed using predesigned PCR Focus Panels.

Sensitivity is defined by the limit of detection

Cell-free 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 C_q values from qPCR assays relate to higher sensitivity. A C_q value has to be compared either to another C_q value in another sample (to calculate relative expression), or to a standard curve where known amounts of the target have been analyzed

(for absolute quantification). In addition, any C_q value must be compared to the C_q value obtained in the absence of the target (i.e., the background signal), as determined by RT and PCR control reactions.

To determine the true sensitivity of a qPCR assay, it is necessary to run a dilution series of known input amounts including a negative, no-template control (e.g., a blank purification replacing the biofluid with water). The limit of detection is usually defined as the last point on a curve within the linear range, excluding any data point outside of the linear regression curve. An example of a dilution series used to compare the sensitivity of different qPCR assays is shown in Figure 18.

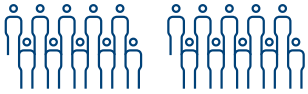
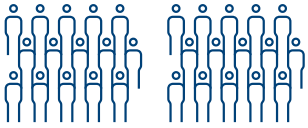
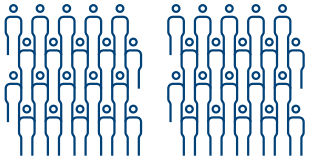
	Initial screening	**Profiling**	**Validation**
	Limited sample size: 	Medium sample size: 	Large sample size: 
	Identify subset of relevant miRNAs	Profile miRNAs and reference genes	Validate differentially expressed miRNAs
Serum/plasma	**miRNA PCR Panels:** miRNome Serum/Plasma Focus	**miRNA PCR Panels:** Serum/Plasma Focus Custom Panels	**miRNA PCR Panels:** Custom Panels Individual assays
Urine	**miRNA PCR Panels:** miRNome Urine Exosome Focus	**miRNA PCR Panels:** Urine Exosome Focus Custom Panels	**miRNA PCR Panels:** Custom Panels Individual assays
CSF	**miRNA PCR Panels:** miRNome CSF Exosome Focus	**miRNA PCR Panels:** CSF Exosome Focus Custom Panels	**miRNA PCR Panels:** Custom Panels Individual assays

Table 9. Overview of miRCURY LNA miRNA PCR Panels for miRNA profiling in biofluids. The PCR panels are available as orderable products or as a service through QIAGEN Genomic Services. Custom PCR Panels can be fully customized to include any pre-designed or custom miRCURY LNA miRNA PCR Assay. Validation of initial NGS or PCR screening results can be performed using customized PCR panels or individual assays.

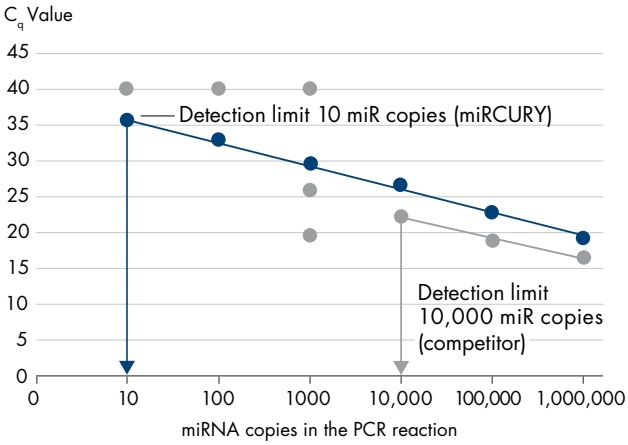


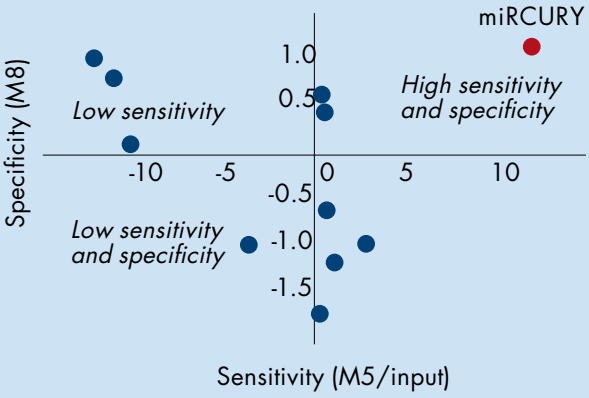
Figure 18. Sensitivity is defined by the limit of detection, not the C_q value. The sensitivity of miRNA PCR assays are compared using a dilution series of synthetic miRNA template to identify the limit of detection. Using a dilution series of hsa-let-7a, the C_q values from the competitor's miRNA PCR assay are lower than those of the miRCURY LNA miRNA PCR Assay. Despite this, the competitor assay is less sensitive than the miRCURY assay, because the linear regression curve from the dilution series can only be extended to 10,000 copies and not 10 copies.

The best combination of sensitivity and specificity

Biofluid samples may contain fragments or degradation products of other RNA species, so it is important to use a highly specific detection method able to discriminate miRNAs both from contaminating RNA fragments, and from other closely related miRNA family members. Accurate miRNA quantification requires a linear relationship between signal and template concentration. When compared to other miRNA profiling systems, the miRCURY LNA miRNA PCR System was the only one offering both high sensitivity and high specificity, as demonstrated in the miRQC study, the largest comparison of miRNA profiling platforms, which was published in Nature Methods. (16)

miRCURY performs best in miRQC – the largest miRNA benchmarking study to date

In a large study published in Nature Methods, Pieter Mestdagh et al. (16) compare the performance of commercially available miRNA profiling platforms in key areas. The miRCURY platform was the only top performer in all areas, combining both high sensitivity and specificity.



Validated PCR assays

Over 1200 of the PCR assays available in the miRCURY LNA miRNA PCR System have been wet-bench validated to ensure that they meet strict performance criteria, and over 95% of the assays are sensitive down to less than 10 RNA copies in the PCR reaction (Figure 19). Below 10 copies, variation in C_q values can be affected simply by stochastic variance in the number of template molecules pipetted into the qPCR reaction.

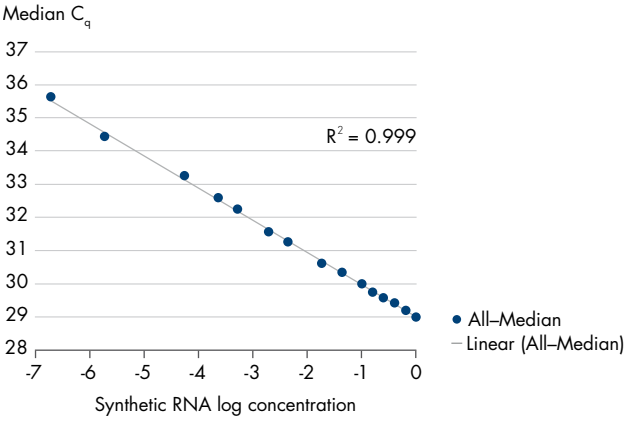


Figure 19. miRCURY LNA miRNA PCR Assays demonstrate excellent sensitivity and linearity over a wide range of template concentrations. A pool of synthetic templates for 647 miRNAs was subjected to serial dilution (15–1500 copies of each template RNA in the PCR reaction) in a background of MS2 bacteriophage total RNA and assayed by RT-qPCR. The median C_q value for all assays was plotted against template concentration.

Normalization

Data normalization in miRNA qPCR

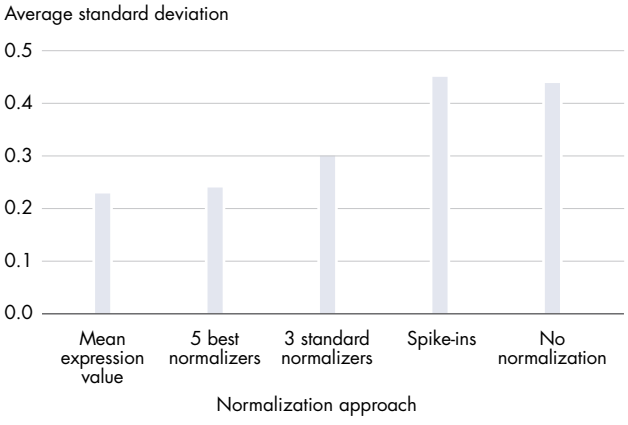
The purpose of normalization is to remove technical variation in data not related to the biological changes under investigation. Proper normalization is critical for the correct analysis and interpretation of results. The most commonly used methods for normalization are to:

- Use the mean expression value of all commonly expressed miRNAs in a given sample as normalization factor. (17) This is usually the best approach when analyzing PCR panels containing a larger number of miRNA assays.
- Identify and use stably expressed reference genes.

The GeneGlobe® data analysis center supports both of these approaches, and it is recommended to investigate which method provides the best normalization of a given dataset. 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 20).

We recommend using stably expressed endogenous miRNAs as reference genes, rather than larger small RNA species, such as 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 miRNAs. Larger RNAs may also behave differently during RNA purification.

Furthermore, the number of miRNAs detected in cell-free biofluid samples is usually rather low. When using mean expression values for normalization, a high number of miRNAs needs to be expressed. Therefore, as a general



Normalization approach	Normalization to the average of:	Conclusion
Mean expression value	90 miRNAs detected in all samples	Superior method for normalization
5 best normalizers	The 5 most stably expressed miRNAs 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 miRNAs 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 20. Normalization of miRNA qPCR data from biofluids to the mean expression value was most successful in minimizing standard deviation across samples. RNA isolation was performed using 120 plasma samples in duplicate, adding RNA spike-ins to the lysis buffer. miRNA profiling was performed on all RNA samples using the miRCURY LNA miRNA miRNome Human Panel I+II (742 miRNAs).

guideline, consider identifying stably expressed reference genes for use in normalization if the number of detected miRNAs is below 20–50.

»Stably expressed endogenous miRNAs are recommended for normalization«

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 into cell-free biofluids, affecting the overall miRNA content and thus impacting the mean expression value. Hence, it is crucial to monitor RNA samples for signs of cellular contamination or hemolysis (see section "Basic QC – standard human serum & plasma samples" on page 20) and ensure that the miRNAs selected for use as normalizers are not affected by hemolysis. (18)

Selecting reference genes

When using stably expressed reference genes for normalization, it is recommended to test 5–6 candidates before setting up the actual miRNA 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, these candidates are typically chosen based on the literature or pre-existing data (e.g., qPCR panel screening). Several miRNAs are available on the miRCURY LNA miRNA PCR panels as candidate reference genes. These are typically medium- to highly expressed genes and may be stably expressed, but their use as reference genes needs to be evaluated on a study-to-study basis. We have identified a number of miRNAs usually stably expressed in serum/plasma (Table 6).

Challenges particular to biofluids

Storage and/or transportation of non-stabilized blood as well as certain treatments can result in dramatic changes in the overall miRNA content of biofluid samples (e.g., as seen with drug-induced toxicity). In these situations, it may be very challenging to identify stably expressed miRNAs, and the mean expression value of all miRNAs may not be stable either, so alternative strategies such as use of miRNA ratios may need to be considered. External factors that are difficult to quantify, such as sample transportation, can be best avoided by using stabilization tubes like PAXgene Blood RNA or PAXgene Blood ccfDNA Tubes (see section "Sample collection and stabilization" on page 5).

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, the state of hydration as well as potential disease state. Therefore, care should be taken when selecting stable reference genes, and it may also be necessary to normalize to urinary volume or creatinine levels.

For other biofluid samples, we recommend to empirically determine the best reference miRNAs by doing an initial pilot study to identify the best candidates. For further details regarding experimental setup and data analysis of miRNA qPCR experiments, refer to the *miRCURY LNA miRNA PCR – Exosomes, Serum/Plasma and Other Biofluid Samples Handbook,* which is available at www.qiagen.com.

Data QC for miRNA qPCR analysis

In challenging samples such as biofluids, characterized by low levels of miRNAs combined with the presence of PCR inhibitors, the C_q values obtained will often be at the high end (in the range of 30–35). This is acceptable as long as the signal can be easily distinguished 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 parameters as part of the quality control procedure:

- 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 shows no signs of PCR inhibition
- Sample C_q is clearly discerned from any background (e.g., at least 5 C_q below negative control / blank purification)

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

»SYBR® Green-based qPCR systems enable important quality control of the data by melting curve analysis«

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Ordering Information

Product	Contents	Cat. no.
Sample stabilization and isolation		
exoRNeasy Midi Kit	For purification of RNA from exosomes and other extracellular vesicles out of serum or plasma samples	77144
exoRNeasy Maxi Kit	For purification of RNA from exosomes and other extracellular vesicles out of serum or plasma samples	77164
exoEasy Maxi Kit	For purification of exosomes and other extracellular vesicles (EVs) from plasma, serum and cell culture supernatant	76064
miRCURY Exosome Serum/Plasma Kit	For enrichment of exosomes and other extracellular vesicles from serum/plasma samples	76603
miRCURY Exosome Cell/Urine/CSF Kit	For enrichment of exosomes and other extracellular vesicles from cell/urine/CSF samples	76743
miRNeasy Serum/Plasma Advanced Kit	For phenol-free isolation of total RNA including miRNA from serum and plasma samples	217204
miRNeasy 96 Advanced QIAcube HT Kit	For automated purification of total RNA, including miRNA, from serum and plasma samples	217261
PAXgene Blood RNA Tubes*	For collection, transport and storage of blood and stabilization of intracellular RNA for molecular testing	762125
PAXgene Blood ccfDNA Tubes*	For collection of whole blood samples and stabilization of ccfDNA	768165
PAXgene Blood miRNA Kit	For purification of miRNA and total RNA from whole blood	763134
QIAsymphony PAXgene Blood RNA Kit	For automated purification of intracellular RNA including miRNAs from stabilized blood	762635
NGS solutions		
QIAseq miRNA Library Kit	For gel-free miRNA NGS library prep for differential expression analysis and novel discovery	331502
QIAseq miRNA Library QC PCR Array Kit	For evaluating RNA sample quality prior to miRNA/small RNA NGS library preparation and for assessing NGS performance post-sequencing	331541
QIAseq miRNA Library QC Spike-Ins	For evaluating RNA sample quality prior to miRNA/small RNA NGS library preparation and for assessing NGS performance post-sequencing	331535
CLC Genomics Workbench	For analyzing, comparing and visualizing NGS data	832000
Ingenuity Pathway Analysis (IPA)	For modeling, analyzing and understanding complex 'omics data	830000

* Product is intended for in vitro diagnostic use.

Ordering Information

Product	Contents	Cat. no.
PCR analysis solutions		
miRCURY LNA RT Kit	For optimal first-strand cDNA synthesis with the miRCURY LNA miRNA PCR System	339340
RNA Spike-In Kit, for RT	For QC of the RNA isolation and cDNA synthesis steps of miRCURY LNA miRNA PCR experiments	339390
miRCURY LNA SYBR® Green PCR Kits	For high-performance PCR with the miRCURY LNA miRNA PCR System	339345–339347
miRCURY LNA miRNA PCR Starter Kit	For setup and optimization of miRNA quantification experiments	339320
miRCURY LNA miRNA PCR Assay	For extremely sensitive and specific miRNA quantification using LNA-optimized miRNA PCR	339306
miRCURY LNA miRNA PCR Focus Panels (Serum/Plasma, Cancer, CSF-Exosome, and Urine-Exosome)	For application-based miRNA profiling using LNA-enhanced miRNA qPCR	339325
miRCURY LNA miRNA miRNome PCR Panels	For exceptionally sensitive and specific miRNA profiling using LNA-enhanced miRNA qPCR	339322
miRCURY LNA Custom PCR Panel	Custom-formatted 96- and 384-well qPCR plates for the miRCURY LNA miRNA PCR System	339330
miRCURY LNA miRNA QC PCR Panel	For quick and easy RNA quality control of miRNA samples prior to qPCR profiling	339331

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