October 2017

miRCURY® LNA® miRNA QC PCR Panel Handbook

For performing quality control of the RNA purification process and isolated miRNA

Sample to Insight

QIAGEN

Contents

Kit Contents

The miRCURY LNA miRNA QC PCR Panel (cat. no. 339331) consists of either 96-well or 384-well PCR plates containing dried-down LNA PCR assays for one 10 µl real-time PCR reaction per well. The 96-well format contains assays for analyzing 8 samples, and the 384-well format contains assays for analyzing 32 samples.

The miRCURY LNA miRNA QC PCR Panel is available in six different plate formats:

Plate format	Product number	Suitable real-time cyclers
A	YAHS-999YA-2	Applied Biosystems® models 5700, 7000, 7300, 7500, 7700, 7900HT, ViiA 7 (96-well block); Bio-Rad® models iCycler®, iQ5, MyiQ, MyiQ2; Bio-Rad / MJ Research Chromo4; Eppendorf® MasterCycler® ep realplex models 2, 2s, 4, 4s; Stratagene® models Mx3005P®, Mx3000P®; Takara: TP-800
C	YAHS-999YC-2	Applied Biosystems models 7500 (Fast block), 7900HT (Fast block), StepOnePlus, ViiA 7 (Fast block)
D	YAHS-999YD-2	Bio-Rad CFX96; Bio-Rad / MJ Research models DNA Engine Opticon®, DNA Engine Opticon 2; Stratagene Mx4000®
E	YAHS-999YE-1	Applied Biosystems models 7900HT (384-well block), ViiA 7 (384-well block); Bio-Rad CFX384
F	YAHS-999YF-2	Roche® LightCycler® 480 (96-well block)
G	YAHS-999YG-1	Roche: LightCycler 480 (384-well block)

Storage

The miRCURY LNA miRNA QC PCR Panel is shipped at room temperature with the DNA primers dried down. Upon arrival the panel(s) should be stored at –15 to –30ºC. Under these conditions, all components are stable for at least 12 months.

Intended Use

The miRCURY LNA miRNA QC PCR Panel is intended for molecular biology applications. These products are not intended for the diagnosis, prevention or treatment of a disease.

All due care and attention should be exercised in the handling of the product. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety** where you can find, view and print the SDS for each QIAGEN kit and kit component.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of miRCURY LNA miRNA QC PCR Panel is tested against predetermined specifications to ensure consistent product quality.

Introduction

The miRCURY LNA miRNA QC PCR Panel lets you analyze the robustness of the RNA isolation process and quality of isolated miRNA. The panel consists of either 96-well or 384-well PCR plates containing dried-down LNA PCR assays for one 10 µl real-time PCR reaction per well. The LNA PCR assays are designed for optimal performance with the miRCURY LNA RT Kit (cat. no. 339340) and the miRCURY LNA SYBR® Green PCR Kit (cat. nos. 339345, 339346, 339347). The performance of these PCR panels will be affected if they are used in combination with other reagents.

Principle and workflow

First, the panel contains matching locked nucleic acid (LNA) PCR assays for detection of the RNA Spike-In Kit, for RT (cat. no. 339390), UniSp2, UniSp4 and UniSp5. The RNA Spike-In Kit, for RT provides a control for the quality of the RNA isolation in any miRNA quantitative reverse-transcription PCR (qRT-PCR) experiment. Reproducible RNA isolations can be difficult from certain types of samples. One way to check for differences in isolation yields is by adding known RNA spike-ins to the sample prior to RNA isolation. Use of RNA spike-ins may also reveal presence of nucleases. After conducting the qRT-PCR but before progressing into data analysis, wells detecting the RNA spike-ins are compared and outlier samples may be identified and considered for exclusion in the downstream processing and analysis. For detailed information see the *RNA Spike-In Kit, for RT Handbook*.

The panel also contains matching LNA PCR assays for detecting the spike-ins UniSp6 and cel-miR-39-3p to monitor cDNA synthesis and UniSp3 IPC (inter-plate calibrator) to check if the qPCR was successful. Some RNA samples may contain compounds that inhibit the cDNA synthesis or the qPCR reaction, even though the RNA has been purified using the best standard procedures. This may result in efficiency differences of the reverse transcription or PCR between samples, causing variation.

In addition, the panel contains matching LNA PCR assays for detecting six miRNAs to evaluate the biological samples that are being interrogated with regards to downstream processing. These miRNAs include:

- miR-103-3p and miR-191-5p, which are well expressed in most tissues
- miR-451a and miR-23a-3p, which are found in plasma and serum and serve as a hemolysis marker and an internal control, respectively
- miR-30c-5p, which is well expressed in kidney and found in urine samples
- miR-124-3p, which is well expressed in central nervous system tissues and found in cerebrospinal fluid (CSF)

This set of assays can indicate not only if the RNA isolation was successful, but also if the biological samples are of similar quality with regards to miRNAs expected to be present in a given sample set.

The workflow involves a two-step procedure:

- First-strand cDNA synthesis using the miRCURY LNA RT Kit (cat. no. 339340) and the RNA Spike-In Kit, for RT (cat. no. 339390)
- Quantitative, real-time PCR using the miRCURY LNA miRNA QC PCR Panel (cat. no. 339331) and the miRCURY LNA SYBR Green PCR Kit (cat. nos. 339345, 339346, 339347)

RNA quality assessment PCR panel reagents

The miRCURY LNA miRNA QC PCR Panel contains the following LNA PCR assays for use with the miRCURY LNA miRNA PCR System.

Table 1. Assays included in the miRCURY LNA miRNA QC PCR Panel.

	Assay (common name*)	Human, miRbase v20	Mouse, miRbase v20
miRNA assay	miR-103	hsa-miR-103a-3p	mmu-miR-103-3p
miRNA assay	miR-191	mmu-miR-191-5p	mmu-miR-191-5p
miRNA assay	miR-451	hsa-miR-451a	mmu-451
miRNA assay	miR-23a	hsa-miR-23a-3p	mmu-miR-23a-3p
RT assay	UniSp6, v2*	–	–
Spike-in assay	UniSp2	–	–
Spike-in assay	UniSp4	–	–
Spike-in assay	UniSp5, v2*	–	–
RT assay	cel-miR-39-3p	–	–
Inter-plate calibrator / PCR control	UniSp3	–	–
miRNA assay	miR-124	hsa-miR-124-3p	mmu-miR-124-3p
miRNA assay	miR-30c	hsa-miR-30c-5p	mmu-miR-30c-5p

* In the species rno, cfa and mml (rat, dog and rhesus), the common names are used for these miRNAs.

† New, optimized UniSp5 and UniSp6 assays designed for V4 panels target same RNA template as previous assays.

The miRCURY LNA miRNA QC PCR Panel only comprises the LNA PCR assays. It is designed to be used together with the RNA Spike-In Kit, for RT. For instructions on how to use the Spike-In Kit, for RT with RNA isolation procedure, see the *RNA Spike-In Kit, for RT Handbook*. A full description of the miRCURY LNA miRNA PCR System can be found in the *miRCURY LNA miRNA PCR Handbook* and the *miRCURY LNA miRNA PCR – Exosomes, Serum/Plasma and Other Biofluid Samples Handbook*.

Layout of the plate

The 12 assays in the miRCURY LNA miRNA QC PCR Panel are arranged in the plate as shown in Figure 1. The 96-well format contains assays for analyzing 8 samples, and the 384-well format contains assays for analyzing 32 samples.

Figure 1. Layout of the 12 assays in the plates. The miRCURY LNA miRNA QC PCR Panel contains 6 LNA-enhanced PCR assays for quality control of RNA samples (light gray) and PCR efficiency (dark gray). In addition, 5 LNA PCR assays that target miRNAs are included. These assays target the following: miRNAs expressed in most tissues (blue), miRNAs expressed in kidney (purple) and brain (green). PCR assays targeting blood miRNAs are also part of the panel (red). These assays can be used as controls of hemolysis.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, consult the appropriate safety data sheets (SDSs) available from the product supplier.

Reagents:

- miRCURY LNA miRNA PCR System materials
- miRCURY LNA SYBR Green PCR Kit (cat. nos. 339345, 339346, 339347)
- miRCURY LNA RT Kit (cat. no. 339340)
- RNA Spike-In Kit, for RT (cat. no. 339390)

Materials and equipment:

- Nuclease-free PCR tubes or plates for use with individual LNA PCR assays
- Nuclease-free, aerosol-barrier pipette tips
- Nuclease-free, low nucleic acid binding (siliconized) microcentrifuge tubes
- Sealing foil for PCR plates
- Microcentrifuge and plate centrifuge
- Heating block, thermal cycler or other incubators
- Real-time PCR instrument

Important Notes

RNA input

The miRCURY LNA miRNA QC PCR Panel protocol is optimized for using 10 ng total RNA per cDNA synthesis reaction. The exact amount of total RNA needed depends on whether the downstream application is individual assays or panels. Furthermore, the amount of total RNA to be used may also vary depending on the miRNA expression levels in the cells or tissue to be analyzed. For highly expressed miRNAs, it is possible to use down to 10 pg total RNA as starting material. For weakly expressed miRNAs, it may be possible to use up to 200 ng of total RNA; however, in samples with high amounts of PCR inhibitors (e.g., FFPE tissue samples), this may not be feasible. Finally, inhibitors may be present in RNA preparations from certain samples (e.g., serum and plasma). Prior to conducting a larger miRNA profiling study, it is recommended to optimize the amount of input RNA to the RT reaction to avoid conducting a larger study where inhibition occurs sporadically throughout the dataset.

Information on how to extract and handle RNA can be found in the *miRCURY LNA miRNA PCR Handbook*. Briefly, total RNA should be prepared using a method that preserves small RNA species. DNase treatment may be necessary. When using commercially available kits, ensure that the total RNA preparation is guaranteed to contain miRNAs.

miRNA from serum and plasma

Serum and plasma are complex sample types that require special RNA isolation procedures, and the amount of RNA present in the samples usually cannot be accurately determined. Due to the low levels of miRNAs and potentially high levels of inhibitors in samples derived from serum and plasma, we have prepared specific recommendations for setting up experiments with these sample types; refer to the *miRCURY LNA miRNA PCR Handbook – Exosomes, Serum/Plasma and Other Biofluid Samples* for complete details.

Passive reference dye

For certain real-time cyclers, the presence of ROX passive reference dye in real-time PCR compensates for non-PCR-related variations in fluorescence detection. Fluorescence from ROX dye does not change during the course of real-time PCR, but provides a stable baseline to which PCR-related fluorescent signals are normalized. Thus, ROX dye compensates for differences in fluorescence detection between wells due to slight variations in reaction volume or to differences in well position. ROX dye does not interfere with real-time PCR, since it is not involved in the reaction and has an emission spectrum different from the SYBR Green dye.

The use of ROX dye is necessary for instruments from Applied Biosystems. The miRCURY SYBR Green PCR Kit includes a separate tube of ROX Reference Dye, which can be added to the real-time PCR if you are using a real-time cycler that uses ROX as a passive reference dye. For instruments requiring a high concentration of ROX dye, use the ROX Reference Dye as a 20x concentrate. For instruments requiring a low concentration of ROX dye, use the dye as a 200x concentrate. Refer to Table 2 for details on real-time cyclers that require low or high ROX concentrations. If desired, ROX Reference Dye can be diluted with 2x miRCURY SYBR Green PCR Master Mix for long-term storage (Table 3). For details, see "Adding ROX dye to the PCR master mix" on page 13.

Table 2. Real-time cyclers requiring high/low concentrations of ROX.

High ROX concentration (ROX Reference Dye to be used at a 20x dilution)	Low ROX concentration (ROX Reference Dye to be used at a 200x dilution)
ABI PRISM® 7000	Applied Biosystems 7500
Applied Biosystems 7300	Applied Biosystems ViiA™ 7
Applied Biosystems 7900	Applied Biosystems QuantStudio® Systems
Applied Biosystems StepOne™	
Applied Biosystems StepOne Plus	

Adding ROX dye to the PCR master mix

If you only use cyclers from Applied Biosystems with the miRCURY SYBR Green PCR Kit, you can add ROX Reference Dye to the 2x miRCURY SYBR Green PCR Master Mix for long-term storage, if desired (Table 3). For information on the concentration of ROX required for Applied Biosystems instruments, refer to Table 2.

Table 3. Addition of ROX Reference Dye to master mix.

Volume of 2x miRCURY SYBR Green PCR Master Mix (without ROX Reference Dye)	Volume of ROX Reference Dye for high ROX concentration/low ROX concentration
1 ml	100 µl / 10 µl
10 ml	1 ml / 100 µl

ABI instruments

The default settings on ABI real-time PCR cyclers are not suitable for running miRCURY LNA miRNA PCR Assays. Settings must be changed from automatic to manual background and threshold settings to obtain valid PCR data. Furthermore, if the dataset is to be analyzed using the GenEx analysis software, it is important that the experiment is set up as an AQ experiment, instead of RQ.

Protocol: First-Strand cDNA Synthesis

This protocol describes performing first-strand cDNA synthesis reactions using the miRCURY LNA RT Kit (cat. no. 339340) and the RNA Spike-In Kit, for RT (cat. no. 339390).

Important points before starting

- Keep reagents and reactions on ice (or at 4ºC) at all times.
- We recommend using low-nucleic acid binding tubes or plates.
- Storage of diluted cDNA is not recommended.

Things to do before starting

- Gently thaw the 5x Reaction Buffer and Nuclease-free water, and immediately place on ice. Mix by vortexing.
- Re-suspend the RNA spike-in UniSp6 and cel-miR-39-3p according to the description in the *RNA Spike-In, for RT Handbook*.
- Immediately before use, remove the enzyme mix from the freezer, mix by flicking the tubes and place on ice. Spin down all reagents.

Procedure for first-strand cDNA synthesis

1. Dilute each template RNA sample to 5 ng/µl using nuclease-free water.
2. Prepare the reverse transcription reaction mix according to Table 4 and place it on ice.

   If you are setting up multiple reactions, prepare an RT reaction master mix containing the 5x Reaction Buffer, Nuclease-Free Water, Enzyme Mix and Spike-In Mix and dispense 8 µl into nuclease-free tubes. Place on ice.

3. Add 2 µl template RNA to each tube of reaction mix. We highly recommend including a non-template control (NTC) reaction.

   Note: If working with low yield samples (e.g., from plasma or serum), do not exceed 2 µl of RNA in the final cDNA reaction.

Table 4. Reverse transcription reaction setup per sample.

Reagent	Volume
5x Reaction buffer	2 µl
Nuclease-free water	4.5 µl
Enzyme mix	1 µl
Synthetic spike-in mix	0.5 µl
Template total RNA*	2 µl
Total volume	**10 µl**

* Replace with nuclease-free water for non-template controls.

4. Gently mix, briefly centrifuge and place on ice.
5. Incubate for 60 min at 42ºC.
6. Incubate for 5 min at 95ºC to heat inactivate the reverse transcriptase.
7. Immediately cool to 4°C.
8. Store the cDNA samples in low nucleic acid binding tubes for up to 4 days at 4°C or at –15 to –30ºC for up to 5 weeks.

Protocol: Quantitative, Real-Time PCR

This protocol describes performing quantitative, real-time PCR using the miRCURY LNA miRNA QC PCR Panel (cat. no. 339331) and the miRCURY LNA SYBR Green PCR Kit (cat. nos. 339345, 339346, 339347).

Important points before starting

- Keep reagents and reactions on ice (or at 4ºC) at all times.
- We recommend using low-nucleic acid binding tubes or plates.
- We do not recommend storing diluted cDNA.
- This protocol is optimized for detection of miRNA targets with any real-time cycler and conditions for fluorescence normalization. ROX dye is required at the following concentrations:

  No requirement for ROX dye: Rotor-Gene®, Bio-Rad® CFX, Roche® LightCycler® 480 and Agilent® Technologies Mx instruments.

  Low concentration of ROX dye: Applied Biosystems 7500, ViiA 7 and QuantStudio Real-Time PCR Systems.

  High concentration of ROX dye: ABI PRISM® 7000, Applied Biosystems 7300, 7900 and StepOne Real-Time PCR Systems.
- The ROX Reference Dye should be used as a 20x concentrated solution for a 1x reaction when using an instrument requiring a high ROX dye concentration. For instruments requiring a low ROX dye concentration, use the dye as a 200x concentrate.

Things to do before starting

- Place the cDNA (from step 8), nuclease-free water and miRCURY LNA SYBR Green Master Mix on ice and thaw for 15–20 min. Protect the Master mix vials from light. Immediately before use, mix the Master mix by pipetting up and down. Vortex the rest of the reagents and briefly centrifuge.

Procedure for quantitative PCR

1. Prepare the PCR reaction mix. For cDNA from tissues or cells, refer to Table 5. For cDNA from biofluids, refer to Table 6. Mix gently by inverting the tube, and briefly centrifuge.

Table 5. Reaction setup for miRCURY LNA miRNA QC PCR Panels with cDNA from tissues or cells.

Component	QC PCR Panel consisting of 1 x 96 assays	QC PCR Panel consisting of 1 x 384 assays
2x miRCURY SYBR Green Master Mix	500 µl	2000 µl
ROX Reference Dye (ABI instruments only)	50 µl/5 µl*	200 µl/20 µl*
cDNA template from tissue or cell lines	5 µl	20 µl
RNase-free water	495 µl*	1980 µl*
Total reaction volume	**1000 µl**	**4000 µl**

Table 6. Reaction setup for miRCURY LNA miRNA QC PCR Panels with cDNA from biofluids.

Component	QC PCR Panel consisting of 1 x 96 assays	QC PCR Panel consisting of 1 x 384 assays
2x miRCURY SYBR Green Master Mix	500 µl	2000 µl
ROX Reference Dye (ABI instruments only)	50 µl/5 µl*	200 µl/20 µl*
cDNA template from biofluids	10 µl	40 µl
RNase-free water	490 µl*	1960 µl*
Total reaction volume	**1000 µl**	**4000 µl**

* Use ROX Reference Dye as a 20x concentrate for cyclers requiring a high ROX dye concentration (i.e., ABI PRISM 7000, Applied Biosystems 7300, 7900 and StepOne Real-Time PCR Systems) and as a 200x concentrate for cyclers requiring a low ROX dye concentration (i.e., Applied Biosystems 7500, ViiA7 and QuantStudio Real-Time PCR Systems). Adjust the amount of RNase-free water accordingly.

2. Briefly centrifuge the plate(s) containing the PCR panel before removing the plate seal to ensure the primers are at the bottoms of the wells.
3. Add 10 µL of the Master mix:cDNA mixture to each well.

4. Seal the plate with optical sealing as recommended by the instrument manufacturer.

   Note: If necessary, the experiment can be paused at this point. Store the reactions protected from light at 4°C for up to 16 hours.

5. Briefly centrifuge the plate (1500 *x g* for 1 minute) to remove air bubbles.

6. Perform real-time PCR amplification followed by melting curve analysis according to Table 7 below.

Table 7. Cycling conditions for real-time PCR

Step	Settings for LC480 instrument*	Settings for other instruments
Polymerase activation/ denaturation	2 min	2 min
Amplification	45 cycles: 95ºC, 10 s 56ºC, 60 s Optical read	40 cycles: 95ºC, 10 s 56ºC, 60 s Optical read
Melting curve analysis†	yes	yes

* Five additional amplification cycles are required when using the LC480 instrument to allow collection of assay data with C_p values up to 40.

† Melting curve analysis of the PCR product(s) is recommended to verify specificity and identity of the amplification reaction. Melting curve analysis is an analysis step built into the software of the instruments. Follow the instructions provided by the supplier. The T_m of a PCR product depends on buffer composition, salt concentration and the PCR instrument.

7. Perform initial data analysis using the software supplied with the real-time PCR instrument to obtain raw C_q values (C_p or C_T, depending on PCR instrument). If you are using an ABI instrument, note that it is not recommended to use auto C_T settings.

   If you are using a Roche LC480 instrument, we recommend analysis using the 2nd derivative method.

   For interpretation of the miRCURY LNA miRNA QC PCR Panel data, refer to the guideline section on the next page.

miRNA QC PCR Panel Guideline

How to use this guideline

This section describes how to assess the quality of RNA samples prior to a miRNA profiling effort, to avoid spending valuable resources and time analyzing samples that are unlikely to add information to the sample set due to technical or biological challenges. The LNA PCR assays on the miRCURY LNA miRNA QC PCR Panel are aimed at different steps in the qRT-PCR process. RNA spike-ins from the RNA Spike-In Kit, for RT UniSp2, 4 and 5 enable evaluation of the RNA isolation efficiency and yield. The RNA spike-ins cel-miR-39-3p and the UniSp6 (supplied with the miRCURY LNA RT Kit) evaluate the cDNA synthesis step with regards to inhibition, and the UniSp3 (IPC) evaluates the qPCR reaction.

The following six miRNA assays are included on the panel:

- miR-103a-3p and miR-191-5p: well-expressed in most tissues
- miR-451a: highly expressed in serum and plasma and predominantly derived from red blood cells
- miR-23a-3p: more stably expressed in serum and plasma but not affected by hemolysis; together with miR-451a helps monitor hemolysis
- miR-30c-5p: well expressed in in many tissues, including the kidney and is widely found in urine samples
- miR-124-3p: well expressed in central nervous system derived tissues and is widely found in cerebrospinal fluid (CSF)

These six miRNA assays enable analysis of the biological quality of a number of different tissues, along with body fluids such as serum, plasma, urine or CSF. These assays also work with samples from humans, rat, mouse, dog and rhesus monkey.

How to use the spike-ins

In a typical experiment involving samples purified from tissue or biofluids, add the three RNA isolation spike-ins to the RNA isolation lysis buffer, and purify the samples according to the kit handbook. After isolation, prepare cDNA from the samples, adding UniSp6 and cel-miR-39-3p to the reverse transcription (RT) mixture. Analyze the samples using the miRNA QC PCR Panel, according the kit handbook.

An example of a plasma sample, isolated five times with three different RNA isolation kits (A, B and C) is shown below (Figure 2). Here, the RNA spike-in yields can be monitored (UniSp2, 4 and 5), as well as the potential presence of inhibitors (UniSp6) and the overall success of the qPCR reactions (UniSp3).

The UniSp2, UniSp4 and UniSp5 are present at a 100-fold concentration difference in the RNA Spike-in Kit, which results in approximately 5–7 C_q difference between the spike-ins. The exact difference depends on the amount of Spike-in mixture added, the yield of the isolation and the volume of the eluted RNA.

Note that the most dilute Spike-in (UniSp5) corresponds in amount to miRNAs expressed at a very low concentration. Therefore, UniSp5 could be undetected, or its high C_q values (low signal) could be filtered out during data processing. For more information on the RNA Spike-in Kit, for RT, see the *RNA Spike-In Kit, for RT Handbook*.

A more advanced version of the RNA input experiment is to prepare a two-fold serial dilution of the RNA into the cDNA reactions (e.g., add a serially diluted range of 0.5, 1, 2 and 4 µl RNA to the cDNA synthesis). This can give valuable information about how much RNA can be used before inhibition occurs when working with low concentrations of RNA or difficult material that is known to be challenged by inhibitors

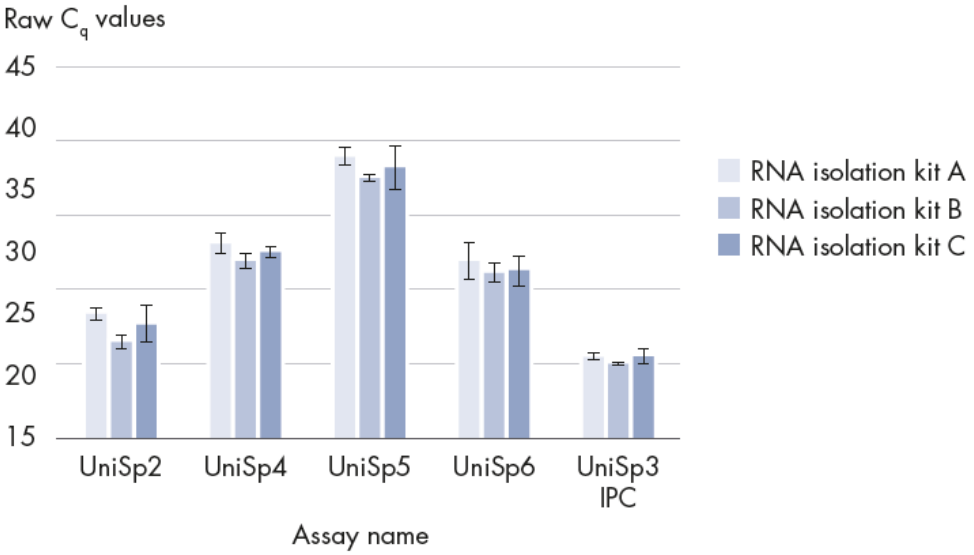


Figure 2. RNA isolation from one plasma sample using three different RNA isolation methods. Spike-in mixture was added to the kit lysis buffers according to the *RNA Spike-in Kit, for RT Handbook*. The amount of starting material was 200 µl, and the elution volume was 50 µl. The spike-in assessment demonstrated that RNA isolation kit B gave the best yields and that there was a potential inhibition with kit A (see the UniSp6), but the UniSp3 IPC (inter-plate calibrator), which monitors overall qPCR efficiency, showed comparable qPCR efficiency. The stepwise difference (ΔC_q) between the RNA isolation spike-ins (2,4,5) was in the expected range of 5–7 C_q. Error bars indicate standard deviations from the five replicate extractions.

Potential problems that can be revealed using the miRCURY LNA miRNA QC PCR Panel are described below.

Observation 1: High variance in the UniSp2, 4 and 5

Problem: High variance (>2–3 C_q difference within a dataset for a given spike-in) in the spike-in data reflects high variance in RNA yields or potential sporadic RNase contamination. Potential solutions include making sure the work area is free of RNases and that buffers and plasticware are RNase free. Make sure that isolation kits are not past their

expiration date and that the solutions are not contaminated. Re-isolate samples, making sure all steps are followed correctly or exclude outlier samples.

Observation 2: UniSp5 not detected

Problem: Potential RNAse contamination or low yields from the RNA isolation method, resulting in loss of data from miRNAs expressed at a low level. For potential solutions, see observation 1. Note however, that the lack of signal in UniSp5 could be normal, for example, if the elution volume is more than 50 µl or if a small amount of RNA was used for cDNA synthesis.

Observation 3: UniSp6 variation

Problem: If the UniSp6 shows high variation in the samples (>1–2 C_qs), and the C_q values suggest lower concentration of the UniSp6 in samples compared to the non-template-controls (NTCs), there may have been some inhibition in the cDNA synthesis. Potential solutions include adding an extra purification step or diluting the RNA more in the cDNA synthesis reaction.

Monitoring hemolysis

Circulating, cell-free miRNAs hold great promise as a new class of biomarkers due to their stability in biofluids such as plasma and serum and their association with various disease states. miRNAs in biofluids are protected in microvesicles such as exosomes or are bound to proteins such as argonaute 2 or high-density lipoproteins.

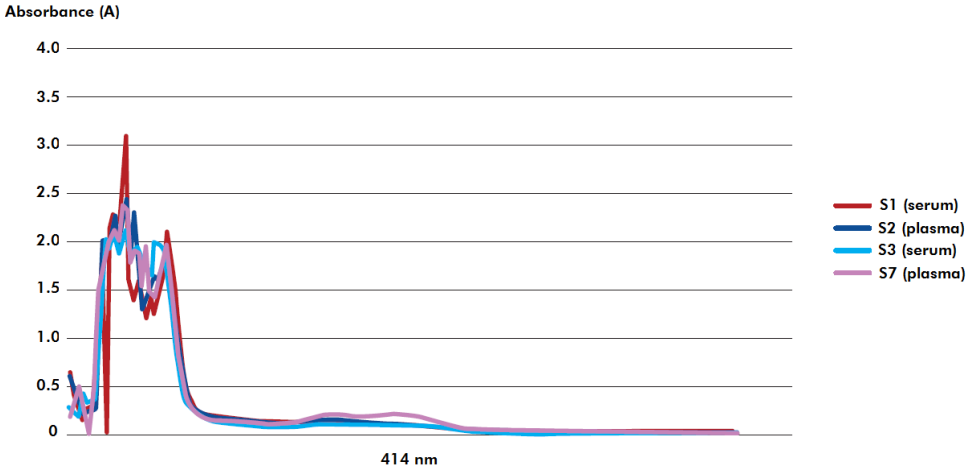
Although measuring miRNA in plasma and serum is straightforward using sensitive and specific methods like the miRCURY LNA miRNA PCR System, a number of technical challenges are associated with analysis of miRNA from biofluids. A major source of variation in plasma and serum comes from contamination from cellular-derived miRNA resulting from hemolysis. The data from the red blood cell specific miR-451a and the stable

miR-23a can be used to monitor hemolysis. After extensive data analysis on human serum and plasma samples, including monitoring hemolysis using spectrophotometric methods, we have found that a ΔC_q(miR-23a – miR-451a) lower than 5 in human serum or plasma represents non-hemolyzed samples. If the ΔC_q is close to or higher than 7, there is an increased risk of hemolysis. In case of high levels of hemolysis, miRNAs from red blood cells will make a significant contribution to the overall miRNA profile identified, and this may or may not disqualify the samples depending on the biological question – detection of altered expression of red blood cell miRNAs may be relevant. Also note that not all miRNAs are affected by hemolysis or the overall change studied may be considerably larger than the effect of hemolysis.

Large variations in the degree of hemolysis within a project may introduce noise to the data interpretation and removal of outlier samples should be considered.

Note: Although the miRCURY LNA miRNA QC PCR Panel contains assays for miRs that are the same in human (hsa), mouse (mmu), rat (rno), dog (cfa) and rhesus macaque (mml), ΔC_qs for other than human have not been experimentally determined. Therefore, we recommend looking at hemoglobin contamination as well as the ΔC_q to determine the best ΔC_qs for your samples, as it might not be the same as for human samples, described above.

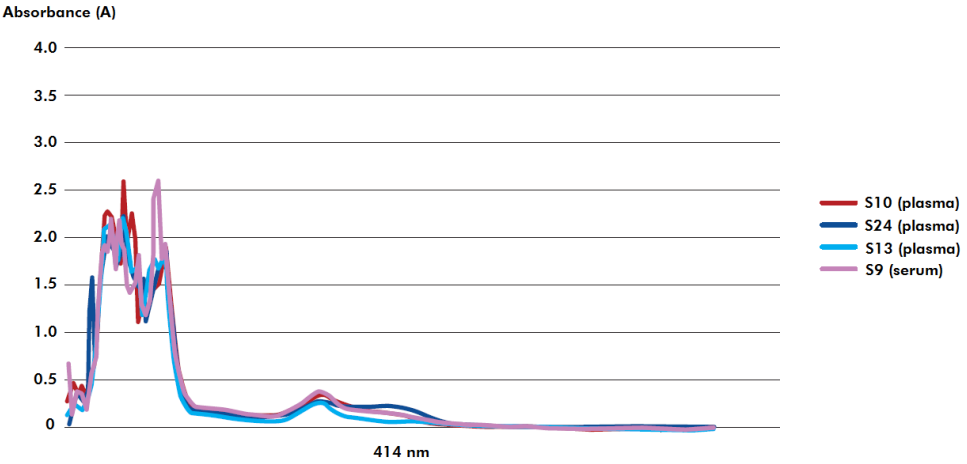
Examples of four human serum/plasma samples that are not affected by hemolysis are shown in Figure 3. All values for the ΔC_q are below 5, and no distinct peak of oxyhemoglobin is detected on the spectra (λ=414 nm).



Name/Assay	hsa-miR-23a-3p	hsa-miR-451a	23a-3p – 451a
S1 (serum)	29.0	24.1	4.9
S2 (plasma)	28.7	24.6	4.1
S3 (serum)	28.9	24.8	4.1
S7 (plasma)	29.0	24.2	4.8

Figure 3. Four human plasma and serum samples were analyzed with regards to miR-23a-3p and miR-451a, and the ΔC_q (miR-23a-3p – miR-451a) was determined. The four samples were also analyzed by spectrophotometer (Nanodrop ND-1000) from Δ220-720 nm. No distinct peak of oxy-hemoglobin (maximum absorbance at 414 nm) was detected in the samples. Sample S7 (plasma) showed potential bilirubin contamination (broad absorbance peak at 350–500nm). All four samples pass the hemolysis criteria.

When there is a hemolysis problem, the expression of miR-451a increases independently of miR-23a. This results in higher ΔC_q values than with non-hemolyzed samples. Figure 4 shows examples of hemolyzed samples and how the ΔC_q is affected. As seen on the spectrophotometer graphs, there is a distinct peak in all samples at λ=414nm, corresponding to oxy-hemoglobin contamination.



Name/Assay	hsa-miR-23a-3p	hsa-miR-451a	23a-3p – 451a
S10 (plasma)	29.5	21.9	7.6
S24 (plasma)	29.8	23.1	6.7
S13 (plasma)	29.1	21.8	7.3
S9 (serum)	30.0	21.5	8.5

Figure 4. Four human plasma and serum samples were analyzed with regards to miRNA-23a-3p and miRNA-451a, and the ΔC_q (miR-23a-3p – miR-451a) was determined. Three of the samples had ΔC_q higher than 7 and one was close to 7. The four samples were also analyzed by spectrophotometer (Nanodrop ND-1000) from λ220–750 nm. A distinct peak of oxy-hemoglobin was detected in all the samples. Sample S24 (plasma) also showed potential bilirubin contamination.

An example of how a human plasma dataset can be evaluated is shown in Figure 5. The ΔC_q of the sample set shows that it is challenged by hemolysis. Thirteen out of 34 samples (40%) have a ΔC_q around or above 7. In this case, we recommend the following actions:

- Consider excluding samples with $\Delta C_q > 7$ if the biomarkers investigated are expected to be very weak.
- If possible, collect and analyze more samples to identify more non-hemolyzed samples.

- Look at the blood collection protocol to try to identify the technical cause for this hemolysis problem.

We recommend following the NCI's Early Detection Research Network (EDRN) standard operating procedures for the collection of serum and plasma:
http://edrn.nci.nih.gov/resources/standard-operating-procedures/standard-operating-procedures.

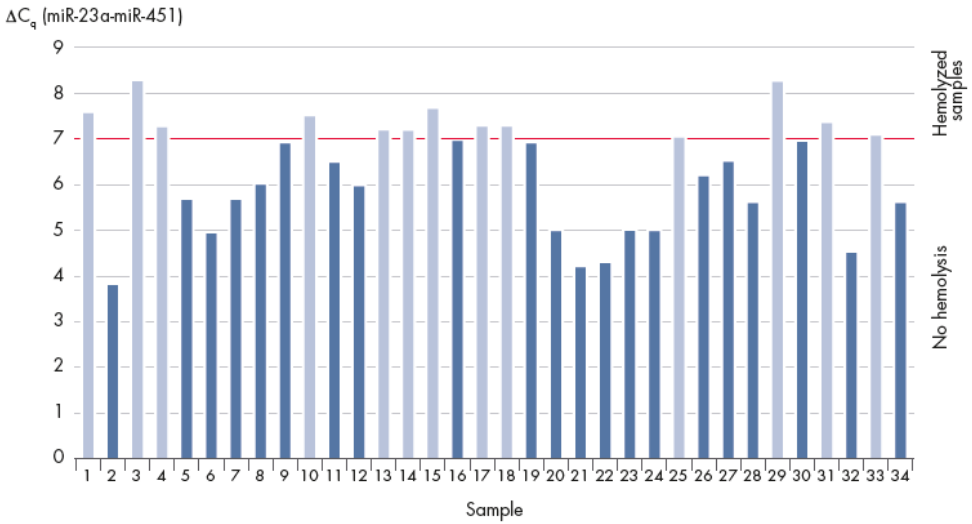


Figure 5. ΔCq (miR-23a-3p – miR-451a) values for a 34 sample cohort. It is clear that this sample set is affected by hemolysis. Almost half of the samples have a ΔC_q around or above 7. Some or all of the samples in red should possibly be removed from the study, if hemolysis is considered critical for the study.

We have also noted that miR-30c-5p is commonly present in urine samples. miR-124-3p is well expressed in the central nervous system, including the brain, and we have noted that it is often present in CSF samples.

The miRNAs on the panel enable monitoring of the overall biological content of the samples and assessment whether a subset of samples has very low miRNA content. This must be evaluated within any given sample set and on a tissue-to-tissue basis.

Figure 6 demonstrates variation of miRNAs isolated from different sample sources. Note that there will be biological variation within as well as between sample sets. Therefore, it is common to see a difference of a few C_qs, especially between normal and disease groups, for example, between tumor and normal adjacent tissue sample groups.

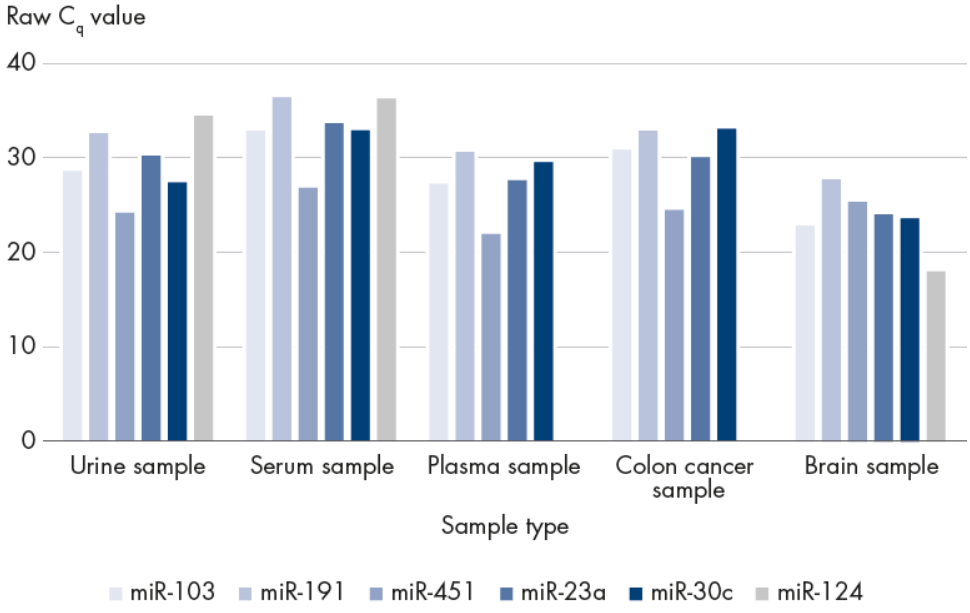
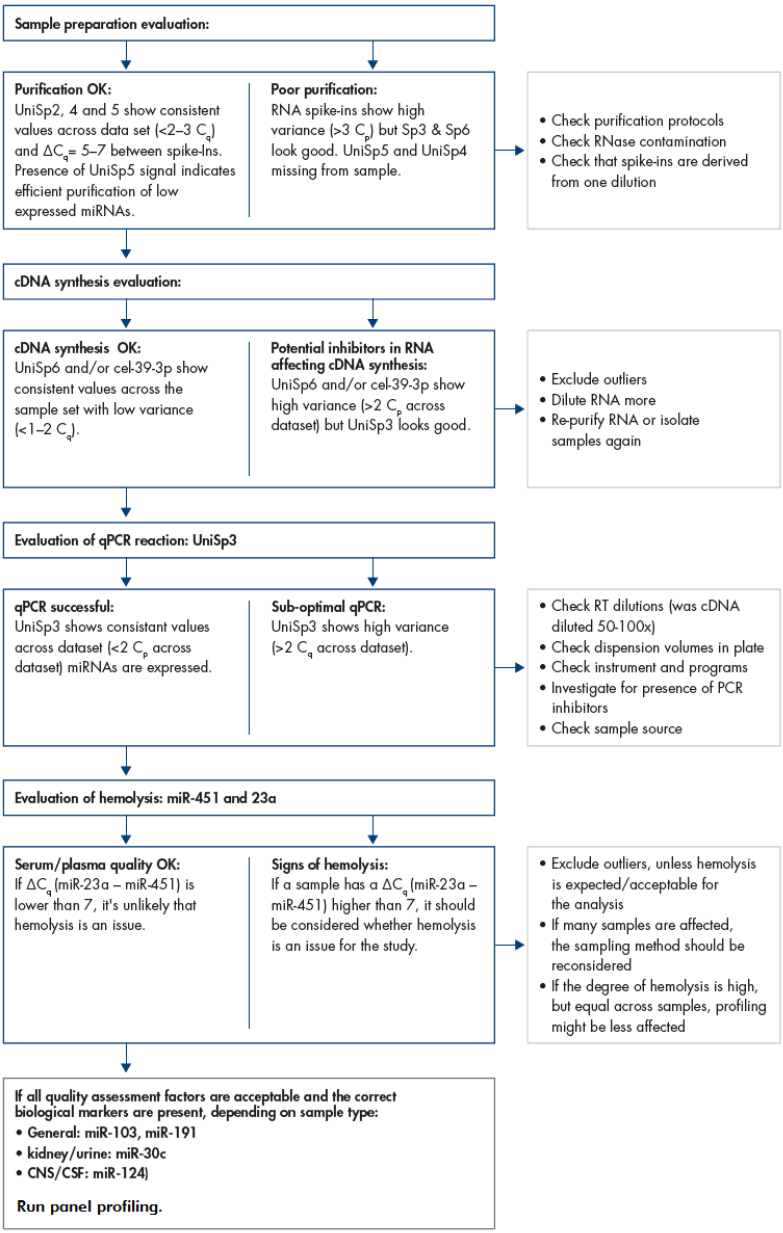


Figure 6. Samples from different sources analyzed with the tissue miRNAs. Samples from human urine, serum, plasma and colorectal cancer (FFPE), along with mouse brain (fresh-frozen) were analyzed. Note both the differential expression of the CNS miR-124-3p and the blood miRNA-451a as well as the overall variation in miRNA content from different sample sources.


Sample preparation evaluation:
Purification OK:
UniSp2, 4 and 5 show consistent values across data set (<2–3 C_q) and ΔC_q= 5–7 between spike-Ins. Presence of UniSp5 signal indicates efficient purification of low expressed miRNAs.
Poor purification:
RNA spike-ins show high variance (>3 C_q) but Sp3 & Sp6 look good. UniSp5 and UniSp4 missing from sample.
• Check purification protocols
• Check RNase contamination
• Check that spike-ins are derived from one dilution
cDNA synthesis evaluation:
cDNA synthesis OK:
UniSp6 and/or cel-39-3p show consistent values across the sample set with low variance (<1–2 C_q).
Potential inhibitors in RNA affecting cDNA synthesis:
UniSp6 and/or cel-39-3p show high variance (>2 C_q across dataset) but UniSp3 looks good.
• Exclude outliers
• Dilute RNA more
• Re-purify RNA or isolate samples again
Evaluation of qPCR reaction: UniSp3
qPCR successful:
UniSp3 shows consistant values across dataset (<2 C_q across dataset) miRNAs are expressed.
Sub-optimal qPCR:
UniSp3 shows high variance (>2 C_q across dataset).
• Check RT dilutions (was cDNA diluted 50-100x)
• Check dispension volumes in plate
• Check instrument and programs
• Investigate for presence of PCR inhibitors
• Check sample source
Evaluation of hemolysis: miR-451 and 23a
Serum/plasma quality OK:
If ΔC_q (miR-23a – miR-451) is lower than 7, it's unlikely that hemolysis is an issue.
Signs of hemolysis:
If a sample has a ΔC_q (miR-23a – miR-451) higher than 7, it should be considered whether hemolysis is an issue for the study.
• Exclude outliers, unless hemolysis is expected/acceptable for the analysis
• If many samples are affected, the sampling method should be reconsidered
• If the degree of hemolysis is high, but equal across samples, profiling might be less affected
If all quality assessment factors are acceptable and the correct biological markers are present, depending on sample type:
• General: miR-103, miR-191
• kidney/urine: miR-30c
• CNS/CSF: miR-124)
Run panel profiling.

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Product	Contents	Cat. no.
miRCURY LNA miRNA QC PCR Panel	Pre-made panel of LNA PCR assays for miRNA quality control; for one 10 µl qPCR reaction per well; 96-well or 384-well format	339331*
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miRCURY LNA RT Kit	5x RT Reaction Buffer, 10x RT Enzyme Mix, UniSp6 RNA Spike-in template, RNase-free water; for 8–64 reactions	339340
miRCURY LNA miRNA PCR Starter Kit	2 miRCURY LNA PCR Assays of your choice, UniSp6 Spike-in control assay, miR-103-3p endogenous control assay, 5x RT Reaction Buffer, 10x RT Enzyme Mix, UniSp6 RNA Spike-in template, RNase-free water, 2x miRCURY SYBR Green Master Mix; for 20 RT reactions and 100 PCR amplifications	339320
miRCURY LNA SYBR Green PCR Kit (200)	2x miRCURY SYBR Green Master Mix, RNase-free water; for 200 reactions	339345
miRCURY LNA SYBR Green PCR Kit (600)	2x miRCURY SYBR Green Master Mix, RNase-free water; for 600 reactions	339346
miRCURY LNA SYBR Green PCR Kit (4000)	2x miRCURY SYBR Green Master Mix, RNase-free water; for 4000 reactions	339347
RNA Spike-in Kit, for RT	UniSp2, UniSp4, UniSp5 RNA Spike-in template mix, cel-miR-39-3p RNA Spike-in template; for 50 reactions	339390

Product	Contents	Cat. no.
Related PCR assays and panels		
miRCURY LNA miRNA PCR Assay	LNA-optimized PCR assay for miRNA quantification; for 200 reactions	339306*
miRCURY LNA miRNA Custom PCR Assay	Custom-designed and LNA-optimized PCR assay for miRNA quantification; for 200 reactions	339317*
miRCURY LNA miRNA miRNome PCR Panels	Pre-made panels of LNA PCR assays for miRNome profiling; for one 10 µl qPCR reaction per well; 96-well or 384-well format	339322*
miRCURY LNA miRNA Focus PCR Panel	Pre-made panel of LNA PCR assays focused on disease, pathway or sample type; for one 10 µl qPCR reaction per well; 96-well or 384-well format	339325*
miRCURY LNA miRNA Custom Bulk Plate (200)	Custom-configured plate of LNA-optimized PCR assays for miRNA quantification; 96-well format; for 200 reactions per well	339319*
miRCURY LNA miRNA Custom PCR Panel	Custom panel of LNA-optimized PCR assays for miRNA quantification; 96- or 384-well plate format	339330*

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HB-2440-001 1107944 10/2017