



# miRCURY™ microRNA QC PCR Panel

## Instruction manual v2.0

#203887-203892 September 2014

Supplement to Exiqon's Instruction Manuals for:

miRCURY™ RNA isolation kits

miRCURY™ RNA spike-in kit

miRCURY LNA™ Universal RT microRNA PCR

miRCURY LNA™ Universal RT microRNA PCR, serum/plasma samples

# Table of Contents

<b>Product summary</b> .....	3
miRCURY™ microRNA QC PCR panel content .....	3
Storage .....	4
Additional required materials .....	4
Recommended accompanying products .....	4
<b>Protocol description</b> .....	5
miRCURY™ microRNA QC PCR panel .....	5
RNA quality assessment PCR panel reagents .....	6
Layout of the plate .....	7
<b>Before starting the experiment</b> .....	8
<b>Protocol</b> .....	10
<b>microRNA QC PCR Panel Guideline</b> .....	15
How to use this guideline .....	15
How to utilize the spike-ins .....	15
Monitoring hemolysis .....	17
Biologically relevant microRNAs .....	21
<b>Decision tree for identification of outlier samples</b> .....	23
<b>References</b> .....	24

# Product summary

## miRCURY™ microRNA QC PCR Panel content

The miRCURY™ microRNA QC PCR panels consist of either 96-well or 384-well PCR plates containing dried down LNA™ primer sets for one 10 µl real-time PCR reaction per well. The LNA™ primer sets are designed for optimal performance with the Universal cDNA Synthesis Kit and the SYBR® Green master mix. The performance of the LNA™ primer sets will be affected if they are used in combination with less than optimal reagents.

**Table 1. Contents.**

Product	Product name	Product description
203887	microRNA QC PCR Panel, 96 well (V4.R0)	miRCURY LNA™ Universal RT microRNA PCR, 16 Ready-to-Use microRNA QC PCR Panels, supplied in two 96-well plates. For use with Roche LightCycler 480 instruments.
203888	microRNA QC PCR Panel, 96 well (V4.AF)	miRCURY LNA™ Universal RT microRNA PCR, 16 Ready-to-Use microRNA QC PCR Panels, supplied in two 96-well plates. For use with ABI Fast instrument series and ABI StepOne Plus.
203889	microRNA QC PCR Panel, 96 well (V4.MI)	miRCURY LNA™ Universal RT microRNA PCR, 16 Ready-to-Use microRNA QC PCR Panels, supplied in two 96-well plates. For use with BioRad, Eppendorf, Stratagene Mx4000 and AB 7000 series instruments.
203890	microRNA QC PCR Panel, 96 well (V4.ST)	miRCURY LNA™ Universal RT microRNA PCR, 16 Ready-to-Use microRNA QC PCR Panels, supplied in two 96-well plates. For use with Stratagene Mx3000P series.
203891	microRNA QC PCR Panel, 384 well (V4.R)	miRCURY LNA™ Universal RT microRNA PCR, 32 Ready-to-Use microRNA QC PCR Panels, supplied in one 384-well plate. For use with Roche LightCycler 480 instruments.
203892	microRNA QC PCR Panel, 384 well (V4.M)	miRCURY LNA™ Universal RT microRNA PCR, 32 Ready-to-Use microRNA QC PCR Panels, supplied in one 384-well plate. For use with ABI 7900HT and other instruments.

## Storage

The RNA quality assessment PCR panel is shipped at room temperature with the DNA primers dried down. Upon arrival the panel(s) should be stored at -20°C. Under these conditions, all components are stable for at least 12 months.

## Additional required materials

### Exiqon reagents not supplied

- miRCURY LNA™ Universal RT microRNA PCR system materials
- SYBR Green® Master mix (#203403 or #203421)
- Universal cDNA synthesis Kit II (#203301)
- RNA Spike-In Kit (#203203)

### Other reagents not supplied

- ROX or other passive reference dye (required on some PCR cyclers)

### Materials and Equipment not supplied

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

## Recommended accompanying products

Exiqon GenEx qPCR analysis software  
miRCURY™ RNA Isolation Kit – Cell & Plant (#300110)  
miRCURY™ RNA Isolation Kit - Tissue (#300111)  
miRCURY™ RNA Isolation Kit - Biofluids (#300112)

# Product description

## miRCURY™ microRNA QC PCR Panel

The main purpose of the microRNA QC PCR panel is to analyze the robustness of the RNA isolation process and quality of isolated microRNA.

Firstly, it contains matching LNA™ primer sets for detection of the miRCURY™ RNA Spike-in kit (#203203), UniSp2, UniSp4 and UniSp5. The RNA Spike-In Kit provides a control for the quality of the RNA isolation in any microRNA RT-qPCR 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 RT-qPCR 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 instruction manual (#203203).

Secondly, it contains matching LNA™ primer sets for detection of the spike-ins UniSp6 and cel-39-3p to monitor cDNA synthesis, and UniSp3 (IPC) 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.

Thirdly, it contains matching LNA™ primer sets for detection of six microRNAs to evaluate the biological samples that are being interrogated with regards to downstream processing. These miRNAs are miR-103 and miR-191 which are well expressed in most tissues, miR-451 and miR-23a which are found in plasma and serum and serve as a hemolysis marker and an internal control, respectively. miR-30c which is well expressed in kidney and found in urine samples and miR-124 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.



## RNA quality assessment PCR panel reagents

This kit contains the following RT-qPCR assays for use with the miRCURY LNA™ Universal microRNA PCR system. Primer pairs are desiccated in the bottom of the wells of either 96 well or 384 well plates.

**Table 2. Components of the microRNA QC PCR panel.**

	Assay (common name <sup>1)</sup> )	Human, miRbase v18	Mouse, miRbase v18
MicroRNA assay	miR-103	hsa-miR-103a-3p	mmu-miR-103-3p
MicroRNA assay	miR-191	mmu-miR-191-5p	mmu-miR-191-5p
MicroRNA assay	miR-451	hsa-miR-451a	mmu-451
MicroRNA assay	miR-23a	hsa-miR-23a-3p	mmu-miR-23a-3p
RT assay	UniSp6, v2 <sup>2)</sup>	-	-
Spike-in assay	UniSp2	-	-
Spike-in assay	UniSp4	-	-
Spike-in assay	UniSp5, v2 <sup>2)</sup>	-	-
RT assay	cel-miR-39-3p	-	-
Inter plate calibrator/PCR control	UniSp3	-	-
MicroRNA assay	miR-124	hsa-miR-124-3p	mmu-miR-124-3p
MicroRNA assay	miR-30c	hsa-miR-30c-5p	mmu-miR-30c-5p

<sup>1)</sup> In the species: *rno*, *cfa* and *mmi* the common names are used for these microRNAs.

<sup>2)</sup> New optimized UniSp5 and UniSp6 assays designed for V4 panels target same RNA template as previous assays.

## Layout of the plate

The layout of the two panels are a replicate of the 12 assays as seen in figure 1. The 96 well format contains assays for 8 samples and the 384 well format contains assays for 32 samples.

---

**Figure 1. Layout of the 12 assays.**



# Before starting the experiment

## Important note

This kit only contains assays. It is designed to be used together with the RNA Spike-In Kit (#203203). For instructions on how to use the Spike-In Kit with RNA isolation procedure see the RNA spike-in instruction manual. Full description of the miRCURY LNA™ Universal RT microRNA PCR system can be found in the corresponding instruction manual.

Before setting up a real-time PCR experiment, there are a number of practical experimental design parameters that should be considered:

**RNA input** - The miRCURY LNA™ microRNA QC PCR protocol is optimized for use of 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 microRNA expression levels in the cells or tissue to be analyzed. For highly expressed microRNAs it is possible to use down to 10 pg total RNA as starting material. For weakly expressed microRNAs 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 microRNA profiling study, it is recommended to optimize the amount of input RNA to the RT reaction in order to avoid conducting a larger study where inhibition occurs sporadically throughout the data set.

Information on how to extract and handle RNA can be found in the tips section of the miRCURY LNA™ Universal microRNA PCR manual. In short, total RNA should be prepared using a method that preserves small RNA species. DNase treatment may be necessary. When using commercially available kits, please ensure that the total RNA preparation is guaranteed to contain microRNAs.

### Note

Blood serum and plasma are particular sample types that require special RNA purification procedures and the amount of RNA present in the samples can usually not be accurately determined. Due to the low levels of microRNAs and potentially high levels of inhibitors in samples derived from serum and plasma, specific recommendations for how to set up experiments using these types of sample can be found in the miRCURY LNA™ Universal RT microRNA PCR, Instruction Manual “Serum/plasma samples”.

**ROX** – ROX is a passive reference dye used by some PCR cycles to obtain a robust read over the entire array of wells in a 96- or 384-well PCR plate. The requirement for ROX is instrument dependent and we recommend to follow the instrument manufactures guidelines on this.

**ABI instruments** – the default settings on ABI real-time PCR cyclers are not suitable for running miRCURY LNA™ Universal RT microRNA PCR. Settings need to be changed from automatic to manual background and threshold settings to obtain valid PCR data (see also Tip 10). Furthermore, if the dataset is to be analyzed using the GenEx software, it is important that the experiment is set up as an AQ experiment, not RQ. To ensure correct settings, download the instrument settings file at [www.exiqon.com/sds](http://www.exiqon.com/sds).

# Protocol

The miRCURY LNA™ Universal RT microRNA PCR protocol is a two-step protocol consisting of:

- 1. First strand cDNA synthesis (Step 1-5)**
- 2. Real-time PCR amplification (Step 6-11)**

## Important

Keep reagents and reactions on ice (or at 4°C) at all times.

### First strand synthesis:

#### Step 1

Dilute template RNA

Adjust each of the template RNA samples to a concentration of 5 ng/ $\mu$ L using nuclease free water. If working with low yield samples e.g. plasma or serum, do not exceed 2  $\mu$ L of RNA in the final cDNA reaction

#### Step 2

Prepare reagents

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-39-3p according to the description in the Spike-In manual. Immediately before use, remove the enzyme mix from the freezer, mix by flicking the tubes and place on ice. Spin down all reagents.

**Step 3**

Combine reagents according to Table 3

When performing first-strand cDNA synthesis on multiple RNA samples, it is recommended to prepare an RT working solution of the 5x Reaction buffer, water, enzyme mix and spike-in mix.

The following procedure is recommended:

1. Prepare the required amount of RT working solution and place it on ice.
2. Dispense RT working solution into nuclease free tubes.
3. Dispense template RNA in each tube. The use of a non-template control (NTC) is highly recommended.

**Table 3 - Reverse transcription reaction setup per sample.**

Reagent	Volume (µL)
5x Reaction buffer	2
Nuclease-free water	4.5
Enzyme mix	1
Synthetic spike-in mix	0.5
Template total RNA <i>Replace with H<sub>2</sub>O if omitted</i>	2 (5ng/µL)
Total volume	10

**Step 4**

Mix and spin reagents<sup>1)</sup>

Mix the reaction by very gentle vortexing or pipetting to ensure that all reagents are thoroughly mixed. After mixing, spin down.

**Step 5**

Incubate and heat inactivate<sup>2)</sup>

- Incubate for 60 min at 42°C
- Heat-inactivate the reverse transcriptase for 5 min at 95°C
- Immediately cool to 4°C
- Store at 4°C or freeze

<sup>1)</sup> The volumes suggested here provides excess amount of cDNA, which ensures the highest possible reproducibility because these volumes can be pipetted with great accuracy.

<sup>2)</sup> Although not recommended, the protocol can be interrupted at this stage. The undiluted cDNA may be kept at -20°C for up to 5 weeks (optional store at 4°C for up to 4 days). It is recommended that synthesized cDNA be stored in "low-nucleic acid binding" tubes or plates.

## qPCR protocol:

### Step 6

Prepare reagents for real-time PCR

Place cDNA (from Step 5), nuclease free water and 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. The rest of the reagents are mixed by vortexing and spun down.

### Step 7

Dilute cDNA template in nuclease free water:

Tissue/cell lines:  
1:100 dilution

Biofluid: 1:50 dilution

Dilute 2  $\mu$ L of the cDNA from the RT reactions in 198  $\mu$ L nuclease free water to reach 100x dilution if working with tissue or samples from cell lines and dilute 4  $\mu$ L in 196  $\mu$ L nuclease free water to reach 50x dilution if working with biofluids, such as serum, plasma, urine or other low yield samples.

**Recommendation:** It is recommended that "low-nucleic acid binding" tubes or plates are used. It is not recommended to store the diluted cDNA. Include a passive reference dye in the cDNA dilution if advised by your instrument manufacturer. Please note that the SYBR Green® Master mix does not include ROX. The amount of ROX required is instrument dependent and it is important to refer to the manufacturer's recommendations.

**ABI instrument user?**

Apply manual baseline and threshold settings! Go to [www.exiqon.com/sds](http://www.exiqon.com/sds) to download settings file

**Step 8**

Combine cDNA and SYBR Green® Master mix in 1:1 ratio and add to PCR plates

The following procedure is recommended:

1. Before removing the plate seal, briefly spin down the plate(s) in a plate centrifuge.
2. Combine 2x Master mix and diluted cDNA 1:1 (e.g. 60 µL 2x Master mix and 60 µL diluted cDNA).
3. Mix gently by inverting the tube, spin down.
4. Add 10 µL Master mix:cDNA mix to each well.<sup>3)</sup>
5. Seal the plate with optical sealing as recommended by the instrument manufacturer.

The experiment can be paused at this point. Store the reactions protected from light at 4°C for up to 16 hours.

**Step 9**

Spin plate

Spin the plate briefly in a plate centrifuge (1500g for 1 minute), to remove air bubbles.

**Step 10**

Real-time PCR amplification

Perform real-time PCR amplification followed by melting curve analysis according to Table 4 below.

**Table 4 - Real-time PCR cycle conditions.**

Process step	Settings, LC480 instrument <sup>4)</sup>	Settings, other instruments <sup>5)</sup>
Polymerase Activation/ Denaturation	95°C, 10 min	95°C, 10 min
Amplification	45 amplification cycles at 95°C, 10 s 60°C, 1 min ramp-rate 1.6°C/s <sup>6)</sup> Optical read	45 amplification cycles at 95°C, 10 s 60°C, 1 min ramp-rate 1.6°C/s <sup>6)</sup> Optical read
Melting curve Analysis <sup>7)</sup>	Yes	Yes

**Step 11**

Analyze data

Perform initial data analysis using the software supplied with the real-time PCR instrument to obtain raw Cq values (Cp or Ct, depending on PCR instrument). If you are using an ABI instrument, please note that it is not recommended to use auto Ct settings. For a guide on how to set manual baseline and threshold, refer to Tip 10, page 55 in the tips section of the miRCURY LNA™ Universal RT microRNA PCR

Instruction Manual.

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

For interpretation of the microRNA QC Panel data, go to the guideline section on the next page

<sup>3)</sup> Corresponding to 0.05ng total RNA starting material pr. PCR reaction.

<sup>4)</sup> Five additional amplification cycles are required when using the LC480 instrument to allow collection of assay data with Cp-values up to 40.

<sup>5)</sup> If using a 96-well cycler with a minimum recommended volume of 20 µL (like some ABI instruments), then use 10 µL reaction volume and set the instrument settings at 20 µL.

<sup>6)</sup> The ramp-rate of cooling from 95°C to 60°C should be set to 1.6°C/s. This is equivalent to 100% under standard cycling conditions on the ABI 7500, 7900 and Vii7 instruments. If the ramp rate of cooling is too rapid, performance may be compromised.

<sup>7)</sup> 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. Please follow the instructions provided by the supplier. Note: The  $T_m$  of a PCR product depends on buffer composition, salt concentration and the PCR instrument.



# microRNA QC PCR Panel Guideline

## How to use this guideline

This section describes how to assess the quality of RNA samples prior to a microRNA profiling effort, in order to avoid spending valuable resources and time on analyzing samples that are unlikely to add information to the sample-set due to technical or biological challenges. The assays on the microRNA QC PCR Panel are aimed at different steps in the RT-qPCR process. RNA spike-ins from the RNA Spike-In kit: UniSp2, 4 and 5 are aimed at evaluating the RNA purification efficiency and yield. The RNA spike-ins cel-39-3p and the UniSp6 (supplied with the cDNA synthesis kit) evaluate the cDNA synthesis step with regards to inhibition and the UniSp3 (IPC) evaluates the qPCR reaction. The six microRNA assays on the panel are: miR-103 and miR-191 (well expressed in most tissues), miR-451 is highly expressed in serum and plasma and predominantly derived from red blood cells while miR-23a is more stably expressed in serum and plasma but not affected by hemolysis. Together they can be used to monitor hemolysis. miR-30c is well expressed in many tissues including the kidney and is widely found in urine samples. Lastly, miR-124 is well expressed in central nervous system derived tissues and is widely found in cerebrospinal fluid (CSF). Those six microRNA assays can analyze the biological quality of a number of different tissues including body fluids like serum, plasma, urine or CSF. Also these assays will work on samples from humans, rat, mouse, dog and rhesus monkey.

## How to utilize the spike-ins

In a typical experiment where tissue or biofluid derived samples are purified, the three RNA purification spike-ins should be added to the RNA isolation lysis buffer and the samples purified according to the manufacturer's instructions. After purification, cDNA is made from the samples with UniSp6 and cel-39-3p added to the reverse transcription (RT) mixture and the samples should be analyzed on the microRNA QC PCR Panel according the instruction manual.

An example of a plasma sample, isolated 5 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).

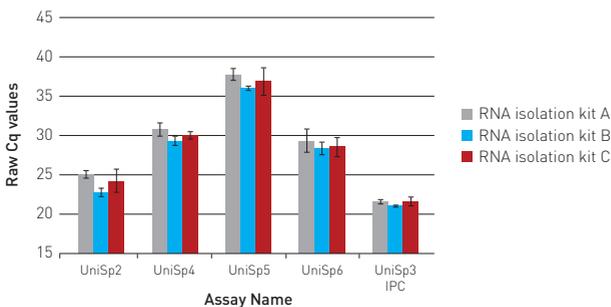


The UniSp2, UniSp4 and UniSp5 are present at a 100 fold concentration difference in the RNA Spike-in kit, which results in approximately 5-7 Cq difference between the spike-ins. This however depends on the amount of Spike-in mixture added, the yield of the purification and the volume of the eluted RNA.

Note specifically that the most dilute Spike-in (UniSp5) corresponds in amount to microRNAs expressed at a very low concentration and therefore could be undetected or its high Cq values (low signal) filtered out during data processing. For more information on the RNA Spike-In kit see the instruction manual (#203203).

Also a more advanced version of the RNA input experiment is to make a two-fold serial dilution of the RNA into the cDNA reactions. For example by adding a serial range of 0.5, 1, 2 and 4  $\mu\text{L}$  of RNA to the cDNA synthesis. This can give valuable information on how much RNA can be used before inhibition set in when working with low concentrations of RNA or difficult material, known to be challenged by inhibitors

**Figure 2. One plasma sample was purified 5 times with three different RNA isolation methods.** Spike-in mixture was added to the lysis buffers of the kits according to the RNA Spike-in kit instruction manual. The starting material was 200  $\mu\text{L}$  and the elution volume was 50  $\mu\text{L}$ . The spike-in assessment demonstrated that the RNA isolation kit B showed the best yields and that there was a potential inhibition in the kit A (see the UniSp6) but the UniSp3 (inter plate calibrator) which monitors overall qPCR efficiency, showed comparable qPCR efficiency. The stepwise difference (dCq) between the RNA isolation Spike-ins (2,4,5) was in the expected range of 5-7 Cqs. Error bars are standard deviations from the 5 replicate extractions.



Below are potential problems that can be revealed using the microRNA QC PCR panel:

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

Problem: High variance (>2-3 Cq 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 purification kits are not past their expiry 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, which results in loss of data from microRNAs expressed at a low level. Potential solutions see problem 1. Note however that the lack of signal in UniSp5 could be normal, for example, if elution volume is more than 50 µL or 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 Cqs) and the Cq 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 microRNAs hold great promise as a new class of biomarkers due to their stability in biofluids e.g. plasma and serum and association with various disease states. microRNA in biofluids are protected in microvesicles like exosomes or bound to proteins e.g. argonaute 2 or high density lipoproteins. Even though measuring microRNA in plasma and serum is straight forward using sensitive and specific methods like the miRCURY LNA™ Universal RT microRNA PCR platform, technical challenges are associated with microRNA analysis in biofluids. A major source of variation in plasma and serum is potential cellular derived microRNA contamination including hemolysis (1-5). The data from the red blood cell specific miR-451 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  $\Delta Cq$ (miR-23a - miR-451) lower than 5 in human serum or plasma represents non-hemolysed samples. If the  $\Delta Cq$  is close to or higher than 7 there is an increased risk of hemolysis. In case of high levels of hemolysis, microRNAs from red blood cells will make a significant contribution to the overall microRNA 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.

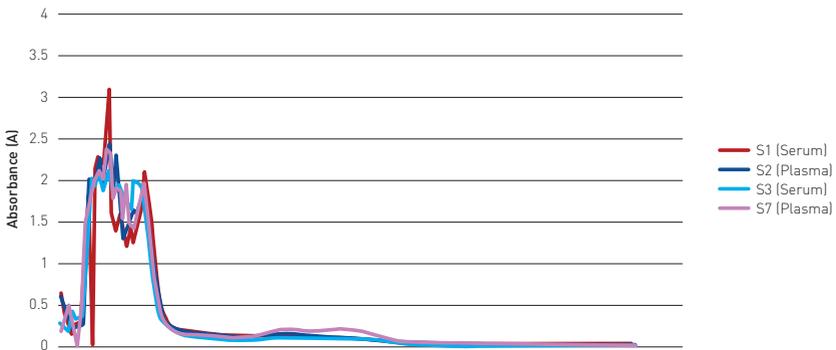
**Tip!**

Although the microRNA QC PCR Panel contains assays for miRs that are the same in human (hsa), mouse (mmu), rat (rno), dog (cfa) and rhesus macaque (mml),  $\Delta Cq$ s for other than human have not been experimentally determined. Therefore we recommend looking at hemoglobin contamination as well as the  $\Delta Cq$  to determine the best  $\Delta Cq$ s for your samples, as it might not be the same as for human samples, described in this guideline.

Examples of 4 human serum/plasma samples that are not affected by hemolysis are shown on the next page (figure 3). All values for the  $\Delta Cq$  are below 5 and no distinct peak of oxy-hemoglobin is detected on the spectra ( $\lambda=414$  nm).



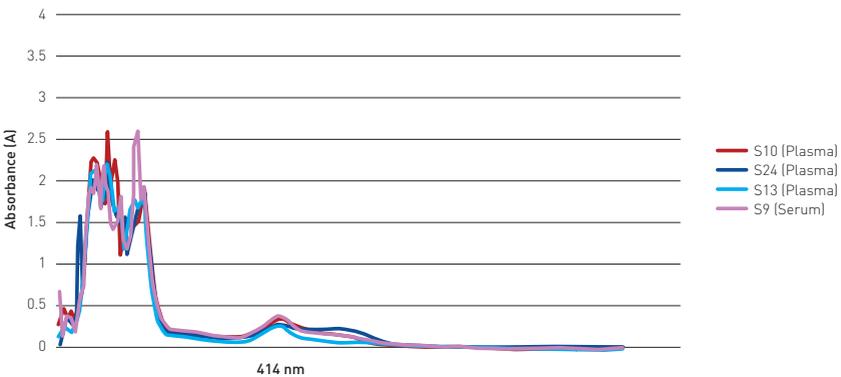
**Figure 3. Four human plasma and serum samples were analyzed with regards to miR-23a and miR-451 and the  $\Delta Cq$  (miR-23a - miR-451) determined.** The four samples were also analyzed by spectrophotometer (Nanodrop ND-1000) from  $\Delta 220$ -720 nm. No distinct peak of oxy-hemoglobin (maximum 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.



Name / assay	hsa-miR-23a	hsa-miR-451	23a-451
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

When there is a hemolysis problem, the expression of miR-451 increases independently of miR-23a. This results in higher  $\Delta Cq$  values than with non-hemolysed samples. Below are examples of hemolysed samples (figure 4) and how the  $\Delta Cq$  is affected. As seen on the spectrophotometer graphs, there is a distinct peak in all samples at  $\lambda=414$ nm, corresponding to oxy-hemoglobin contamination.

**Figure 4. Four human plasma and serum samples were analyzed with regards to miRNA-23a and miRNA-451 and the  $\Delta Cq$  (miR-23a - miR-451) determined.** Three showed higher  $\Delta Cq$  than 7 and one close to 7. The four samples were also analyzed by spectrophotometer (Nanodrop ND-1000) from  $\lambda$ 220-750 nm. A distinct peak of oxy-hemoglobin was detected in all the samples. Sample S24 (plasma) showed potential bilirubin contamination also.

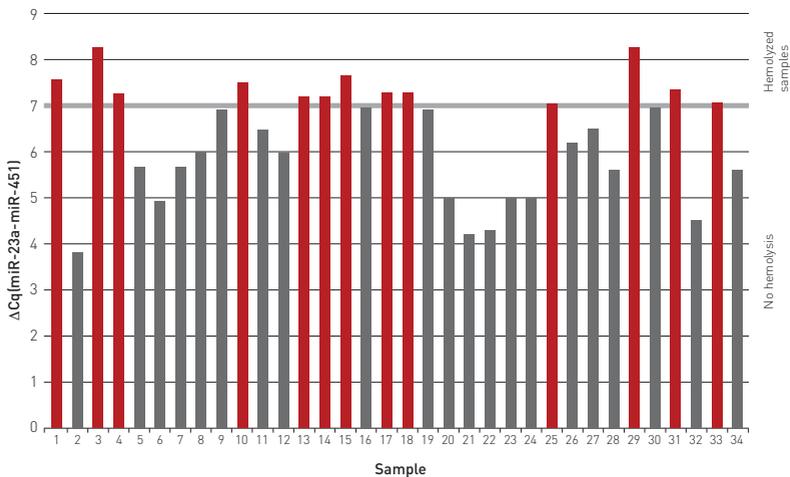


Name / assay	hsa-miR-23a	hsa-miR-451	23a-451
S10 (Plasma)	29.5	21.9	7.6
S24 (Plasma)	29.8	23.1	6.7
S13 (Plasma)	29.1	21.8	7.3
S9 (Serum)	30.0	21.5	8.5

An example of how a human plasma dataset can be evaluated is shown in figure 5. By looking at the  $\Delta Cq$  of the sample set it is clear that it is challenged by hemolysis. 13 out of 34 samples (40%) have  $\Delta Cq$  around or above 7. In this case we would recommend to a) consider excluding samples showing  $\Delta Cq > 7$  if the biomarkers investigated are expected to be very weak and b) if possible, collect and analyze more samples to identify more non-hemolyzed samples and c) look at the blood collection protocol to try to identify the technical cause for this hemolysis problem.

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

**Figure 5.**  $\Delta Cq$  (miR-23a - miR-451) values for 34 sample cohort. It is clear that the sample-set is affected by hemolysis. Almost half of the samples have the  $\Delta Cq$  around or above 7. Some or all of the samples in read should maybe be removed from the study, if hemolysis is considered critical for the study.



## Biologically relevant microRNAs

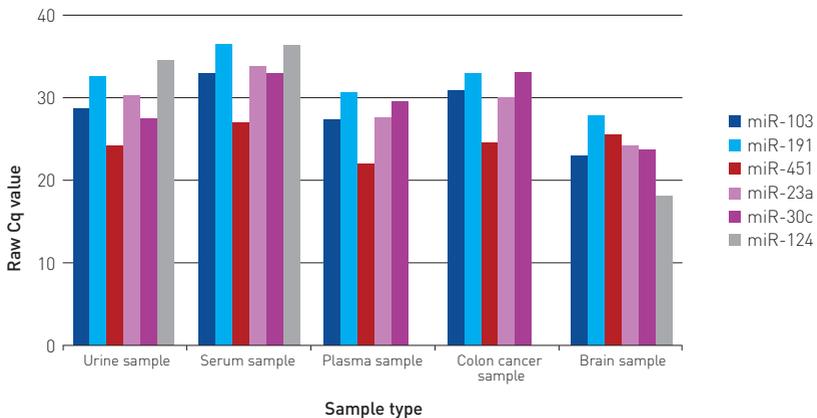
While the spike-ins serve as technical quality controls and miR-451 and miR-23a are used to monitor hemolysis, a few biologically relevant microRNAs are present on the panel and serve a more general purpose to identify outlier samples within a sample set. The microRNAs, miR-103 and miR-191, are relatively stable and well expressed in most tissues analysed to date and serve as general microRNA markers. miR-30c is also well expressed in many tissues including the kidney.

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

The microRNAs on the panel are included so that the overall biological content of samples can be monitored and to evaluate if a subset of the samples show very low microRNA content. This however has to be evaluated within any given sample-set and on a tissue-to-tissue basis.

To show the variation of microRNAs between sample sources, a few samples from different sources are shown below (figure 6). Note that there will be biological variation within as well as between sample-sets, therefore a few Cq difference is commonly seen, especially between normal and disease groups, for example between tumor and normal adjacent tissue sample groups.

**Figure 6. Different sources of samples analyzed with the tissue microRNAs.** This includes Urine sample (human), Serum sample (human), Plasma sample (human), Colorectal Cancer sample (human, Formalin-Fixed-paraffin-embedded) and Brain sample (Mouse, Fresh-Frozen). Note both the different expression of e.g. the CNS miR-124 and the blood miRNA-451 and overall different microRNA content of different sample sources.



# Decision tree for identification of outlier samples



# References

- Mitchell P.S. *et al.* (2008) Circulating microRNAs as stable blood-based markers for cancer detection. *Proc. Natl. Acad. Sci. USA.* 105:10513-8
- Kroh E.M. *et al.* (2010) Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). *Methods.* 50:298-301
- Vickers K.C. *et al.* (2011) MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nature Cell Biology* 13, 423–433
- Arroyo J.D. *et al.* (2011) Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc. Natl. Acad. Sci. USA.* 2108:5003-8
- Pritchard C.C. *et al.* (2012) Blood cell origin of circulating microRNAs: a cautionary note for cancer biomarker studies. *Cancer. Prev. Res.* 2012 Mar; 5(3):492-7
- Eldh M. *et al.* (2012) Importance of RNA isolation methods for analysis of exosomal RNA: Evaluation of different methods. *Mol Immunology* 50:278-286

# Notes





### Literature citations

Please refer to miRCURY LNA™ Universal RT microRNA PCR when describing a procedure for publication using this product.

### Notice to purchaser

Exiqon, LNA™ and miRCURY are registered trademarks of Exiqon A/S, Vedbaek, Denmark. SYBR® Green is a licensed trademark of Invitrogen. All other trademarks are the property of their respective owners.

Locked-nucleic Acids (LNA™s) are protected by US Pat No. 6,268,490, US Pat No. 6,770,748, US Pat No. 6,639,059, US Pat No. 6,734,291 and other applications and patents owned or licensed by Exiqon A/S. Products are provided to buyers for research use only. The products in their original or any modified form may be used only for the buyer's internal research purposes and not for commercial, diagnostic, therapeutic, or other use, including contract research. The buyer may not provide products to third parties in their original or any modified form. The purchase of products does not include or carry an implied right or license for the buyer to use such products in their original or any modified form in the provision of services to third parties, and a license must be obtained directly from Exiqon A/S for such uses.

### Limited license

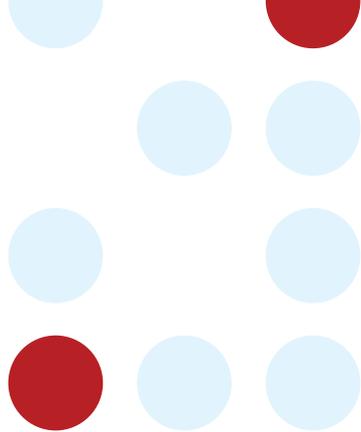
Use of this product is covered by one or more of the following US patents and corresponding patent claims outside the US: 5,994,056 and 6,171,785. The purchase of this product includes a limited, nontransferable immunity from suit under the foregoing patent claims for using only this amount of product solely in Contract Research, including reporting results of purchaser's activities for a fee or other commercial consideration, and also for the purchaser's own internal research. No right under any other patent claim is conveyed expressly, by implication, or by estoppel. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

Furthermore, this product is provided under an agreement between Molecular Probes, Inc., a wholly owned subsidiary of Invitrogen Corporation, and EXIQON and the manufacture, use, sale or import of this product is subject to one or more U.S. Patents and corresponding international equivalents. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer, where such research does not include testing, analysis or screening services for any third party in return for compensation on a per test basis. The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. For information on purchasing a license to this product for purposes other than research, contact Molecular Probes, Inc., Business Development, 29851 Willow Creek Road, Eugene, OR 97402. Tel: (541) 465-8300, Fax: (541) 335-0354.

Further, the purchase of this product includes a limited, non-transferable license under specific claims of U.S. Patent Nos. 6,174,670 and 6,569,627, owned by the University of Utah Research Foundation and licensed to Roche Diagnostics GmbH and Idaho Technology, Inc., to use only the enclosed amount of product according to the specified protocols. No right is conveyed, expressly, by implication, or by estoppel, to use any instrument or system under any claim of U.S. Patent Nos. 6,174,670 and 6,569,627, other than for the amount of product contained herein.

For life science research use only. Not for use in diagnostic procedures.

© Copyright 2012 Exiqon. All rights reserved.



### **Outside North America**

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

### **North America**

Exiqon Inc. · 12 Gill Street, Suite 1650  
Woburn, MA 01801 · United States  
Phone (781) 376 4150  
Fax (781) 376 4152

[exiqon.com](http://exiqon.com)

**EXIQON**  
Seek Find Verify