Exiqon
Services

A guide to the miRCURY™ microRNA qPCR Profiling Service

Guidelines v3.4
September 2017

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Consultation and experimental design

How do I get started?

- Please contact us by filling in a request at www.exiqon.com/contact. If you are able, please provide an outline of the experiment you want performed
- After receiving the Request Form, we will contact you for further discussion. This is a no obligation discussion to agree on the details of the experimental design. At this stage we will also make a no obligation quote for the total cost of the experiment
- If you decide to use Exiqon's microRNA qPCR profiling service, experimental details should be submitted on the Sample Submission Form (SSF) available at www.exiqon.com/ssf, in order for Exiqon to obtain all the information necessary to perform the experiment
- A copy of the SSF is sent to Exiqon where it will be reviewed and approved by an Exiqon scientist. You will be given a unique project reference code. Please print out and sign the approved form. Enclose the signed form with the samples upon shipping

Designing the optimal experiment

- When you engage in microRNA qPCR Service projects with Exiqon, you are assured direct communication with the scientists performing your experiments throughout the duration of the project
- Each project begins with a free consultation with a microRNA profiling expert. Together we design an experimental setup that best satisfies your research needs and budget. Along with the complete and detailed Sample Submission Form we ensure that all experimental details and subsequent analysis are clearly defined and understood by both parties

RNA sample submission

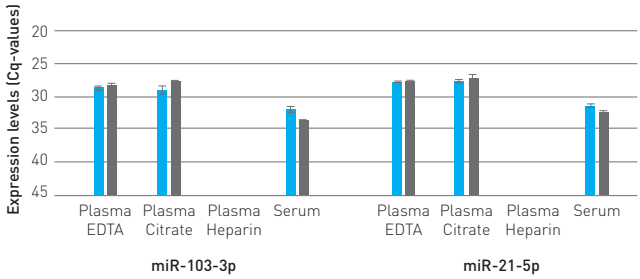
How do I isolate RNA?

High quality samples are important for accurate microRNA profiling. During the initial consultation, we offer recommendations on suitable extraction and clean-up methods. Some of our recommendations on isolation of RNA are listed below. Exiqon also offers RNA isolation in addition to its profiling service. (Details on Exiqon's isolation services can be found below in section "If I want Exiqon to isolate the total RNA", page 5).

Total RNA

- Total RNA should be prepared using a method that preserves small RNA species. When using commercially available kits, please note that they often have two alternative protocols. One is for isolation of total RNA (including microRNA) and one is for enrichment of small RNA. We recommend using the protocol for total RNA if it also retains the small RNA species
- The purified total RNA should be eluted or dissolved in RNAse-free H_2O
- Avoid heparin in collection tubes and cell culture media as it inhibits the PCR reaction (figure 1)

Figure 1. Serum and plasma samples collected in different tubes and profiled on qPCR platform.



FFPE

We recommend the Exiqon miRCURY™ RNA Isolation Kit - FFPE for purification of RNA from FFPE material, but other kits preserving small RNAs may also be used.

Tissue and cells

We recommend the Exiqon miRCURY™ RNA Isolation kits (www.exiqon.com/rna-isolation) for purification of RNA from tissue or cells.

Exosomes

We recommend the Exiqon miRCURY™ Exosome Isolation Kits (http://www.exiqon.com/exosome-isolation-kits) in combination with the Exiqon miRCURY™ RNA isolation kits for purification of RNA from exosomes.

Blood serum and plasma

All samples should preferably be collected using the same protocol to minimize technical variation. Examples of good protocols for collection of samples can be found at:
Plasma SOP:
http://edrn.nci.nih.gov/resources/standard-operating-procedures/standard-operating-procedures/plasma-sop.pdf
Serum SOP:
http://edrn.nci.nih.gov/resources/standard-operating-procedures/standard-operating-procedures/serum-sop.pdf

Upon collection of plasma or serum - prior to freezing - spin at 3000 g for 5-10 min to collect debris. Take the supernatant from the vial and leave some plasma / serum on top of debris. This reduces the potential contamination of cellular components (from e.g. buffy coat) even further.

We recommend using the Exiqon miRCURY™ RNA Isolation Kit - Biofluids (www.exiqon.com/mirna-isolation-biofluids) for purification of RNA from serum, plasma, urine and CSF.

Further recommendations can be found in Exiqon's guideline "Profiling of microRNA in serum/plasma and other biofluids (www.exiqon.com/ls/Documents/Scientific/microRNA-serum-plasma-guidelines.pdf)

How much purified RNA is needed?

Exiqon generally recommends to send RNA samples with a concentration of minimum 25 ng/µl and a sample volume of 10 µl. For samples with very lillte RNA, or for biofluids samples, please see recommended input volumes in table 1. The table below shows how much total RNA should be submitted for different types of samples:

Table 1. Amounts of purified RNA from a 50 µL elution needed for qPCR analysis Services. Note: assumes Exiqon recommended protocol is used for exosomes and all biofluids (including MS2 as carrier) (http://www.exiqon.com/ls/Documents/Scientific/microRNA-serum-plasma-guidelines.pdf).

Sample type	Amount needed for QC or qPCR QC	Amount needed pr panel	Amounts needed in total
Total RNA + FFPE	40 ng	65 ng	105-165 ng
Biofluids, all species (serum, plasma, urine, lymph, saliva etc.). See note	5 µL*	15 µL	15-35 µL

*qPCR QC optional

If I want Exiqon to isolate the total RNA

Exiqon offers an RNA isolation service in addition to its profiling services. Due to personal health and safety legislation, we do not accept any form of contagious material, or samples shipped in glass tubes. All samples, except biofluids for exosome precipitation, must be shipped to Exiqon Services in clearly labeled 1.5-2.0 mL plastic tubes. Biofluid samples for exosome precipitation and RNA isolation with more than 1.8 mL input volume should be sent in 5 or 15 mL plastic tubes. If you want Exiqon to isolate the total RNA, please see the chart on the next page for amounts needed:

Table 2. Amounts of sample needed for purification and subsequent microRNA qPCR Services.

Sample type	Amount needed	Comment
Plasma / serum (human)	250 µL	
Plasma / serum (rodent)	75 µL	
Urine, saliva and other biofluids	250 µL	
Plasma / serum for exosome isolation	500 - 3500 µL	1500 µl recommended
Urine for exosome isolation	2500 -3500 µL	3500 µL recommended
CSF for exosome isolation	500 - 3500 µL	1500 µL recommended
Other biofluids for exosome isolation	500 - 3500 µL	
LCM	>1000 cells	
FFPE sections	Min 2 x 10 µm sections of 1 cm^2	Not mounted on slides
Fresh-frozen tissue	1-5 mg	
Cells	10^6 cells pelleted and frozen	Spin cells down gently, take the medium off, rinse gently in cold PBS once, take of the PBS, and freeze quickly (e.g. liquid nitrogen) and store at -80 degrees Celsius.

Please consult us for instruction on how to collect samples for purification prior to shipping samples to Exiqon Services.

How do I assess the RNA quality?

Exiqon will perform RNA quality control prior to profiling, but we recommend that you check your RNA yourself prior to submission. We recommend measuring the OD260/230 ratio as well as the OD260/280 ratio. See details on page 7 for the rationale behind these measurements. If any of these ratios are lower than 1.6, it may be advisable to perform additional column purification that preserves small RNA in order to be absolutely sure of superior performance.

If possible, we also recommend checking the integrity of the total RNA prior to shipment, to avoid increased turn-around-time (TAT) due to resubmission of RNA samples.

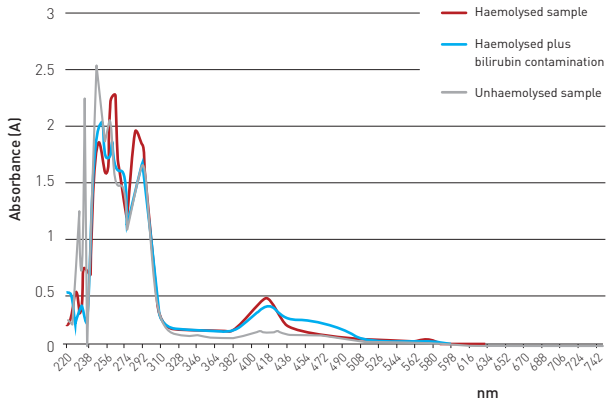
For serum or plasma samples, it can be advantageous to perform a hemolysis check on the samples prior to purification. To monitor hemolysis we recommend two steps:

1) Visual inspection. Pink or red samples are hemolysed. White or yellow samples are usually not hemolysed. But the range from yellow to pink can be difficult to interpret.

2) Spectrophotometry. By measuring the absorbance in the range from 230-600 nm of the undiluted plasma/serum sample, the absorbance of hemoglobin can be analyzed. If there is a distinct peak at 414 nm, then there is oxyhaemoglobin in the sample indicating hemolysis. Examples of hemolysed and non-hemolysed samples are shown below. Note also that some samples have high lipid content (cloudy white under visual inspection) that can interfere with spectrophotometric measurements. It can be difficult to purify RNA with sufficient yields from high lipid samples.

   Exiqon Services also offer hemolysis analyses (extra charge).

Figure 2. Examples of three spectra obtained from different plasma samples. An un-hemolysed sample (gray), a hemolysed sample (red) and a hemolysed sample with a shoulder (a bilirubin contamination (blue)).



How does Exiqon assess the RNA quality?

When RNA samples arrive at Exiqon they undergo a quality assessment prior to the profiling analysis. The standard quality assessment includes absorbance measurements and RNA integrity measurements.

Absorbance spectrum

To assess the purity of the samples, we examine the absorbance spectra to identify potential contaminations and differences between the samples in the same project. Differences in purity, or obvious contaminations, may affect the downstream results. For most projects these contaminations do not have an effect, but they could for projects in which very minute biological differences are being investigated.

- OD260/230 nm <1.6: Indicates potential contamination with Guanidinium isothiocyanate or other chaotropic agent absorbing at 230 nm. This is seen if the wash buffer is carried through in column purifications
- OD260/ 280 nm < 1.6: indicates potential contamination with phenol absorbing at 270 nm. This is seen if part of the phenol phase is aspirated when collecting the aqueous phase in a phenol:chloroform extraction

Both contaminations may reduce the performance of the expression profiling. This is however not seen very often.

RNA integrity

To assess the integrity of the ribosomal subunits, a Bioanalyzer profile is run and an RNA integrity number, RIN, value is assigned by the software. The RIN value is a measurement of the intactness of the two ribosomal bands. Please note that for samples with RNA concentrations below 25 ng/µL, the robustness of RIN values is poorer. Best results are obtained for concentration values above 50 ng/µL.

For FFPE samples, we do not measure RIN values. RNA from FFPE samples yields excellent profiling results on the qPCR platform.

- RIN value >7: High quality RNA
- RIN value 5-7: Partial degradation
- RIN value <5: Degraded RNA

Recommended
1 - High quality RNA
2 – RNA of similar quality (e.g. all degraded)

Avoid
1 – Big differences in RIN values between samples
2 – One biological group with high quality RNA, one with degraded RNA.

qPCR-based RNA sample quality control (qPCR QC)

In Exiqon Services, we offer two types of qPCR-based RNA sample QC. The two products are designed to examine whether samples are suitable for panel profiling.

- We do not recommend pooling of samples, neither from sample preparation or purified RNA. This usually does not give valuable information about the samples.

- The microRNAs to be tested are pre-selected by Exiqon.

Basic qPCR QC
This product is designed to find outlier samples in larger projects prior to running these on panels.

- The QC screen is based on running all samples using the standard experimental setup for the miRCURY LNA™ Universal RT microRNA PCR System. Five pre-selected microRNAs and three synthetic RNA spike-ins are assayed. A positive and a negative control sample are included
- The levels of microRNAs will be evaluated across all samples based on experience and compared to prior data from similar sample sources. For plasma and serum samples, the degree of hemolysis will be evaluated. Based on amplification of the RNA spike-ins the level of qPCR inhibition will be assessed. For RNA purifications executed at Exiqon, the performance of the extraction will be evaluated
- This RNA QC allows removal of outlier samples from the sample cohort, but optimizing the experimental setup is not possible
- We recommend including this product for projects where standard RNA quality control is not applicable (e.g. low RNA samples, such as exosome derived RNA, plasma/serum RNA, or samples where the RNA quality/quantity is expected to be a challenge)

Extended qPCR QC
This product is designed to be used only when there is a strong potential for qPCR inhibition, or when optimization of the experimental setup is needed (e.g. when evaluating a new RNA source or new sample preparation protocol). This is a much more thorough QC than the basic qPCR QC.

- The qPCR QC profile is based on testing only a few samples (i.e. 2 from each experimental group or similar)
- The selected samples will be analyzed in three different dilutions and tested for the expression of five pre-selected miRNAs and three synthetic RNA spike-ins. A positive and a negative control sample are included
- Exiqon Services will choose the range of dilutions tested based on experience and available input amounts
- The level of microRNAs as well as the correlation of the three dilutions with the signals obtained will be evaluated
- Based on amplification of the synthetic RNA, the level of qPCR inhibition will be assessed. The experimental setup is optimized based on these results
- For RNA purifications executed at Exiqon, the performance of the extraction will be evaluated

For both types of QC, Exiqon will forward recommendations regarding the experimental set up based on the results of these QC tests

- If a sample is thought to be unacceptable for profiling, you will be given the option of sending replacement sample(s)

How many biological replicates do I need?

- The number of biological replicas needed for microRNA profiling depends on the objectives of the experiments. Inclusion of at least three biological replicates per sample group will allow statistical tests of data comparisons, but we recommend minimum four
- Overall the reproducibility of our assays is such that we recommend prioritizing biological replicates over technical replicates for most screening purposes. If validating e.g. array results on limited number of samples, we recommend doing RT replicates

How do I send my samples?

- Please ensure that you include a signed copy of the sample submission form with your samples
- Ensure that samples are labeled clearly and with unique numbers using a permanent marker. Pack the samples arranged in the same order as listed on the SSF in a cryo storage box or similar.
- Please use the fastest available shipping service. For international shipping please use courier service such as FedEx
- If you are shipping from outside Europe and North America, please only send your samples out Monday or Tuesday to avoid weekend deliveries
- RNA samples should be shipped on dry ice in insulated boxes. Please make sure you use adequate amounts of dry ice. We recommend minimum 3,5 kg for shipments with a duration of 1-2 days (e.g within North America or from Northern Europe to Denmark), minimum 6 kg for shipments with a duration of 2-3 days (e.g. from Southern Europe or Middle-East to Denmark) and minimum 9 kg for shipments with a duration of 3-5 days (e.g. from Asia, Australia and South America to Denmark).

For North America, Mexico and Canada, please ship samples to
QIAGEN Genomic Services
Attn. Krishna Amin
QIAGEN Americas
6951 Executive Way
Frederick, Maryland 21703
USA
Phone: +1 301 673 5045

For all other countries, please ship samples to:
QIAGEN GmbH
R&D Life Science Key Account Service
Attn. Andre Bahr / Anke Singer / Holger Wedler
Qiagen Str. 1
40724 Hilden
Germany
Phone +49 2103 29 11649

What about customs regulations?

In order to avoid delays in customs, please make sure that you describe the content accurately: i.e. Purified RNA dissolved in water/for research purpose only.

- The commercial invoice should state a value of 0 (or lowest amount possible)
- For non-human samples, please contact your local representative

Receipt of Samples

Upon receipt of your samples we immediately transfer them to a secure –80°C freezer. You will receive an e-mail confirming that your samples arrived in good condition.

Data quality control

After the microRNA qPCR profiling has been completed, extensive data quality control is performed to ensure that the data used for the data analysis is reliable.

- Melting curve analysis is performed to verify that amplifications are specific and within the expected range. Data is filtered based on Cq values obtained from the negative controls. If there is less than 5 Cq difference between the sample and the negative control, then that particular data point is excluded from the analysis
- The synthetic RNA spike-ins and inter-plate calibrators are used to monitor the RT efficiency and the qPCR reaction
- For each qPCR reaction performed, we determine the efficiency of the qPCR amplification curve using an algorithm based on the LinRegPCR algorithm (J. M. Ruijter, J.M. *et al.*, 2009, Nucleic Acid Research, vol. 37, No. 6) and check that amplification efficiencies are within the acceptable range. No amplification efficiency correction is performed when using the panels

Normalization

- To identify the most appropriate normalization strategy we use NormFinder (Andersen, C.et al. (2004) Cancer Res., 64, 5245-5250). If panels I and II have been used, the data is first combined, before performing global plate normalization

Data analysis

- We provide comprehensive data analysis customized to your project and individual needs. Our experienced scientists have in-depth knowledge about our qPCR platform and a profound understanding of how to handle and interpret microRNA qPCR data. This allows them to adapt the best methods of normalization and statistical data analysis to the project at hand. Our data analysis includes statistical analysis appropriate to the design of your experiment (such as T-test or ANOVA, unsupervised or supervised clustering illustrated with two dimensional heat maps, PCA plots and analysis of correlation with array data)
- In connection with large service projects we also offer fully extensive customized bioinformatics analysis

Report and final consultation

The data is presented in a summary report containing a description of the project, assessments of sample and data quality and an overview of the results of the data analysis with publication-grade illustrations. An extensive Excel file with all raw data is also included. An example of a report on microRNA qPCR Service can be downloaded from our webpage (www.exiqon.com/microRNA-pcr-services).

- Upon completion of the microRNA profiling service, we will send you an e-mail with a link to a secure web-server from which you may download the final report and all associated files. Exiqon offers a free consultation with one of our microRNA qPCR experts to discuss the data
- For standard projects please allow approximately 4-6 weeks for completion of the entire analysis from receipt of samples to delivery of the final report. For larger projects (> 70 samples) longer TATs are to be expected. We will keep you informed about the progress of your profiling experiments throughout this time
- Exiqon will keep all information confidential and will not use any data generated for purposes other than customer statistics and troubleshooting
- Exiqon will store data for at least 3 months after delivery of the final report
- The unused portion of any samples will be disposed of 3 months after completion of the experiments unless specifically agreed otherwise

Selected Publications by service customers

Bye *et al*. Circulating microRNAs and aerobic fitness--the HUNT-Study. PLoS One. 2013;8(2):e57496. PMID: 23469005

Schietinger et at. Rescued tolerant CD8 T cells are preprogrammed to reestablish the tolerant state. Science. 2012 Feb 10;335(6069):723-7. PMID: 22267581

Eulalio *et al*. Functional screening identifies miRNAs inducing cardiac regeneration. Nature. 2012. Dec 20;492(7429):376-81. PMID: 23222520

Xhemalce *et al*. Human RNA methyltransferase BCDIN3D regulates microRNA processing. Cell. 2012 Oct 12;151(2):278-88. PMID: 23063121

Jorde *et al*. Plasma profile of microRNA after supplementation with high doses of vitamin D3 for 12 months. BMC Res Notes. 2012. May 17;5:245. PMID: 22594500

Gusscott *et al*. Notch-mediated repression of miR-223 contributes to IGF1R regulation in T-ALL. Leuk Res. 2012. 36(7): 905-11. PMID: 22424712

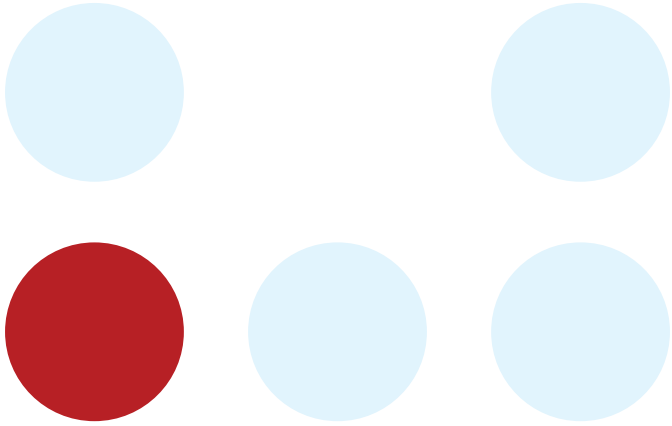
Jorde *et al*. Plasma profile of microRNA after supplementation with high doses of vitamin D3 for 12 months. BMC Res Notes. 2012. 5(1): 245. PMID: 22594500

Barrey *et al*. Pre-microRNA and mature microRNA in human mitochondria. PLoS One. 2011. 6(5): e20220. PMID: 21637849

Haflidadóttir *et al*. miR-148 regulates Mitf in melanoma cells. PLoS One. 2010. 5(7): e11574. PMID: 20644734

Nylander *et al*. Changes in miRNA expression in sera and correlation to duration of disease in patients with multifocal mucosal lichen planus. J Oral Pathol Med. 2011. [Ahead of print]. PMID: 21777290

Ralfkiaer *et al*. Diagnostic microRNA profiling in cutaneous T-cell lymphoma (CTCL). Blood. 2011. 118: 5891-900. PMID: 21865341



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