Expression Analysis

ExiSEQ NGS sample QC kit – small RNA/microRNA

Instruction manual v1.0

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Product summary

The primary purpose of the ExiSEQ NGS sample QC kit is to provide a control for the quality of the RNA isolation in any small RNA Next Generation Sequencing (NGS) experiment. Reproducible RNA isolation may be difficult from some types of samples. Some RNA samples may contain compounds that inhibit the downstream enzymatic reactions even though RNA has been purified using the best standard procedures. This may result in different efficiencies of the library preparation.

One way to check for differences in efficiencies in isolation, is by adding known RNA templates during isolation prior to analysis. Use of RNA spike-ins may also reveal potential presence of nucleases. After conducting the RT qPCR based QC, using PCR plates containing corresponding LNA™ primers to detect the spike-ins and endogenous controls, the data are compared and outlier samples may be identified and considered for exclusion in the further library processing.

In addition, the comprehensive set of ExiSEQ NGS Spike-ins allow thorough QC of the NGS data by assessing the reproducibility and linearity of the reads mapped to these exogenous sequences.

The ExiSEQ NGS sample QC kit consists of the components described in Table 1 and 2.

Kit components, 48 rxns (#800101+800102)	Amount supplied
Enzyme mix	2x24 µL
5× Reaction buffer	128 µL, 5 x concentrated
Nuclease-free water	1 mL
384 well plate with 48x8 assays	1 plate
UniSp6, RNA spike-in	Dried down in 1 tube, re-dissolve in 80 µL water
ExiSEQ NGS Spike-ins, 52 5' phosphorylated RNA molecules	Dried down in 1 tube, re-dissolve in 500 µL water

Table 1.

Kit components, 24 rxns (#800103+800105)	Amount supplied
Enzyme mix	24 µL
5× Reaction buffer	128 µL, 5x concentrated
Nuclease-free water	1 mL
96 well plate with 12x8 assays	2 plates
UniSp6, RNA spike-in	Dried down in 1 tube, re-dissolve in 80 µL water
ExiSEQ NGS Spike-ins, 52 5' phosphorylated RNA molecules	Dried down in 1 tube, re-dissolve in 500 µL water

Table 2.

The ExiSEQ NGS spike-ins are available separately (see table 3).

Kit components, Spike-in kit (#800100)	Amount supplied
ExiSEQ NGS Spike-ins, 52 5' phosphorylated RNA molecules	Dried down in 1 tube, re-dissolve in 500 µL water
Nuclease-free water	1 mL

Table 3.

ExiSEQ NGS Spike-ins

1 vial containing a mix of 52 synthetic 5' phosphorylated microRNAs of different concentrations. The ExiSEQ NGS Spike-ins are dried down. Each vial is sufficient for maximum of 500 purifications of standard 500 µL Serum or Plasma samples. A vial containing 1 mL nuclease-free water is provided for re-suspension of the ExiSEQ NGS Spike-ins. The 52 ExiSEQ NGS Spike-ins are synthetic microRNAs of plant origin and bear no significant homology to microRNAs from the following species: Human (hsa), Mouse (mmu), Rat, (rno), Rhesus Monkey (mml), Orangutan (ppy), Chimpanzee (ptr) or Pig (ssc).

The complete list of spike-in sequences are found in appendix A and is also available to download as a FASTA file from **www.exiqon.com/mirna-NGS-QC**

Plate layouts

miRCURY LNA™ PCR primer sets are available in 96 and 384 well plates. Plate layouts can be downloaded from **www.exiqon.com/mirna-NGS-QC**

Additional required materials

- ExiLENT SYBR® Green master mix
  - ExiLENT SYBR® Green master mix,1.0 ml (#203401) recommended for ExiSEQ NGS sample QC kits 800103, 800104 and 800105
  - ExiLENT SYBR® Green master mix, 2.5 ml (#203403) recommended for ExiSEQ NGS sample QC kits 800101 and 800102
- Benchtop microcentrifuge
- Pipettes (and RNase free tips)
- Vortexer / multi-vial vortex shaker
- Refrigerator and freezer
- Heating block or thermocycler
- Sealing foils for PCR plates
- Real-time PCR instrument
- RNA isolation kit (miRCURY™ RNA Isolation Kits)

Instructions for re-suspension and storage of ExiSEQ NGS Spike-ins

Prior to use, the ExiSEQ NGS Spike-ins must be dissolved in 500 µL nuclease-free water and the UniSp6 RNA spike-in must be dissolved in 80 µL. Vortex for 5 seconds and leave the suspension on ice for 30 minutes to dissolve. Vortex for 5 seconds again and spin to collect tube contents. Exiqon recommends to aliquot the dissolved ExiSEQ NGS Spike-ins, and to avoid repeated freeze/thawing. The re-suspended ExiSEQ NGS Spike-ins can be stored at -20 °C short term. For long-term storage, keep the vials at -80 °C after re-suspension.

All other reagents should be stored at -20 °C. Do not store in a frost-free freezer.
The PCR panels can be stored between +4 °C and -20 °C. Under these conditions, all components are stable until expiry date on the package or vial.

Important note – Cautions

Ensure that a suitable lab coat and disposable gloves are worn and standard safety precautions are followed when working with chemicals.

For more information, please consult the appropriate Material Safety Data Sheets (MSDSs). Body fluids like serum or plasma of all human and animal subjects are considered potentially infectious. All necessary precautions recommended by the appropriate authorities in the country of use should be taken when working with Body fluids. See tips 2 and 3 for more information on how to handle RNA.

Product description

Addition of ExiSEQ NGS Spike-ins during RNA isolation enables the comparability and reproducibility of the whole process from RNA isolation to sequencing to be monitored. The protocol given here is intended for biofluid samples, and is expected to result in NGS Spike-ins representing 1-3 % of total reads when added during RNA isolation starting with 400-500 µL serum/plasma. The kit is compatible with many samples types, but is especially useful for challenging samples with low RNA content such as serum, plasma, urine and other biofluids. Note that the small RNA content of samples can differ considerably from sample to sample and source to source. ExiSEQ NGS Spike-ins can also be added directly to RNA samples before small RNA library preparation. For more accurate ratios of spike-ins *vs* endogenous microRNAs in other sample types or when using isolated RNA samples, experimental titration of ExiSEQ NGS Spike-ins is recommended.

The protocol consists of 5 simple steps (see also Figure 1):

1. Addition of 52 ExiSEQ NGS Spike-ins to the samples during RNA isolation
2. cDNA synthesis, including UniSp6 spike-in
3. qPCR reactions
4. Sample evaluation and NGS of accepted samples
5. Evaluation of the ExiSEQ NGS Spike-in data

Protocol overview of the ExiSEQ NGS sample QC kit - small RNA/microRNA

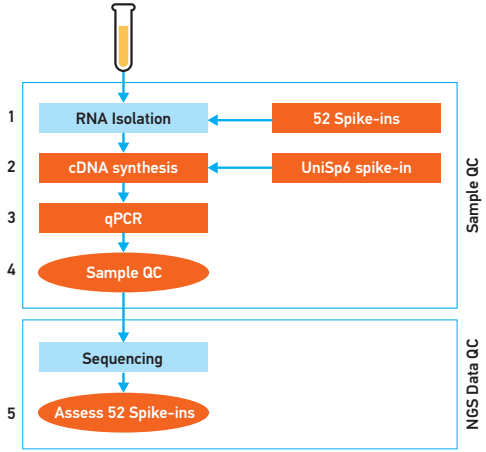


Figure 1. Reagents/ primers used in the orange boxes are included in the kit (except PCR mastermix) and tips on sample evaluation and NGS Data QC using the ExiSEQ NGS Spike-ins are found in this manual.

1. Addition of ExiSEQ NGS Spike-ins reveals variation in RNA isolation efficiency
The 52 ExiSEQ NGS Spike-ins which are introduced during RNA isolation by adding them to the lysis solution enable quality control of the RNA isolation efficiency prior to library preparation and offer the possibility to reject samples before downstream library preparation and sequencing analysis. Alternatively the ExiSEQ NGS Spike-ins can be added directly to the RNA after RNA isolation, however this means any information on RNA isolation, especially with regard to the small RNA fraction, will be lost.

2-4. RT qPCR based QC
The ExiSEQ NGS sample QC kit is based on miRCURY LNA™ Universal RT microRNA PCR, which offers the best available combination of performance and ease-of-use on the microRNA real-time PCR market because it unites two important features (Figure 2):

1. Universal RT – One first-strand cDNA synthesis reaction provides template for all spike-in and microRNA real-time PCR assays. This saves precious sample, reduces technical variation, requires use of less reagents, and saves time in the laboratory.
2. LNA™ PCR amplification – Both PCR amplification primers (forward and reverse) are specific to the spike-in or microRNA and optimized with LNA™. The result is: 1) exceptional sensitivity as well as extremely low background enabling accurate quantification of very low spike-in or microRNA levels and 2) highly specific assays that allow discrimination between closely related sequences.

Schematic outline of the miRCURY LNA™ Universal RT microRNA PCR System

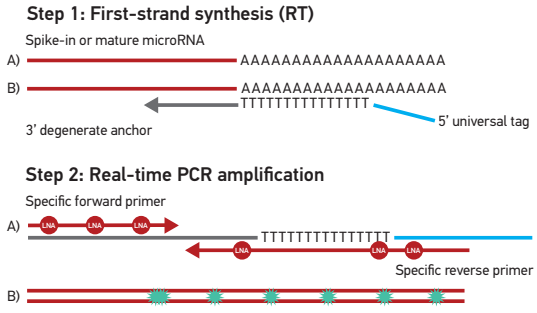


Figure 2. A poly-A tail is added to the spike-in or mature microRNA template (step 1A). cDNA is synthesized using a poly-T primer with a 3' degenerate anchor and a 5' universal tag (step 1B). The cDNA template is then amplified using LNA™-enhanced specific forward and reverse primers (step 2A). SYBR® Green is used for detection (step 2B).

An RNA spike-in (UniSp6) is added during cDNA synthesis in order to monitor cDNA and qPCR efficiency. A total of 8 miRCURY LNA™ PCR primer sets amplify spike-ins added during RNA isolation (2) or cDNA synthesis (1), as well as endogenous microRNA controls (5). These endogenous microRNA controls are: miR-103 and miR-191 which are well expressed in most tissues; miR-451a 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. The signals from these assays are used to qualify samples prior to NGS. See Table 4 for details.

qPCR assay	Recommended usage
UniSp100	RNA Isolation efficiency assessment
UniSp101	RNA Isolation efficiency assessment
miR-103a-3p	Endogenous control, serum/plasma
miR-191-5p	Endogenous control, serum/plasma
miR-30c-5p	Endogenous control, biofluids including urine
miR-451a	Hemolysis indicator serum/plasma
miR-23a-3p	Hemolysis indicator serum/plasma
UniSp6	Monitoring presence of inhibitory compounds

Table 4.

5. Evaluation of the ExiSEQ NGS Spike-in data

The 52 ExiSEQ NGS Spike-ins added during RNA isolation are present at different concentrations, representing microRNAs ranging from very low to very high endogenous levels. They are very useful for evaluating NGS performance and can be used to assess the technical reproducibility across samples and verify the linearity of the NGS reads mapped to these exogenous sequences across samples.

Before starting the experiment

Sample input: The ExiSEQ NGS sample QC kit is optimized for using 400-500 µL of biofluid. Using the recommended ExiSEQ NGS Spike-in amount is expected to result in ExiSEQ NGS Spike-ins representing 1-3 % of total reads. This may however vary from project to project and it may be necessary to adjust the amount of ExiSEQ NGS Spike-ins added for optimal results. The sample input volume to the cDNA synthesis reaction may have to be adjusted, depending on sample quality/yield.

RNA handling: Information on how to handle RNA can be found in the tips section of the 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.

Please 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 our biofluid guidelines: www.exiqon.com/ls/Documents/Scientific/microRNA-serum-plasma-guidelines.pdf

Master mix: For optimal performance it is highly recommended to use ExiLENT SYBR® Green master mix (not included in kit) in order to obtain optimal sensitivity and specificity.

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 manufacturer's guidelines on this. See tip 6

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 tip 10

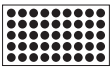
Excess volumes required for pipetting: Liquid handling with pipettes or pipetting robots require excess volumes of reagents due to loss during pipetting. The loss depends on the available pipetting system but losses in the range from 10-25 % are not uncommon. All protocols in the current instruction manual reflect the required reaction volumes, and pipetting volumes should be adjusted according to accommodate the pipetting loss of the available pipetting system.

Protocol & notes

This protocol describes how to add the ExiSEQ NGS Spike-ins to a biofluid sample during RNA isolation, and conduct the first strand cDNA synthesis and real-time PCR using the supplied LNA™ PCR plate, for qualification of biofluid samples before library preparation and NGS.

Checklist:

- Have you considered excess volumes required for using liquid handling robotics?
- Have you dissolved the spike-ins as recommended: ExiSEQ NGS Spike-ins in 500 µL nuclease-free water and UniSp6 in 80 µL nuclease-free water
- ROX: The ExiLENT SYBR® Green master mix does not include the ROX passive reference dye. Please follow instrument manufacturer's recommendations
- ABI instruments: The use of manual background and threshold settings is necessary for obtaining correct PCR data. Make sure to have the optimal settings by downloading the instrument settings file at www.exiqon.com/sds. Furthermore, if the data is to be analyzed using GenEx, the experiment must be set up as an AQ experiment, not RQ.

Overview of experimental workflow for qualification of samples for NGS analysis:	
	**Phase 1:** RNA isolation and addition of ExiSEQ NGS Spike-ins to the sample
	**Phase 2:** cDNA synthesis
	**Phase 3:** Real-time PCR amplification
	**Phase 4:** Sample QC

Phase 1: RNA isolation and addition of ExiSEQ NGS Spike-ins to the samples:

Step 1.1 Sample preparation	In order to reduce the effect of inhibitors/nucleases, the starting material (biofluid) should be centrifuged at no more than 3000 x g for 5 minutes. This should be done prior to storage rather than immediately prior to isolation. Omitting this clearance step can affect PCR detection (use excess sample in order to secure sufficient supernatant available for the next steps). If working with urine or CSF samples an initial exosome isolation step is highly recommended.
Step 1.2 Addition of ExiSEQ NGS Spike-ins	Thaw solution of ExiSEQ NGS Spike-ins on ice, spin quickly and keep on ice until frozen again. A mastermix of lysis buffer is made for all samples to be purified. For each purification of biofluid sample to be performed add 1 µL ExiSEQ NGS Spike-in solution (typically per a 400-500 µL serum/plasma volume). Mix well and add the spiked lysis buffer to the biofluid samples and mix well. Continue the RNA isolation according to the manufacturer's instructions. **Note:** To avoid RNA degradation, make sure the lysis buffer inhibits all RNase activity in the biofluid immediately, by thoroughly homogenizing after addition of the lysis buffer.

Phase 2: cDNA synthesis:

Step 2.1
RNA input volume

Since the RNA concentration in extractions from serum and plasma and other biofluids cannot be determined with accuracy, we recommend using the amount of starting material as a measure for input amount.

See tip 1

Step 2.2
Prepare reagents for cDNA synthesis

Gently thaw the 5x Reaction buffer and nuclease-free water and immediately place on ice. Mix by vortexing. Re-suspend the UniSp6 spike-in as recommended. Immediately before use, remove the Enzyme mix from the freezer, mix by flicking the tubes and place on ice. Spin down all reagents.

Step 2.3
Combine reagents according to Table 5

Note: remember to calculate necessary excess volume for pipetting and robotic dead volume

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 RNA spike-ins (in the proportions indicated in the first four lines of Table 5). 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.

Reagent	Volume (µL), RT reaction
5x Reaction buffer	2
Nuclease-free water	4.5
Enzyme mix	1
UniSp6 spike-in	0.5
Template RNA[1)]	2
Total volume	10

Table 5. Reverse transcription reaction setup

Step 2.4 Mix and spin reagents	Mix the reaction by very gentle vortexing or pipetting to ensure that all reagents are thoroughly mixed. Then spin down.

Step 2.5 Incubate and heat inactivate[2)]	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.

1) Optimal volumes may need to be determined depending on sample.
2) 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 is stored in "low-nucleic acid binding" tubes or plates. See tip 5.

Phase 3: Real-time PCR amplification

Step 3.1
Prepare reagents for real-time PCR

Place cDNA (from Step 2.5), nuclease-free water and ExiLENT SYBR Green® PCR Master mix on ice and thaw for 15-20 minutes. Protect the ExiLENT SYBR Green® PCR Master mix vials from light. Immediately before use, mix the PCR Master mix by pipetting up and down. The rest of the reagents are mixed by vortexing and spun down.

Step 3.2
Dilute cDNA template 1:50 in nuclease free water

Dilute 4 µL in 196 µL nuclease free water to reach 50x dilution.

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. Please note that the ExiLENT 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.

See tip 6

Step 3.3
Combine cDNA and ExiLENT 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 in 1:1 v/v ratio (e.g. 40 µL 2x Master mix and 40 µL diluted cDNA).
3. Mix gently by inverting the tube, spin down.
4. Add 10 µL Master mix:cDNA mix to each well.
5. Seal the plate with optical sealing as recommended by the instrument manufacturer.

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

Step 3.4
Spin plate

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

Step 3.5
Real-time PCR amplification

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

Process step	Settings, LC480 instrument[3)]	Settings, other instruments[4)]
Polymerase Activation/ cDNA Denaturation	95 °C, 10 min	
Amplification	45 amplification cycles at 95 °C, 10 s 60 °C, 1 min ramprate 1.6 °C/s[5)] Optical read	45 amplification cycles at 95 °C, 10 s 60 °C, 1 min ramprate 1.6 °C/s[5)] Optical read
Melting curve Analysis[6)]	Yes	Yes

Table 6. Real-time PCR cycle conditions

3) Five additional amplification cycles are required when using the LC480 instrument to allow collection of assay data with Cp-values up to 40.
4) 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.
5) 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 Viia7 instruments. If the ramp rate of cooling is too rapid, performance may be compromised.
6) 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 Tm of a PCR product depends on buffer composition, salt concentration and the PCR instrument.

Phase 4: Sample qualification

Step 4.1
Cq data extraction

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, please see tip 7.

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

See tip 7

Step 4.2
Assessment of sample quality

The ExiSEQ NGS Spike-in controls are utilized to monitor the technical quality of RNA isolation, cDNA synthesis, and presence of PCR inhibitors in the sample. The UniSp100 and UniSp101 assays are included on the PCR plate to monitor RNA isolation efficiency. The concentration of UniSp100 spike-in corresponds to moderately abundant microRNAs. The concentration of UniSp101 spike-in corresponds to highly abundant microRNA in a biofluid. Cq values will vary depending on experimental settings of the experiment and the method used as well as the elution volume and RNA input, but the typical Cq value for UniSp100 is in the 31-34 range and UniSp101 is in the 25-28 range. The deltaCq for the two spike-ins should be around 5-7.

The ExiSEQ NGS Spike-ins (UniSp100 and UniSp101) were added at a fixed amount per isolation, and a fixed volume of isolated RNA was used in the cDNA synthesis. Thus, the main factors affecting the amplification signals of the two controls are isolation efficiency, cDNA synthesis efficiency, and amplification efficiency. If the isolation controls UniSp100 and UniSp101give comparable values across all samples, the interpretation would be that all isolations were performed with similar efficiencies. If, however, one or more samples give higher Cq values for the RNA isolation efficiency controls, it suggests a problem in one of the steps of the RNA isolation procedure.

The UniSp6 assay is included on the PCR plate to monitor cDNA synthesis and qPCR efficiency. UniSp6 is added in a fixed amount at the cDNA synthesis step, and is therefore unaffected by RNA isolation efficiency and yield, but is affected by downstream inhibitors of cDNA synthesis or qPCR. The Cq values for UniSp6 are usually below 20.

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Step 4.2
Assessment of sample quality

If the isolation controls (UniSp100 and 101) and the 5 endogenous microRNAs genes are affected in a few samples, but the cDNA synthesis control (UniSp6) is stable across all samples, it is likely that the affected RNA samples were isolated with a lower efficiency than the remaining samples. It should be considered to re-isolate these samples, or alternatively exclude them from the study. If the isolation controls, cDNA synthesis controls and endogenous microRNAs are all affected by elevated Cqs in a few samples, this could suggest presence of inhibitors of enzymes such as Reverse transcriptase or DNA polymerase in these samples. It should be considered whether the samples should be excluded from the study, or alternatively re-isolated in the hope of obtaining a purer RNA. If the endogenous microRNAs are affected by high Cqs while none of the spike-ins are affected, this would indicate that the samples in question had a lowered microRNA content to begin with. In this case, consider excluding the samples from the study.

Note: See decision tree for identification of outlier samples in Appendix B

Step 4.3
Interpretation of the hemolysis indicator

A major source of variation in plasma and serum is potential cellular derived microRNA contamination including 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 we have found that a ΔCq (miR-23a-miR-451a) lower than 5 in human serum or plasma represents non-hemolysed samples. If the ΔCq 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.

For mouse and rat samples the ΔCq (miR-23a-miR-451a) levels will be different.

NGS analysis

This protocol will provide sufficient reads derived from the ExiSEQ NGS Spike-ins for post-sequencing analysis using a standard small RNA NGS analysis pipeline. The sequences of the 52 ExiSEQ NGS Spike-ins can be downloaded as a single FASTA file (www.exiqon.com/mirna-NGS-QC). Reads should be mapped to the ExiSEQ NGS Spike-in sequences (using Bowtie2 or similar mapping algorithm) and spike-in reads should be filtered out from the rest of the data. We recommend "perfect match" settings when mapping, filtering and counting ExiSEQ NGS Spike-in reads in a dataset (FASTQ files). Following counting of the ExiSEQ NGS Spike-in reads, they should be normalized to the total number of reads per sample. If TPM (tags per million reads) are to be used, then use the following formula.

For each ExiSEQ NGS Spike-in, calculate TPM based on each sample:

$$\mathbf{TPM} = \frac{\mathbf{\#\ Spike\text{-}in\ reads}}{\mathbf{Total\ reads}} * \mathbf{1000000}$$

After this simple normalization to individual sample reads has been done for all spike-ins in all samples, correlation matrices should be plotted for all sample-to-sample comparisons. This is done to evaluate the sample-to-sample correlation in the sample set. Expected correlation should be R^2 of 0.95-0.99. If comparing day-to-day correlation, the correlation is usually weaker than within a batch of samples purified on the same day. If samples deviate from these values, they could be technical outliers and should potentially be excluded from downstream analysis. Note that a few of the ExiSEQ NGS Spike-ins are present at low concentrations to represent very low TPM counts and will therefore give low numbers of reads, especially if the sequencing depth is not high. They should be excluded from the correlation analysis. A rule of thumb would be to exclude ExiSEQ NGS Spike-in data lower than 1 TPM if read depth is approximately 10 Million reads per sample.

Tips for success

Tip 1. Guidelines for blood serum and plasma samples

Plasma and serum are essentially cell free liquid samples. This means that only circulating RNA is extracted from these sample types, resulting in low total RNA concentrations, even if the microRNA fraction is readily detectable. The result of this is that measuring correct RNA concentrations is difficult, and that there is a high risk of increased loss during extraction. For this reason, we recommend using RNA amounts based on starting volume rather than RNA quantity. Comprehensive guidelines for microRNA profiling in blood serum/plasma can be downloaded at www.exiqon.com/serum-plasma-guidelines.

Tip 2. General guidelines for handling and storage of RNA

The following precautions should be taken to prevent RNase contamination and degradation of the RNA sample and reagents:

- Always wear disposable gloves
- Use nuclease-free, low nucleic acid binding plasticware and filter barrier pipette tips
- Keep tubes closed when possible. Always spin tubes before opening
- For long-term storage, RNA should be stored at -80 °C. Avoid repeated freezing and thawing cycles

Tip 3. Good PCR laboratory practice

To reduce the risk of contaminating PCRs with "old" PCR amplicons, and consequently obtaining false results:

- Always wear a clean lab coat. Use separate lab coats for RNA sample preparation, cDNA synthesis and when setting up PCR reactions or handling PCR products
- Change gloves often, especially whenever you suspect they may have been contaminated
- Establish and maintain designated areas for PCR set-up, PCR amplification, and gel electrophoresis of PCR products
- Never bring amplified PCR products into the PCR set-up area
- Spin down all reaction and sample tubes before opening. Open and close all reagent and sample tubes carefully, trying not to splash or spray PCR samples
- Already run qPCR plates contain up-to millions of PCR products per well. Discard them properly, immediately after the real-time PCR instrument is finished. Never move or open them in any pre-PCR area
- Keep reactions and components capped whenever possible
- Use filter barrier pipette tips to avoid aerosol-mediated contamination of your pipetting device
- Clean laboratory benches and equipment regularly

Tip 4. Keep reagents and reactions cool at all times

In order to ensure optimal performance of the miRCURY LNA™ Universal RT microRNA PCR system it is important that the reagents and reactions are kept on ice (or at 4 °C) as much as possible during the protocol (apart from reaction steps specifically involving raised temperatures).

Tip 5. First strand cDNA synthesis

Store undiluted cDNA samples in nuclease-free low nucleic acid-binding micro centrifuge tubes, e.g. Eppendorf DNA LoBind tubes. Storage of diluted cDNA is not recommended.

Tip 6. Passive reference dye (ROX)

Many real-time PCR instruments will only produce reliable results when a passive reference dye such as ROX is added to the PCR reaction. The reference dye is used to normalize signals from individual PCR wells in order to enable comparison of real-time PCR amplification signals across an entire PCR-plate. It is recommended to determine whether your real-time PCR instrument has this type of requirement. The amount of ROX to include in the PCR reaction depends on the requirements of the real-time PCR instrument and must be adjusted accordingly. Please follow the supplier's instructions for preparation and concentrations of ROX solutions. Instruments that allow excitation at individual wavelengths for individual dyes (most filter wheel based instruments) require less ROX than instruments that use a single excitation wavelength for all fluorophores (most laser based instruments use excitation at 488 nm). It is important to note that excessive amounts of ROX may inhibit the PCR reaction. It may be recommended to perform a titration of ROX amounts in order to determine the optimal concentration for a particular instrument-system combination. It is possible to use the spike-in template (provided in the Universal cDNA synthesis kit II) and the Control primers (provided in the ExiLENT SYBR® Green master mix kit) to perform such titrations. Please contact us (exiqon.com/contact) for the most up-to-date information regarding passive reference dyes. Recommended ROX concentrations are found here:

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Instrument	ROX concentration
ABI 7900HT	300-500 nM
ABI 7900HT FAST	300-500 nM
ABI Viia7	30-50 nM
ABI Viia7 FAST	30-50 nM
ABI 7500 FAST	30-50 nM
ABI StepOnePlus	300-500 nM
ABI 7300	300-500 nM
ABI 7500	30-50 nM
ABI 7700	300-500 nM
ABI 7000	300-500 nM

Tip 7. Guidelines for real-time PCR data collection using ABI instruments

On cyclers using baseline and threshold values for Cq (Ct) calculations, such as ABI 7900HT, it is important that the proper settings are used. Use of the automatic function of the software for these settings does not seem to produce optimal results for SYBR® Green based assays. Often the baseline is set erroneously on non-detected assays, and this in turn gives false positives, therefore do not use automatic settings. Another issue to consider when using automatic settings is that the settings may differ between plates resulting in data that cannot be compared directly. Inter-plate calibration may not fully resolve this issue, since each assay has a separately calculated baseline and threshold. Instead, both threshold and baseline should be set manually, applying the same settings for all assays on the plate. The following principles should be applied to manual baseline and threshold settings:

Baseline: The baseline should be calculated in the cycle interval before the amplification takes off (see Figure 3).

Threshold: The threshold should then be set with the Y-axis in log scale where all assays are in the log linear phase, and the threshold above background for all assays (see Figure 3).

Note: The optimal threshold value may vary between individual machines and experiments.

Important note

If ROX passive reference dye has not been used in the PCR reactions, make sure the SDS software is set-up without reference dye correction.

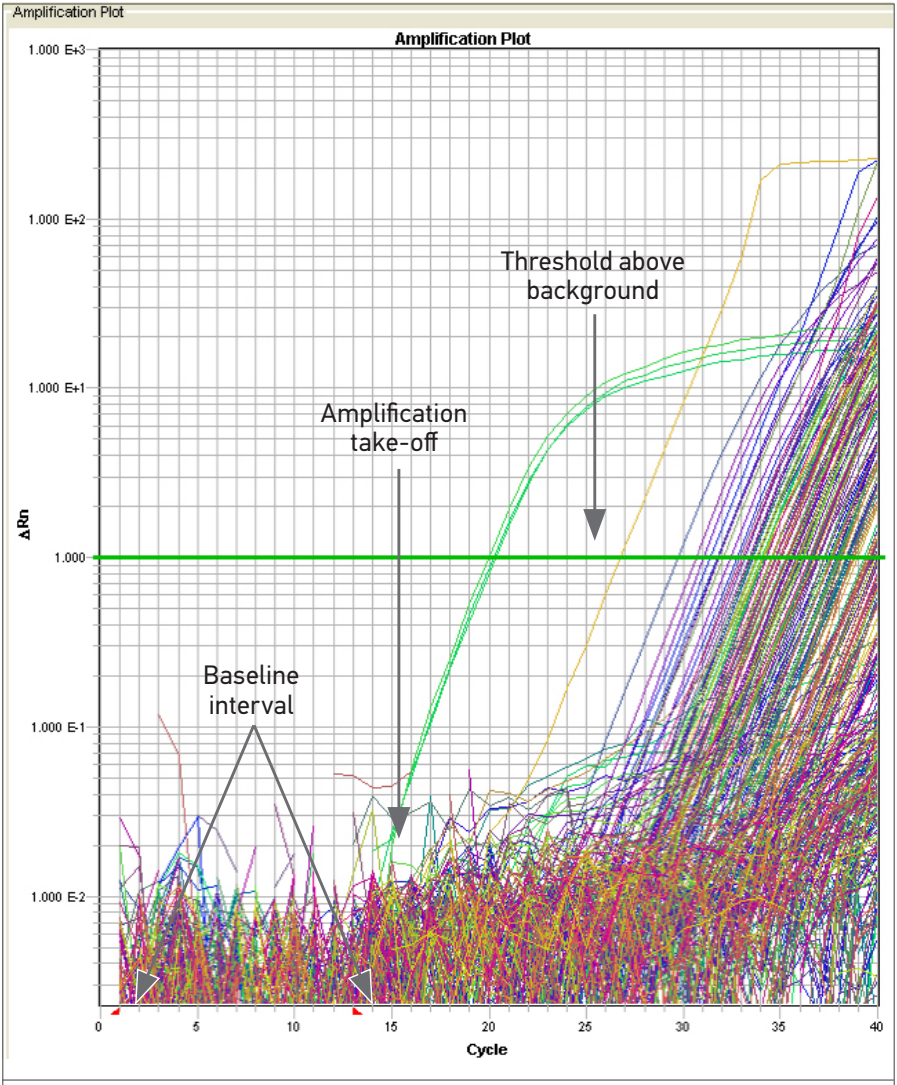


Figure 3. Baseline setting.

Troubleshooting guide

Problem	Suggestion
PCR signal in samples amplified from first-strand synthesis reactions performed without reverse transcriptase	This typically indicates contamination of the template RNA with genomic DNA. Perform DNase treatment of the RNA sample. If this does not solve the problem RNA samples or other reagent may be contaminated with PCR products.
PCR signal in no-template PCR reaction	This typically indicates contamination of the cDNA template or PCR reagents with amplified PCR product. Exposing the reactions to elevated temperatures (i.e. room temperature) during any part of the protocol increases the risk of background signals. It is important that the reagents and assembled reactions are kept cool (on ice or 4 °C) at all times (see tip 4).
Generated signals are weak	On some real-time PCR cyclers, gain-settings are adjustable. Make sure the gain settings of your realtime PCR cycler have been set to accommodate the signals generated from the specific assay. RNA samples may contain PCR inhibitors. Further purification or an alternative RNA extraction method may be necessary. Check positive controls.
No fluorescent signal is detected during the PCR	Confirm that you have a PCR product by running an aliquot of your PCR reaction on an agarose gel.
No fluorescent signal detected during the PCR, but a PCR amplicon can be detected by agarose gel electrophoresis	Check that the filter in the real-time PCR cycler was set to either SYBR® Green or FAM/FITC. Check that the optical read is at the correct step of the real-time PCR cycles. Adjust the baseline in the real-time PCR cycler software.
ExiSEQ NGS Spike-ins detected at very low (<1 %) or very high (>10 %) counts	Adjust the amount of ExiSEQ NGS Spike-ins added accordingly.

Related products

miRCURY™ RNA Isolation Kit – Biofluids (300112)
Small RNA preparations from serum or plasma samples or from exosomes from serum or plasma. Suitable for miRCURY LNA™ Universal RT microRNA PCR.

miRCURY™ Exosome Isolation Kit – Serum and plasma (300101)
Isolate Exosomes from serum or plasma for RNA isolation or other downstream applications.

Exosome Isolation Kit – Cells, urine and CSF (300102)
Isolate Exosomes from diverse biofluids or conditioned cell culture media for RNA isolation or other downstream applications.

miRCURY LNA™ Universal RT microRNA PCR
Exiqon's microRNA qPCR system offers the best available combination of performance and ease-of-use on the microRNA real-time PCR market. The combination of a Universal RT reaction and LNA™-enhanced PCR primers results in unmatched sensitivity and specificity. The Ready-to-use microRNA PCR panels enable fast and easy microRNA expression profiling. Take advantage of the tailored Universal RT microRNA PCR spike in kit to monitor the performance of your PCR.

miRCURY LNA™ Universal RT microRNA PCR – Pick-&-Mix
Exiqon's unique product, which allows customers to design custom qPCR ready-to-use plates, containing assays targeting either known or novel targets – Ideal for validation of microRNA NGS data.

XploreRNA - Online NGS data analysis platform
XploreRNA is the most capable online RNA NGS data analysis platform ever. It's easy and fast to use, and available 24/7. Upload your data files, and let the XploreRNA data analysis pipeline conduct the analysis. Results are presented in a detailed report. Analysis includes everything from Data QC, Mapping, Normalization, Differential expression analysis and statistical analysis, to Gene Ontology Enrichment analysis. XploreRNA also makes validation easy by the Gene Sorting Wizard that allows you to sort and filter gene transcripts to validate.

Appendix A

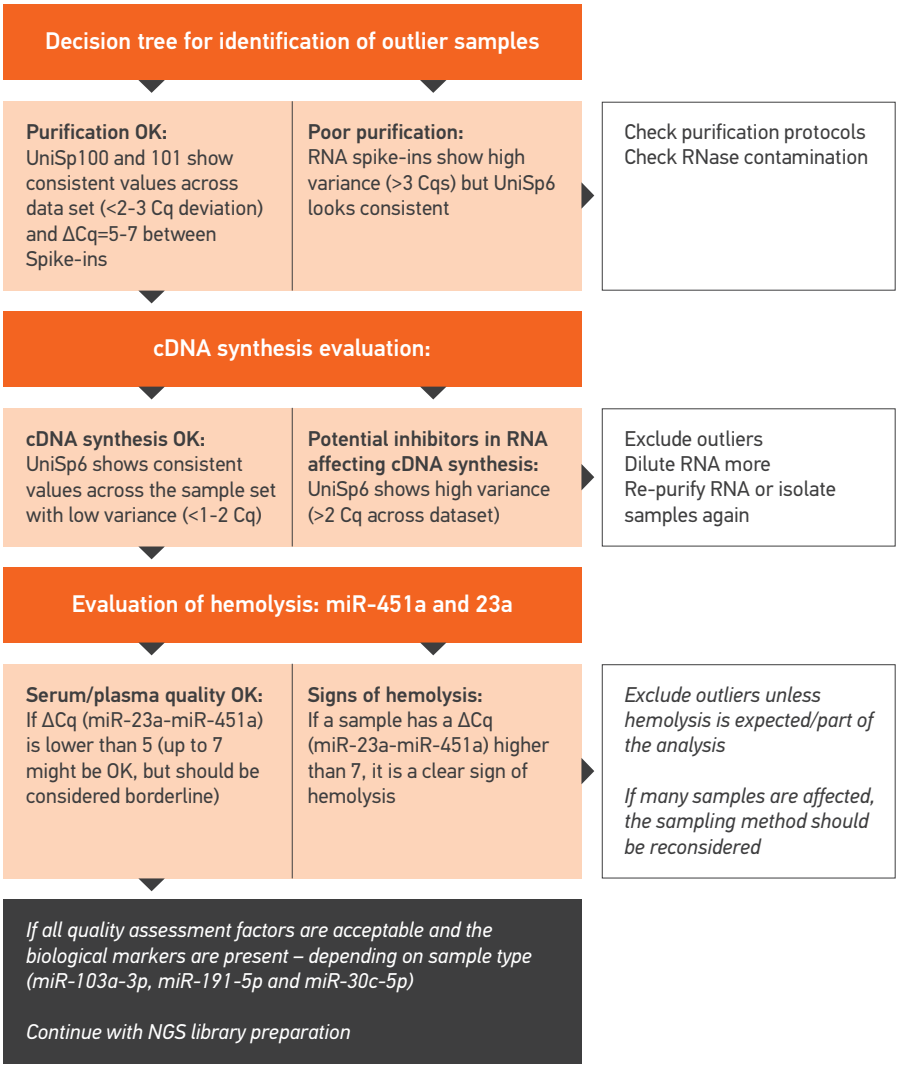
ExiSEQ NGS Spike-in sequences

All 52 ExiSEQ NGS Spike-ins are 5' phosphorylated synthetic microRNAs with sequence length in the range 20-24 nucleotides. The sequences are of plant origin bear no significant homology to microRNAs from the following species: Human (hsa), Mouse (mmu), Rat, (rno), Rhesus Monkey (mml), Orangutan (ppy), Chimpanzee (ptr) or Pig (ssc).

NGS Spike-in name	Sequence	NGS Spike-in name	Sequence
UniSp100	uugauucccaauccaagcaag	UniSp126	acaaacaccuuggauguucuu
UniSp101	uaccaaccuuucaucguuccc	UniSp127	aagcuuugcucguucauguuc
UniSp102	ucccaaauguagacaaagca	UniSp128	uaguccgguuuuggauacgug
UniSp103	ugaagcugccagcaugaucua	UniSp129	uuagaugaccaucaacaaacu
UniSp104	cagccaaggaugacuugccgg	UniSp130	ucuugcuuaaaugaguauucca
UniSp105	uccggcaaguugaccuuggcu	UniSp131	agcucugauaccaaaugauggaau
UniSp106	agaaucuugaugaugcugcau	UniSp132	ugaucucuucguacucuucuug
UniSp107	uuggcauucuguccaccucc	UniSp133	uguuuguuguacucggucuagu
UniSp108	uguguucucaggucaccccuu	UniSp134	uucuugcauauguucuuuauc
UniSp109	cgaaacuggugucgaccgaca	UniSp135	uccuguguuuccuuugaugcgugg
UniSp110	uucgaggccuauuaaaccucug	UniSp136	aucaguuucuuguucguuuca
UniSp111	uagaaugcuauuguaauccag	UniSp137	ucauggucagauccgucaucc
UniSp112	gguucguacguacacuguuca	UniSp138	ucgcucugauaccaaauugaug
UniSp113	uaaacuaaucacggaaaugca	UniSp139	uugaauugaagugcuugaauu
UniSp114	uuuuggaaauuuguccuuacg	UniSp140	ugacaugggacugccuaagcua
UniSp115	ugagccucugugguagcccuca	UniSp141	uaacuaaacauugguguagua
UniSp116	uuugcuuccagcuuuugucuc	UniSp142	uaagauccggacuacaacaaag
UniSp117	uugguuacccauauggccauc	UniSp143	uaauccuaccaauaacuucagc
UniSp118	uucgaugucuagcagugcca	UniSp144	gauggauaugucuucaaggac
UniSp119	ucuaagucuucuauugauguu	UniSp145	ccuuggagaaauaugcgucaa
UniSp120	uacgcauugaguuucguugcuu	UniSp146	uuaugucuuguugaucucaau
UniSp121	uggcuugguuuauguacaccg	UniSp147	uaaagucaauaauaccuugaag
UniSp122	uucugcuauguugcugcucau	UniSp148	uuuuuccucaaauuuauccaa
UniSp123	ugauuggaaauuucguugacu	UniSp149	augaauuuggaucuaauugag
UniSp124	ucuagcagcuguugagcaggu	UniSp150	auugguucaauucugguguug
UniSp125	uucuucgugaauaucuggcau	UniSp151	uaauuugguguuucuucgauc

Appendix B

Decision tree for identification of outlier samples



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