Expression
Analysis

miRCURY LNA™ Universal RT microRNA PCR, RNA Spike-in kit

Instruction manual v2.3

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Supplement to Exiqon's Instruction Manuals for:
miRCURY™ RNA Isolation Kit – Cell & Plant
miRCURY™ RNA Isolation Kit - Tissue
miRCURY™ RNA Isolation Kit - Biofluids
miRCURY™ Exosome Isolation Kit - Serum and Plasma
miRCURY™ Exosome Isolation Kit - Cells, Urine and CSF
miRCURY LNA™ Universal RT microRNA PCR
miRCURY LNA™ Universal RT microRNA PCR, biofluid samples
miRCURY™ microRNA QC PCR Panel

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Product summary

Reagent kit

miRCURY LNA™ Universal microRNA PCR system, RNA Spike-in kit (product# 203203)
This kit contains RNA spike-in templates for use with the miRCURY LNA™ Universal microRNA PCR system.

Contents

	Reagent	Amount	Conc. (re-suspended)
UniSp2, UniSp4, UniSp5 RNA Spike-in template mix	Synthetic UniSp2 RNA (22 nt)	160 fmole	2 fmole/μL
	Synthetic UniSp4 RNA (22 nt)	1.6 fmole	0.02 fmole/μL
	Synthetic UniSp5 RNA (22 nt)	0.016 fmole	0.0002 fmole/μL
	MS2 total RNA	50 ng	0.625 ng/μL
cel-miR-39-3p RNA Spike-in template	Synthetic cel-miR-39-3p RNA	0.16 fmole	0.002 fmole/μL
	MS2 total RNA	50 ng	0.625 ng/μL

Storage

The RNA Spike-in kit is shipped at room temperature with the RNA content dried down. Upon arrival, the spike-in kit should be stored at -20°C. Under these conditions, all components are stable until the expiry date on the vial. It is recommended to store the RNA spike-ins in aliquots at -20°C after re-suspension to avoid repeated freeze-thaw cycles.

Additional required materials

miRCURY LNA™ Universal RT microRNA PCR system materials

- Individual primer sets for the various RNA Spike-in templates
- ExiLENT SYBR® Green master mix
- Universal cDNA synthesis kit II
- Individual primer set(s) or ready-to use panels

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
miRCURY™ RNA Isolation Kit - Tissue
miRCURY™ RNA Isolation Kit - Biofluids

Product description

RNA spike-in kit (synthetic control templates)

The primary purpose of the RNA spike-in kit and the matching LNA™ primer sets for detection of the RNA spike-ins is to provide a control for the quality of the RNA isolation, cDNA synthesis and PCR amplification in any microRNA qPCR experiment. This will help to identify experimental and technical outliers. Reproducible RNA isolation may be difficult from some types of samples. Some RNA samples may contain compounds that inhibit the cDNA synthesis or the PCR even though the RNA has been purified using the best standard procedures. This may result in different efficiencies of the reverse transcription or PCR amplification between compared samples. One way to check for differences in efficiencies in isolation, cDNA synthesis and PCR amplification is by adding known RNA spike-ins to the sample prior to RNA isolation and cDNA synthesis, respectively. Use of RNA spike-ins may also reveal potential presence of nucleases.

After conducting the PCR, but before progressing into data analysis, wells detecting the RNA spike-ins are compared and outlier samples may be identified and considered for exclusion in the further data analysis. Exiqon have designed the RNA spike-in kit for this purpose. The kit provides several RNA spike-ins. Three of the RNA spike-in templates (UniSp2, UniSp4 and UniSp5) are provided pre-mixed in one vial, each at a different concentration with 100-fold increments. This mix is meant as an RNA isolation control. A second vial contains a synthetic version of a *C. elegans* microRNA, cel-miR-39-3p. The cel-miR-39-3p RNA Spike-in template is meant to be used in combination with the UniSp6 RNA Spike-in template provided with the miRCURY LNA™ Universal cDNA synthesis kit II (product # 203301). This mix is meant as a cDNA synthesis control.

The UniSp6 RNA spike-in control primer set can be found in all Exiqon's pre-defined microRNA PCR panel plates. When configuring Pick-&-Mix microRNA PCR Panels, it is possible to select from all five control primer sets in the layout. For use with our non-plate based PCR primer set products a UniSp6 RNA spike-in control primer set is provided with the ExiLENT SYBR® Green master mix, while UniSp2, UniSp4, UniSp5 and cel-miR-39-3p primer sets can be purchased as individual primer sets.

In all Exiqon's pre-defined microRNA PCR panel plates and Pick-&-Mix microRNA PCR Panels, a minimum of 3 UniSp3 assays with template are found. These can be used both as interplate calibrators as well as for PCR amplification control.

Before starting the experiment

Important

The synthetic RNA spike-ins are controls for isolation and cDNA synthesis/PCR efficiencies. They should be used for checking that these technical steps have worked well, but should never be used for normalization. Synthetic RNA spike-ins do not reveal the RNA content and quality in the biological sample. Normalization should always be performed using stably expressed endogenous reference genes or, when applicable, global mean of all expressed microRNAs.

The RNA spike-in templates are shipped dried down and must be re-suspended before use:

RNA isolation spike-in mix (UniSp2, UniSp4 and UniSp5)

1. Spin down vial before opening.
2. Re-suspend the spike-in mix by adding 80 µL nuclease free water to the vial. Leave for 20 minutes on ice to properly dissolve the RNA pellet.
3. Mix by vortexing and spin down. Store in aliquots at -20°C to avoid freeze thaw cycles.
4. Prior to starting the RNA isolation/purification, add 1 µL synthetic UniSp2, UniSp4, UniSp5 RNA spike-in mix per RNA prep (using Exiqon protocols) to the lysis buffer.
   Important note: The spike-in RNA template must be mixed with the lysis buffer before mixing with the sample – if added directly to the sample it may be rapidly degraded.

cDNA synthesis RNA spike-in mix (cel-miR-39-3p and UniSp6)

1. Spin down before opening.
2. Re-suspend the UniSp6 RNA spike-in (from the Universal cDNA synthesis kit II, # 203301) by adding 80 µL nuclease free water to the vial.
3. Mix by vortexing and spin down. Leave for 20-30 min. on ice to properly dissolve the RNA spike-in. Mix by vortexing and spin down.
4. Re-suspend the cel-miR-39-3p RNA spike-in by adding the 80 µL re-suspended UniSp6 RNA spike-in to the vial.
5. Mix by vortexing and spin down. Leave for 20-30 min. on ice to properly dissolve the RNA spike-in. Mix by vortexing and spin down. Store in aliquots at -20°C.
6. Prior to the RT reaction, add 1 µL synthetic spike-in mix per 20 µL cDNA synthesis.
   Note: If the cel-miR-39-3p RNA spike-in is not to be used, follow steps described in the miRCURY LNA™ Universal RT microRNA PCR manual.

RNA isolation protocol modifications for using UniSp2, UniSp4, UniSp5 RNA Spike-in mix

RNA Isolation using the miRCURY™ RNA Isolation Kit - Tissue

The UniSp2, UniSp4, UniSp5 RNA Spike-in mix should be added during Section 1. Lysate preparation from tissue:

Section 1. Lysate preparation from tissue

In Step 2 Homogenize the tissue	**Before adding Lysis Solution** to the tissue sample, prepare a pre-mix of Lysis Solution with RNA spike-in sufficient for the number of extractions performed by mixing: 1 µL spike-in mix per 300 µL Lysis Solution

Then proceed with the protocol as usual.

RNA Isolation using the miRCURY™ RNA Isolation Kit - Cell & Plant

The UniSp2, UniSp4, UniSp5 RNA Spike-in mix should be added during one of the following (depending on which protocol is followed):

Section 1 (A to I)

In Step 2 Cell Lysis (or Homogenize the tissue)	Before adding Lysis Solution to the culture plate or pellet, prepare a pre-mix of Lysis Solution with RNA spike-in sufficient for the number of extractions performed by mixing: 1 µL RNA spike-in mix per x µL Lysis Solution x = amount of Lysis solution needed per sample, either 300, 350 or 600 according to the sub-protocol followed (I-E).

Then proceed with the protocol as usual

RNA isolation using the miRCURY™ RNA Isolation Kit - Biofluids

The UniSp2, UniSp4, UniSp5 RNA spike-in mix should be added during step 2.

In Step 2 Lysis	Before adding Lysis Solution BF to the sample, add 1µL spike-in mix per 60 µL Lysis Solution BF.

Then proceed with the protocol as usual.

RNA isolation from exosomes using a miRCURY™ Exosome Isolation Kit combined with RNA isolation kit

The UniSp2, UniSp4, UniSp5 RNA spike-in mix should be added during the RNA isolation step as described above. Adding the spike-ins during the exosome precipitation may cause degradation of the RNA spike-ins.

RNA isolation using a kit other than Exiqon

In general we advise to use isolation kits from Exiqon, however if another kit is used, the UniSp2, UniSp4, UniSp5 RNA spike-in mix should be added to the first RNA stabilizing solution added to the sample. Typically either a buffer containing chaothopic salts, phenol or a combination. 1 µL RNA spike-in mix should be added per sample.
Then proceed with the protocol as usual.

cDNA synthesis using the UniSp6 and cel-miR-39-3p RNA Spike-ins

cDNA synthesis using the miRCURY LNA™ Universal RT microRNA PCR system
Mix the UniSp6 and cel-miR-39-3p RNA spike-in templates as described in the section: Before starting the experiment, page 6 in this instruction manual.

During the cDNA synthesis, use the mix of UniSp6/cel-miR-39-3p RNA spike-ins as described in Step 2 and 3 of the Protocols A-D in the Instruction Manual for the miRCURY LNA™ Universal RT microRNA PCR system.

In the Protocol, First strand synthesis

Step 2 Prepare reagents	Replace the RNA spike-in re-suspension by the spike-in mixture as described in this manual.
Step 3 Combine reagents according to Table 2	In table 2, the Synthetic RNA spike-ins is replaced by the UniSp6/cel-miR-39-3p mix.

The modification is identical whether using the standard manual or the serum/plasma modified manual

Analysis and interpretation of data

When performing a qPCR experiment it is important to include stably expressed genes in order to be able to perform proper data normalization. In general, we recommend that when studying microRNA expression, the endogenous reference genes used should be stably expressed microRNAs rather than longer RNA species such as snoRNAs and snRNAs including U6. This is because microRNAs are so short that they may have very different behavior during extraction and reverse transcription compared to longer transcripts. Depending on origin of sample, some commonly used reference microRNAs include: hsa-miR-103, hsa-miR-423-3p, hsa-miR-191, hsa-miR-16, hsa-miR-423-5p and hsa-let-7a.

The synthetic RNA spike-ins should **not** be used for normalization. Normalization should always be performed with endogenous microRNA, either as verified stably expressed reference microRNAs or as global mean of all expressed microRNAs (when applicable).

The purpose of the RNA spike-in controls is to monitor the technical quality of RNA isolation, cDNA synthesis, and presence of PCR inhibitors in the sample.

Interpretation of the data can be a challenge, and should be well understood before making conclusions.

The UniSp2, UniSp4, UniSp5 RNA Spike-in mix was designed, so that UniSp2 is present at a concentration 100-fold higher than UniSp4, and UniSp4 is present at a concentration 100-fold higher than UniSp 5. Therefore UniSp2 should amplify at the level of very abundant microRNAs, UniSp4 should amplify approximately 6,6 cycles later than UniSp2, and UniSp5 again approximately 6,6 cycles later than UniSp4. The concentration of UniSp5 corresponds to weakly expressed microRNAs and might not always be detectable.

If UniSp5 is not detected, this could mean that microRNAs expressed at low levels were lost during isolation. It is therefore recommended to use more RNA for the cDNA preparation or improve the yield of the sample preparation.

Further interpretation of the isolation controls depends on the protocol used:

miRCURY LNA™ Universal RT microRNA PCR manual for serum/plasma samples

The RNA spike-in was 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 three controls are isolation efficiency, cDNA synthesis efficiency, and amplification efficiency. If all samples give comparable values for each RNA isolation control, the interpretation would be that all isolations were performed with similar efficiencies. If, however, one or more samples give higher Cq values for the isolation controls, it suggests a problem in one of the steps of the RNA isolation procedure.
If the isolation controls and endogenous reference genes are affected in a few samples, but the cDNA synthesis controls are 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 reference genes are all affected by elevated Cq's in a few samples, this could suggest presence of RT or qPCR inhibitors 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 controls are affected by high Cq's while none of the RNA 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.

Overview of issues and conclusions using serum/plasma samples

Control type	Increased Cq?			
RNA spike-ins in RNA isolation	No	Yes	Yes	No
RNA spike-ins in cDNA synthesis	No	No	Yes	No
Endogenous reference genes	No	Yes	Yes	Yes
Conclusion	All is well	Poor isolation efficiency	Presence of inhibitors	Low microRNA amount in sample
Action to consider	Include in study	Re-isolate or exclude from study	Exclude from study or re-isolate	Exclude from study

miRCURY LNA™ Universal RT microRNA PCR, standard manual

The RNA spike-ins were added with a fixed amount per isolation. However, after isolation the RNA was adjusted to a fixed amount of total RNA per cDNA synthesis, which adjusted the RNA spike-in concentrations in the process.

The interpretation in this case depends on whether or not the sample amount used in each of the RNA isolations was identical.

a. The sample amount was identical in all isolations

If identical sample amount was used in each of the RNA isolations, and each sample contained the same RNA amount, the adjustment of RNA concentrations should reflect isolation efficiencies, and thus also adjust the RNA spike-ins accordingly.

If all samples give comparable values for each control assay (RNA spike-ins and reference microRNAs), the interpretation would be that microRNA was isolated with the same efficiency as longer RNA species, and the adjustment of RNA concentrations before the cDNA synthesis correctly adjusted for any differences in RNA isolation efficiencies.

If the isolation controls and endogenous reference microRNAs are affected by elevated Cq's, but the cDNA synthesis controls are stable across all samples, it is likely that microRNAs were isolated at a lower efficiency compared to longer RNA species than in the remaining samples. It should be considered to re-isolate RNA from these samples, or alternatively exclude them from the study.

If the isolation controls, cDNA synthesis controls and endogenous reference microRNAs are all affected by late Cq's, this could suggest presence of RT or qPCR inhibitors in the sample. It should be considered whether the sample should be excluded from the study, or alternatively re-isolated in the hope of obtaining a purer isolation.

If the endogenous controls are affected by low Cq's while none of the RNA spike-ins are affected, this would indicate that the sample had a lowered microRNA content from start. In this case, consider excluding the sample from the study.

Overview of issues and conclusions

Control type	Increased Cq?			
RNA spike-ins in RNA isolation	No	Yes	Yes	No
RNA spike-ins in cDNA synthesis	No	No	Yes	No
Endogenous reference genes	No	Yes	Yes	Yes
Conclusion	All is well	Poor microRNA isolation efficiency	Presence of inhibitors	Low microRNA amount in sample
Action to consider	Include in study	Re-isolate or exclude from study	Exclude from study or re-isolate	Exclude from study

If different sample amounts were used in each of the RNA isolations, and each sample contains the same RNA amount, the adjustment of RNA concentrations should reflect RNA isolation efficiencies, and thus also adjust the RNA spike-ins accordingly. In this case, all samples should have comparable Cq values for each RNA spike-in, this would mean that the adjustment was performed correctly, and that the total RNA isolation efficiency properly reflects the microRNA isolation efficiency.

b. The sample amount was not identical in all isolations

If the sample amounts used in each of the RNA isolations was not identical, the adjustment of RNA concentrations before the cDNA synthesis will adjust the microRNA level for both input amount and isolation efficiency, while the isolation RNA spike-in concentrations will only be adjusted for RNA isolation efficiency. The effect will be that the sample input amount should be considered when interpreting the isolation spike-in Cq values.

If a high sample input was used in the RNA isolation, the isolation controls can be expected to have elevated Cq's corresponding to the input amount. On the contrary, if a low sample input was used in the RNA isolation the isolation controls can be expected to have lower Cq's.

If isolation efficiencies were identical, the delta Cq of isolation RNA spike-ins between two samples can be estimated with the formula $Cq_2 - Cq_1 = log2(\frac{M_1}{M_2})$
where Cq_1 is the Cq of sample 1, Cq_2 is the Cq of sample two, M1 is the mass of sample 1, and M_2 is the mass of sample two. The Cq value of each isolation spike-ins should be adjusted by the formula $Cq_{adj} = Cq_S - log2(\frac{M_{av}}{M_S})$ where, Cq_{adj} is the adjusted Cq for that spike-in (in that sample), Cq_S is the Cq of that spike-in in the sample, M_{av} is the average sample input mass for all samples compared, and M_S is the input mass of the sample in question.

After adjusting for sample input amounts, the control assays (RNA spike-ins and reference genes) can be interpreted as described for samples with identical input amount.

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

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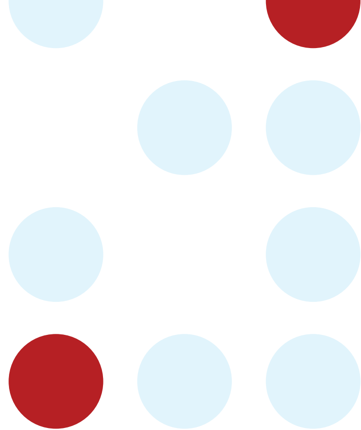

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