

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

Instruction manual v1.0

#203203

January 2012

Supplement to Exiqon's Instruction Manuals for:

miRCURY™ RNA Isolation Kit – Cell & Plant

miRCURY™ RNA Isolation Kit - Tissue

miRCURY LNA™ Universal RT microRNA PCR

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

Protocol for RNA isolation from Blood Plasma & Plasma

EXIQON
Seek Find Verify

Table of contents

Product summary	3
Reagent kit	3
Storage	3
Additional required materials	3
Recommended accompanying products	4
Product description	5
RNA spike-in kit (synthetic control templates)	5
Before starting the experiment	6
RNA isolation protocol modifications for using the UniSp2, UniSp4, UniSp5 RNA spike-in mix	7
Protocol 1	
RNA isolation using the miRCURY™ RNA Isolation Kit - Tissue	7
Protocol 2	
RNA isolation using the miRCURY™ RNA Isolation Kit - Cell & Plant ...	7
Protocol 3	
RNA isolation using the protocol RNA Purification from Blood Plasma & Serum - Human	8
cDNA synthesis using the UniSp6 and cel-miR-39-3p RNA Spike-ins	9
Protocol 1	
cDNA synthesis using the miRCURY LNA™ Universal RT microRNA PCR system	9
Analysis and interpretation of data	10



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
- SYBR® Green master mix, Universal RT
- Universal cDNA synthesis kit
- 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



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 and cDNA synthesis in any microRNA qPCR experiment. 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 between compared samples. One way to check for differences in efficiencies in isolation, cDNA synthesis and PCR is by adding known RNA spike-ins to the sample prior to 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 is meant to be used in combination with the UniSp6 RNA template provided with the miRCURY LNA™ Universal cDNA synthesis kit. Mixing these two RNA templates according to description will provide a 100-fold concentration difference. This this mix is meant as a cDNA synthesis control.

The UniSp6 control (CP) 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 CP PCR primer set is provided with the SYBR® Green master mix, Universal RT, while UniSp2, UniSp4, UniSp5 and cel-miR-39-3p primer sets can be purchased as individual primer sets.



Before starting the experiment

6

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

Important

The synthetic RNA spike-ins are controls for isolation and cDNA synthesis 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:

Isolation RNA spike-in mix

1. Re-suspend the spike-in mix by adding 80 µl nuclease free water to the vial.
2. Mix by vortexing and spin down. Store in aliquots at -20°C.
3. 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

1. Re-suspend the cel-miR-39-3p RNA spike-in by adding 80 µl nuclease free water to the vial.
2. Mix by vortexing and spin down.
3. Re-suspend the UniSp6 RNA spike-in by 40 µl of the re-suspended cel-miR-39-3p RNA spike-in to the vial.
4. Mix by vortexing and spin down. Store in aliquots at -20°C
5. Prior to the RT reaction, add 1 µl synthetic spike-in mix per 20 µl cDNA synthesis (or per 40 µl cDNA synthesis for serum/plasma).

Note: re-suspended cel-miR-39-3p RNA spike-in is enough for re-suspending two vials of UniSp6 RNA spike-in (corresponding to two cDNA synthesis kits).

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

Protocol 1

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.

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 μ l spike-in mix per 300 μ l Lysis Solution

Then proceed with the protocol as usual.

Protocol 2

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 μ l RNA spike-in mix per x μ l 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.



Protocol 3

RNA isolation using the protocol RNA Purification from Blood Plasma & Serum - Human

The UniSp2, UniSp4, UniSp5 RNA spike-in mix should be added during step 5. The modified step is:

5. Make QIAzol master mix: 800 μl QIAzol + 1.25 μl 0.8 $\mu\text{g}/\mu\text{l}$ MS2 RNA + 1 μl RNA spike-in mix per sample. Vortex briefly to mix.

Then proceed with the protocol as usual.



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

Protocol 1

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

During the cDNA synthesis, use the mix of UniSp6/cel-miR-39-3p RNA spike-ins as described for use of UniSp6 RNA spike-in alone.

In the Protocol, First strand synthesis

Step 2

Prepare reagents

replace the RNA spike-in re-suspension by the method described in this manual.

Step 3

Combine reagents according to Table 2

In table 2, the Synthetic spike-in 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

10

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

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

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

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

In the UniSp2, UniSp4, UniSp5 RNA Spike-in mix, UniSp2 is present at a concentration 100-fold higher than UniSp4, and UniSp4 is present at a concentration 100-fold higher than UniSp 5. This means that UniSp2 should amplify at the level of very abundant microRNAs, UniSp4 should amplify approximately 6,6 Cq values later than UniSp2, and UniSp5 again approximately 6,6 Cq values later than UniSp4. The concentration of UniSp5 corresponds to weakly expressed microRNAs.

If UniSp5 is not detected, this could mean that microRNAs expressed at low levels were lost during isolation.

If the UniSp6 and cel-miR-39-3p RNA Spike-ins are mixed according to this manual, UniSp6 will be present at a concentration 100-fold higher than cel-miR-39-3p. This means that cel-miR-39-3p should amplify at approximately 6.6 Cq later than UniSp6.

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 set amount per isolation, and a set 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 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.

If the isolation controls and endogenous reference genes are affected, 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, 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 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 sample had a lowered microRNA content to begin with. In this case, it should be considered if the sample should be excluded 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 set amount per isolation. However, after isolation the RNA was adjusted to a set 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 genes), 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 isolation efficiencies.

If the isolation controls and endogenous reference genes are affected by elevated Cq's, but the cDNA synthesis controls are stable across all samples, it is likely that microRNA was isolated at a lower efficiency compared to longer RNA species than in 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 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, it should be considered if the sample should be excluded 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 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 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 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 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 $Cq_2 - Cq_1 = \log_2\left(\frac{M_1}{M_2}\right)$ where Cq_1 is the Cq of sample 1, Cq_2 is the Cq of sample two, M_1 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 $Cq_{adj} = Cq_s - \log_2\left(\frac{M_s}{M_{av}}\right)$ 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.

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.

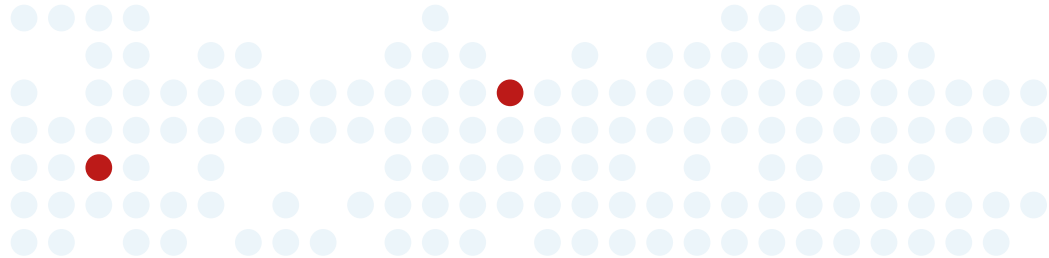
Furthermore, this product is provided under an agreement between Molecular Probes, Inc., a wholly owned subsidiary of Invitrogen Corporation, and EXIQON and the manufacture, use, sale or import of this product is subject to one or more U.S. Patents and corresponding international equivalents. The purchase 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

EXIQON
Seek Find Verify