April 2020

RNA Spike-in Kit, for RT, Handbook

For controlling the quality of RNA isolation, cDNA synthesis and PCR amplification for miRCURY® LNA® miRNA PCR and miRCURY LNA miRNA Probe PCR experiments

Contents

Kit Contents

RNA Spike-in Kit, for RT	
Catalog no.	**339390**
Number of reactions	**50**
UniSp2, UniSp4, UniSp5 RNA Spike-in template mix:	**Conc. (resuspended):**
Synthetic UniSp2 RNA (22 nt), 160 fmol	2 fmol/µl
Synthetic UniSp4 RNA (22 nt), 1.6 fmol	0.02 fmol/µl
Synthetic UniSp5 RNA (22 nt) , 0.016 fmol	0.00002 fmol/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 fmol	0.002 fmol/µl
MS2 total RNA, 50 ng	0.625 ng/µl
Quick-Start Protocol	1

Shipping and Storage

The RNA Spike-In Kit, for RT, is shipped at room temperature with the RNA content dried down. Upon arrival the kit should be stored at –15 to –30°C. Under these conditions, all components are stable until the expiration date on the vial. It is recommended to store the RNA Spike-ins in aliquots at –15 to –30°C after resuspension to avoid repeated freeze-thaw cycles.

Intended Use

The RNA Spike-In Kit, for RT, is intended for molecular biology applications. These products are not intended for the diagnosis, prevention or treatment of a disease.

All due care and attention should be exercised in the handling of the product. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety** where you can find, view and print the SDS for each QIAGEN kit and kit component.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of RNA Spike-In Kit, for RT, is tested against predetermined specifications to ensure consistent product quality.

Introduction

The primary purpose of the RNA Spike-in Kit, for RT, and the matching miRCURY LNA miRNA PCR Assays or miRCURY LNA miRNA Probe PCR Assays for detecting the RNA spike-ins (see Table 1 and below) is to provide a control for the quality of the RNA isolation, cDNA synthesis and PCR amplification for miRCURY LNA miRNA PCR or miRCURY LNA miRNA Probe PCR experiments. This will help to identify experimental and technical outliers.

Table 1. LNA PCR assays for the RNA Spike-in Kit templates

	Corresponding miRCURY LNA miRNA PCR Assay	
Spike-in template	**Catalog number**	**Product number**
Synthetic UniSp2 RNA	339306	YP00203950
Synthetic UniSp4 RNA	339306	YP00203953
Synthetic UniSp5 RNA	339306	YP00203955
cel-miR-39-3p RNA	339306	YP00203952

Table 2. LNA PCR Probe assays for the RNA Spike-in Kit templates

	Corresponding miRCURY LNA miRNA Probe PCR Assay	
Spike-in template	**Catalog number**	**Product number**
Synthetic UniSp2 RNA	339350	ZP00004671
Synthetic UniSp4 RNA	339350	ZP00004673
Synthetic UniSp5 RNA	339350	ZP00004687
cel-miR-39-3p RNA	339350	ZP00004672

Reproducible RNA isolation may be difficult from some sample types, and some RNA samples may contain inhibitors of cDNA synthesis or PCR, even though they were isolated using the best standard procedures. This can lead to 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 amounts

of 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 from further analysis. The RNA Spike-in Kit, for RT, provides several RNA spike-ins. Three of the RNA spike-in templates (UniSp2, UniSp4 and UniSp5) are provided premixed in one vial, each at a different concentration in 100-fold increments. This mix is meant as an RNA isolation control.

A second vial contains a synthetic version of a *C. elegans* miRNA, 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 RT Kit (cat. no. 339340). This mix is meant as a cDNA synthesis control.

The UniSp6 RNA spike-in control PCR assay can be found in all of the predefined miRCURY LNA miRNA PCR Panels and miRCURY LNA miRNA Probe PCR Panels. When configuring custom PCR panels, it is possible to select from all five control PCR assays in the plate layout.

All of our predefined and custom miRCURY LNA miRNA PCR Panels and miRCURY LNA miRNA Probe PCR Panels include a special interplate calibrator. In the miRCURY LNA miRNA PCR Panels, it is a triplicate set of UniSp3 assays with template; in the miRCURY LNA miRNA PCR Probe Panels, it is a triplicate set of UniSp9 assays with template for each plate. These interplate calibrators can also be used for PCR amplification controls.

Important Notes

The synthetic RNA spike-ins in the PCR Spike-in Kit, for RT, are controls for RNA isolation, cDNA synthesis and PCR efficiency. They should be used for checking that these technical steps have worked properly, but they 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, the global mean of all expressed miRNAs.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, consult the appropriate safety data sheets (SDSs) available from the product supplier.

For use with the miRCURY LNA miRNA PCR System

- miRCURY LNA SYBR® Green PCR Kit (cat. nos. 339345, 339346, 339347)
- miRCURY LNA RT Kit (cat. no. 339340)
- Individual miRCURY LNA miRNA PCR Assays or Panels

For use with the miRCURY LNA miRNA Probe PCR System

- miRCURY Probe PCR Kit (cat. nos. 339371, 339372, 339373)
- miRCURY LNA RT Kit (cat. no. 339340)
- Individual miRCURY LNA miRNA Probe PCR Assays or Panels

For use with both systems

- Individual PCR assays for the various RNA Spike-in Kit templates (see Table 1 or Table 2)
- 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 foil for PCR plates
- Microcentrifuge and plate centrifuge
- Heating block, thermal cycler or other incubators
- Real-time PCR instrument

Protocol: Using the UniSp2, UniSp4, UniSp5 RNA Spike-in Mix in RNA Isolation

This protocol describes modifications that must be made if you are using the UniSp2, UniSp4, UniSp5 RNA Spike-in Mix in your RNA isolation procedure.

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

1. Spin down the vial before opening.
2. Resuspend 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 briefly centrifuge. Store in aliquots at –15 to –30°C to avoid repeated freeze–thaw cycles.
4. Prior to starting the RNA isolation procedure, add 1 µl of this UniSp2, UniSp4 and UniSp5 RNA spike-in mix per RNA prep to the lysis buffer.

   Important: The RNA spike-in mix must be combined with the lysis buffer before mixing with the sample. If it is added directly to the sample, it may be rapidly degraded.

RNA isolation from tissue using the miRNeasy® Mini Kit (cat. no. 217004)

The UniSp2, UniSp4, UniSp5 RNA Spike-in mix should be added during the Protocol: Purification of Total RNA, Including Small RNAs, from Animal Tissues, described in the *miRNeasy Mini Handbook*, **www.qiagen.com/HB-1277**.

1. Before adding QIAzol® Lysis Reagent to the tissue sample, prepare a mixture of QIAzol and the UniSp2, UniSp4, UniSp5 RNA Spike-in mix, sufficient for the number of isolations to be performed: mix 1 µl of the Spike-in mix per 700 µl QIAzol.
2. Use this QIAzol:Spike-in mix to lyse your samples.
3. Proceed with the protocol as usual.

RNA isolation from biofluids using the miRNeasy Serum/Plasma Advanced Kit

The UniSp2, UniSp4, UniSp5 RNA Spike-in mix should be added during the Protocol: Purification of Total RNA, Including miRNA, from Serum and Plasma, described in the *miRNeasy Serum/Plasma Advanced Kit Handbook*, **www.qiagen.com/HB-2390**.

1. Before adding Lysis Buffer RPL to the sample, prepare a mixture of Buffer RPL and the UniSp2, UniSp4, UniSp5 RNA Spike-in mix, sufficient for the number of isolations to be performed: mix 1 µl of the Spike-in mix per 60 µl Buffer RPL.
2. Use this Buffer RPL:Spike-in mix to lyse your samples.
3. Proceed with the protocol as usual.

   Note: Do not add the miRNeasy Serum/Plasma Spike-in Control to your samples.

RNA isolation from exosomes using a miRCURY Exosome Kit with an RNA isolation kit

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

RNA isolation using a kit from another supplier

In general, we advise using only the specifically recommended isolation kits. 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, this is a buffer containing chaothopic salts, phenol or a combination of these. In these cases, add 1 µl of the RNA spike-in mix per sample.

Protocol: Using the UniSp6 and cel-miR-39-3p RNA Spike-ins in cDNA Synthesis

This protocol describes first-strand cDNA synthesis using the UniSp6 and cel-miR-39-3p RNA Spike-ins and the miRCURY LNA RT Kit (cat. no. 339340).

Preparation of the UniSp6 and cel-miR-39-3p cDNA synthesis RNA spike-in mix

1. Spin down vials before opening.
2. First resuspend the UniSp6 RNA spike-in from the miRCURY LNA RT Kit (cat. no. 339340) by adding 80 µl nuclease-free water to the vial.
3. Mix by vortexing and briefly centrifuge. Leave for 20–30 min on ice to properly dissolve the RNA spike-in. Mix again by vortexing and briefly centrifuge.
4. Next, resuspend the cel-miR-39-3p RNA spike-in by adding the 80 µl of the resuspended UniSp6 RNA spike-in to the vial.
5. Mix by vortexing and briefly centrifuge. Leave for 20–30 min on ice to properly dissolve the RNA spike-in. Mix again by vortexing and briefly centrifuge. Store in aliquots at –15 to –30°C.
6. When setting up the reverse transcription reactions, add 1 µl of this spike-in mix per 20 µl RT reaction.

   Note: If you do not plan to use the cel-miR-39-3p RNA spike-in, follow the steps described in the *miRCURY LNA miRNA PCR Handbook*, **www.qiagen.com/HB-2431**, or the *miRCURY LNA Probe PCR Handbook*, **www.qiagen.com/HB-2624**.

Analysis and Interpretation of Data

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

The synthetic RNA spike-ins should not be used for normalization. Normalization should always be performed with endogenous miRNAs, either as verified stably expressed reference miRNAs or as global mean of all expressed miRNAs (when applicable). The purpose of the RNA spike-in controls is to monitor the technical quality of RNA isolation and cDNA synthesis and to check for the 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 such 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 miRNAs, UniSp4 should amplify approximately 6.6 cycles later than UniSp2, and UniSp5 approximately 6.6 cycles later than UniSp4. The concentration of UniSp5 corresponds to weakly expressed miRNAs and might not always be detectable.

If UniSp5 is not detected, this could mean that miRNAs expressed at low levels were lost during isolation. If this occurs, we recommend using more RNA for the cDNA preparation or improving the yield of the RNA isolation.

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

Data interpretation with the *miRCURY LNA miRNA PCR – Exosomes, Serum/Plasma and Other Biofluid Samples Handbook*

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 C_q 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. Consider repeating the isolation for 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 C_q values in a few samples, this could suggest presence of RT or qPCR inhibitors in these samples. Consider whether the samples should be excluded from the study, or alternatively, repeat the isolation to see if it yields higher-purity RNA.

If the endogenous controls are affected by high C_q values, while none of the RNA spike-ins are affected, this indicates that the samples in question had a lower miRNA content. 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 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

Data interpretation with the *miRCURY LNA miRNA PCR Handbook* or the *miRCURY LNA miRNA Probe PCR Handbook*

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, adjusting 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.

The sample amount was identical for all isolations:

If the same sample amount was used in each of the RNA isolations, and each sample contained the same amount of RNA, 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 miRNAs), the interpretation is that the miRNA was purified 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 miRNAs are affected by elevated C_q values, but the cDNA synthesis controls are stable across all samples, it is likely that miRNAs were isolated at a lower efficiency than longer RNA species in the remaining samples. Consider repeating the RNA isolation from these samples, or alternatively, exclude them from the study.

If the isolation controls, cDNA synthesis controls and endogenous reference miRNAs are all affected by late C_q values, this could suggest presence of RT or qPCR inhibitors in the sample. Consider whether the sample should be excluded from the study, or repeat the isolation to see if it yields higher-purity RNA.

If the endogenous controls are affected by low C_q values while none of the RNA spike-ins are affected, this indicates that the sample had a lowered miRNA 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 RNA isolation, 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 C_q 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 miRNA isolation efficiency.

The sample amount was not identical for all isolations:

If the sample amounts used in each of the RNA isolations were not identical, the adjustment of RNA concentrations before the cDNA synthesis will adjust the miRNA 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 is that the sample input amount should be considered when interpreting the isolation spike-in C_q values.

If a high sample input was used in the RNA isolation, the isolation controls can be expected to have elevated C_q values 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 C_q values.

If isolation efficiencies were identical, the ΔC_q of isolation RNA spike-ins between two samples can be estimated with the following formula:

$$Cq_2 - Cq_1 = log2\left(\frac{M_1}{M_2}\right)$$

where C_q1 is the C_q of sample 1, C_q2 is the C_q of sample 2, M1 is the mass of sample 1 and M2 is the mass of sample 2.

The C_q value of each isolation spike-in should be adjusted with the following formula:

$$Cq_{adj} = Cq_S - log2(\frac{M_{av}}{M_S})$$

where C_{qadj} is the adjusted C_q for that spike-in (in that sample), C_{qS} is the C_q 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.

Troubleshooting Guide

For more information, see the Frequently Asked Questions page our Technical Support Center: **www.qiagen.com/FAQ/FAQList.aspx**. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information or protocols in this handbook (for contact information, visit **support.qiagen.com**).

Ordering Information

Product	Contents	Cat. no.
RNA Spike-in Kit, for RT	UniSp2, UniSp4, UniSp5 RNA Spike-in template mix, cel-miR-39-3p RNA Spike-in template; for 50 reactions	339390
Related products for reverse transcription and PCR		
miRCURY LNA RT Kit	5x RT Reaction Buffer, 10x RT Enzyme Mix, UniSp6 RNA Spike-in template, RNase-free water; for 8–64 reactions	339340
Related products for Probe- and SYBR Green–detection-based miRNA PCR		
miRCURY LNA miRNA PCR Starter Kit	2 miRCURY LNA PCR Assays of your choice, UniSp6 Spike-in control assay, miR-103-3p endogenous control assay, 5x RT Reaction Buffer, 10x RT Enzyme Mix, UniSp6 RNA Spike-in template, RNase-free water, 2x miRCURY SYBR® Green Master Mix; for 20 RT reactions and 100 PCR amplifications	339320
miRCURY LNA SYBR® Green PCR Kit (200)/(600)/(4000)	2x miRCURY SYBR® Green Master Mix, RNase-free water; for 200, 600, or 4000 reactions	339345 339346 339347
miRCURY Probe PCR Kit (200)/(800)/(4000)	2x QuantiNova Probe PCR Master Mix, miRCURY Probe Universal Reverse Primer, ROX Reference Dye, and RNAse-free Water; for 200, 800, or 4000 reactions	339371 339372 339373

Product	Contents	Cat. no.
Related PCR assays and panels		
miRCURY LNA miRNA Probe PCR Assay	LNA-optimized PCR assay for miRNA quantification; for 200 reactions	339350*
miRCURY LNA miRNA Custom Probe PCR Assay	Custom-designed and LNA-optimized PCR assay for miRNA quantification; for 200 reactions	339351†
miRCURY LNA miRNA Custom Probe PCR Panel	Custom panel of LNA-optimized PCR assays for miRNA quantification; for one 10 µl qPCR reaction per well; 96-well or 384-well format or 20 µl for Rotor-Gene format	339360*
miRCURY LNA miRNA miRNome Probe PCR Panels	Predesigned panels of LNA Probe PCR Assays for miRNome profiling; for one 10 µl qPCR reaction per well; 96-well or 384-well format or 20 µl for Rotor-Gene format	339361*
miRCURY LNA miRNA Focus Probe PCR Panel	Predesigned panel of LNA Probe PCR Assays focused on disease, pathway or sample type; for one 10 µl qPCR reaction per well; 96-well or 384-well format or 20 µl for Rotor-Gene format	339362*
miRCURY LNA miRNA PCR Assay	LNA-optimized PCR assay for miRNA quantification; for 200 reactions	339306
miRCURY LNA miRNA Custom PCR Assay	Custom-designed and LNA-optimized PCR assay for miRNA quantification; for 200 reactions	339317

* Exact catalog number varies depending on the particular assay or panel configuration.

† Exact catalog number varies depending on the particular assay or panel configuration.

Product	Contents	Cat. no.
miRCURY LNA miRNA miRNome PCR Panels	Premade panels of LNA PCR assays for miRNome profiling; for one 10 µl qPCR reaction per well; 96-well or 384-well format	339322
miRCURY LNA miRNA Focus PCR Panel	Premade panel of LNA PCR assays focused on disease, pathway or sample type; for one 10 µl qPCR reaction per well; 96-well or 384-well format	339325
miRCURY LNA miRNA QC PCR Panel	Premade panel of LNA PCR assays for miRNA quality control; for one 10 µl qPCR reaction per well; 96-well or 384-well format	339331 (Product no. varies, YAHS-999Y-)
miRCURY LNA miRNA Custom PCR Panel	Custom panel of LNA-optimized PCR assays for miRNA quantification; 96- or 394-well plate format	339330

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

Document Revision History

Date	Changes
04/2020	Corrected cat. no. for cel-miR-39-3p RNA to YP00203952, from YP00203950. Added references to and information about miRCURY LNA Probe PCR Kit for use with this kit. Inserted a new Table 2 for LNA PCR Probe assays for the RNA Spike-in Kit templates.

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