December 2017

QIAseq™ miRNA Library QC PCR Handbook

For quality control of RNA isolation for small RNA next-generation sequencing

Sample to Insight

QIAGEN

Contents

Kit Contents

QIAseq miRNA Library QC PCR Panel Kit	**(48)**
Catalog no.	**331541**
Number of reactions	**48**
10x miRCURY® RT Enzyme Mix	64 µl
5x miRCURY Reaction Buffer	128 µl
Nuclease-free water	1 ml
384-well plate containing 48x8 assays, or 96-well plate containing 12x8 assays	1 384-well plate, or 2 96-well plates
UniSp6, RNA Spike-In	Dried down in 1 tube
QIAseq miRNA Library QC Spike-Ins (52 5′ phosphorylated RNA molecules)	Dried down in 1 tube
Optical Thin-wall 8 Cap Strips (for A and D format)	24
Optical Adhesive Film (for C and F format)	2
Optical Adhesive Film (for E and G format)	1
384-Easy Load Covers (for E and G format)	1

QIAseq miRNA Library QC PCR Assay Kit	**(48)**
Catalog no.	**331551**
Number of reactions	**48**
10x miRCURY RT Enzyme Mix	64 µl
5x miRCURY Reaction Buffer	128 µl
Nuclease-free water	1 ml
UniSp6, RNA Spike-In	Dried down in 1 tube
QIAseq miRNA Library QC Spike-Ins (52 5′ phosphorylated RNA molecules)	Dried down in 1 tube
103a-3p Assay	Dried down in 1 tube
191-5p Assay	Dried down in 1 tube
UniSp6 Assay	Dried down in 1 tube
451a Assay	Dried down in 1 tube
23a-3p Assay	Dried down in 1 tube
30c-5p Assay	Dried down in 1 tube
UniSp-100 Assay	Dried down in 1 tube
UniSp-101 Assay	Dried down in 1 tube

QIAseq miRNA Library QC Spike-Ins	**(96)**
Catalog no.	**331535**
Number of reactions	**96**
QIAseq miRNA Library QC Spike-Ins (52 5′ phosphorylated RNA molecules)	Dried down in 1 tube
Nuclease-free water	1 ml

Storage

The QIAseq miRNA Library QC Spike-Ins, the UniSp6 RNA Spike-In, the 5x Reaction Buffer and Enzyme Mix should be stored immediately upon receipt at –15 to –30°C in a constant-temperature freezer. After resuspension, store the Spike-Ins in aliquots at –80°C and avoid repeated freeze–thaw cycles. The dried-down PCR panels and assays can be stored at 2–8°C or –15 to –30°C. If stored under these conditions, the kit contents are stable until the date indicated on the box label.

Intended Use

The QIAseq miRNA Library PCR Panel Kit, QIAseq miRNA Library PCR Assay Kit and the QIAseq miRNA Library QC Spike-Ins are 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 products. 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 QIAseq miRNA Library PCR Panel Kit, QIAseq miRNA Library PCR Assay and QIAseq miRNA Library QC Spike-Ins is tested against predetermined specifications to ensure consistent product quality.

Introduction and Principle

The primary purpose of the QIAseq miRNA Library QC PCR Panel 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 isolation efficiencies 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 quality control using PCR panels 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 QIAseq miRNA Library QC Spike-Ins allows thorough QC of the NGS data by assessing the reproducibility and linearity of the reads mapped to these exogenous sequences.

QIAseq miRNA Library QC Spike-Ins

The QIAseq miRNA Library QC Spike-Ins consist of one vial containing a dried-down mix of 52 synthetic 5′-phosphorylated miRNAs of different concentrations. Each vial is sufficient for a maximum of 500 purifications of standard 500 µl serum or plasma samples. A vial containing 1 ml of nuclease-free water is provided for re-suspension of the QIAseq miRNA Library QC Spike-Ins. The 52 QIAseq miRNA Library QC Spike-Ins are synthetic miRNAs of plant origin and bear no significant homology to miRNAs from the following species: human (hsa), mouse (mmu), rat, (rno), rhesus monkey (mml), orangutan (ppy), chimpanzee (ptr) or pig (ssc). Refer to Appendix C for the complete list of Spike-In sequences.

Product description

Addition of QIAseq miRNA Library QC Spike-Ins during RNA isolation enables monitoring of the comparability and reproducibility of the whole process from RNA isolation to sequencing. The protocol provided in this handbook is intended for biofluid samples and is expected to result in NGS Spike-Ins representing 1–3% of total reads when added to an 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.

QIAseq miRNA Library QC Spike-Ins can also be added directly to RNA samples before small RNA library preparation. For more accurate ratios of spike-ins vs. endogenous miRNAs in other sample types or when using isolated RNA samples, experimental titration of QIAseq miRNA Library QC Spike-Ins is recommended.

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

1. Addition of 52 QIAseq miRNA Library QC 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 QIAseq miRNA Library QC Spike-Ins data

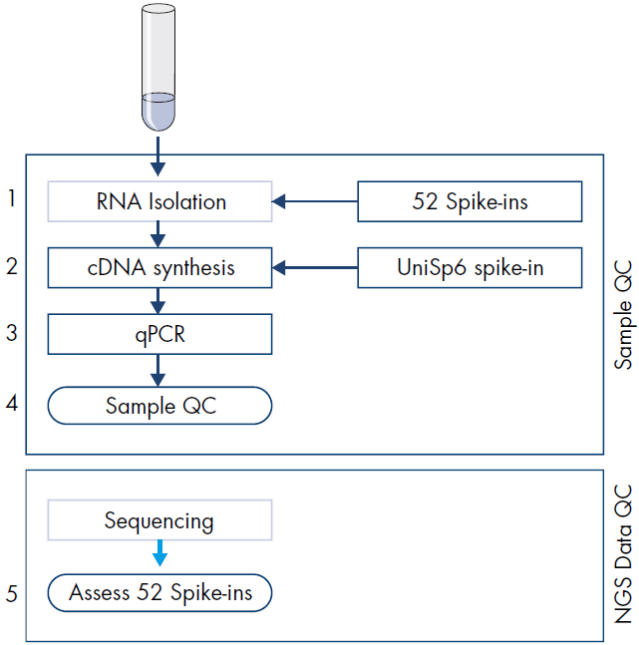

Figure 1. Protocol overview of the QIAseq miRNA Library QC PCR procedure.

Assessing variation in RNA isolation efficiency

Introduction of the 52 QIAseq miRNA Library QC Spike-Ins during RNA isolation, by adding them to the lysis solution, enables quality control of the RNA isolation efficiency prior to library preparation and offers the possibility to reject samples before downstream library preparation and sequencing analysis. Alternatively, the QIAseq miRNA Library QC Spike-Ins can be added directly to the RNA after RNA isolation. However, this means any information on RNA isolation, especially regarding the small RNA fraction, will be lost.

RT-qPCR based quality control

The QIAseq miRNA Library QC PCR Panel Kit is based on miRCURY LNA® miRNA PCR, which unites two important features (Figure 2):

- **Universal RT**: One first-strand cDNA synthesis reaction provides the template for all real-time miRNA PCR assays. This saves precious sample, reduces technical variation, consumes less reagents and saves time in the laboratory.
- **LNA PCR amplification**: Both PCR amplification primers (forward and reverse) are miRNA specific and optimized with LNA. The result is exceptional sensitivity and extremely low background, enabling accurate quantification of very low levels of miRNA. The highly specific assays allow discrimination between closely related miRNA sequences. The miRCURY LNA miRNA PCR System and the outstanding performance of the miRCURY LNA SYBR® Green PCR Kit offer solutions for both high-throughput miRNA expression profiling and for quantification of individual miRNAs.

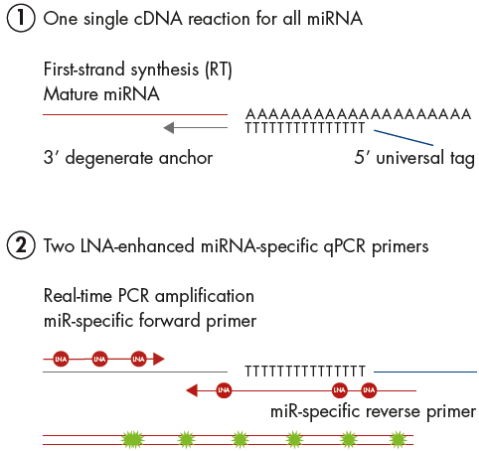

Figure 2. Schematic outline of the miRCURY LNA miRNA PCR System. A poly(A) tail is added to the mature miRNA template (step 1A). cDNA is synthesized using a poly(T) primer with a 3′ degenerate anchor and a 5′ universal tag (step 1B). The cDNA template is then amplified using miRNA-specific and LNA-enhanced forward and reverse primers (step 2A). SYBR Green is used for detection (step 2B).

The UniSp6 RNA Spike-In is added during cDNA synthesis to monitor cDNA and qPCR efficiency. A total of 8 miRCURY LNA PCR primer sets in the PCR panel amplify spike-ins added during RNA isolation or cDNA synthesis, as well as endogenous miRNA controls. These endogenous miRNA controls are: miR-103-3p and miR-191-5p, which are well expressed in most tissues; miR-451a and miR-23a-3p, which are found in plasma and serum and serve as a hemolysis marker and an internal control, respectively; miR-30c-5p, 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 1 for details.

Table 1. Assays included in the PCR panel for measuring spike-ins.

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

Evaluation of the QIAseq miRNA Library QC Spike-In data

The 52 QIAseq miRNA Library QC Spike-Ins added during RNA isolation are present at different concentrations, representing miRNAs 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. Refer to Analysis and Interpretation of Data on page 26 for details.

Important Notes

Sample input

The QIAseq miRNA Library QC PCR Panel Kit is optimized for using 400–500 µl of biofluid. Using the recommended amount of QIAseq miRNA Library QC Spike-Ins is expected to result in QIAseq miRNA Library QC Spike-Ins representing 1–3% of the total reads. This may however vary from project to project, and it may be necessary to adjust the amount of Spike-Ins added for optimal results. The sample input volume to the cDNA synthesis reaction may need to be adjusted, depending on the sample quality and yield.

Plasma and serum are essentially cell-free liquid samples. Therefore, only circulating RNA is extracted from these sample types, resulting in low total RNA concentrations, even if the miRNA fraction is readily detectable. As a result, measuring correct RNA concentrations is difficult, and there is a high risk of increased loss during extraction. For this reason, we recommend using RNA amounts based on starting sample volume, rather than RNA concentration.

RNA input requirements

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 miRNAs. QIAGEN provides a range of solutions for purification of total RNA containing miRNA (Table 2). For more information on miRNA purification, visit **www.qiagen.com/miRNA**.

Table 2. Kits for purification of RNA including miRNA.

Kit	Cat. no.	Starting material
miRNeasy® Mini Kit*	217004	Animal/human tissues and cells
miRNeasy Serum/Plasma Advanced Kit	217204	Serum/plasma samples
miRNeasy FFPE Kit	217504	Formalin-fixed paraffin-embedded (FFPE) tissue samples
miRNeasy 96 Kit*	217061	Animal/human tissues and cells
PAXgene® Tissue miRNA Kit	766134	Animal/human tissues that have been fixed and stabilized in PAXgene Tissue Containers
PAXgene Blood miRNA Kit	763134	Human blood that has been stabilized in PAXgene Blood RNA Tubes

* For quantification of precursor miRNA and mRNA, we recommended performing the on-column DNase digestion step, using the RNase-Free DNase Set (cat. no. 79254), when performing the protocol for total RNA purification using the miRNeasy Mini and miRNeasy 96 Kits. This ensures that any minute traces of genomic DNA are removed from the sample and is especially recommended for quantification of precursor miRNA due to the low levels of this miRNA species.

Good PCR laboratory practice

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

- 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 setup, PCR amplification and gel electrophoresis of PCR products.
- Never bring amplified PCR products into the PCR setup 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 work 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.

Settings for ABI instruments

The default settings on ABI real-time PCR cyclers are not suitable for running miRCURY LNA miRNA PCR. Settings need to be changed from automatic to manual background and threshold settings to obtain valid PCR data.

On cyclers using baseline and threshold values for C_q (C_T) 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: If ROX™ passive reference dye has not been used in the PCR reactions (this is not recommended), make sure the SDS software is set up without reference dye correction.

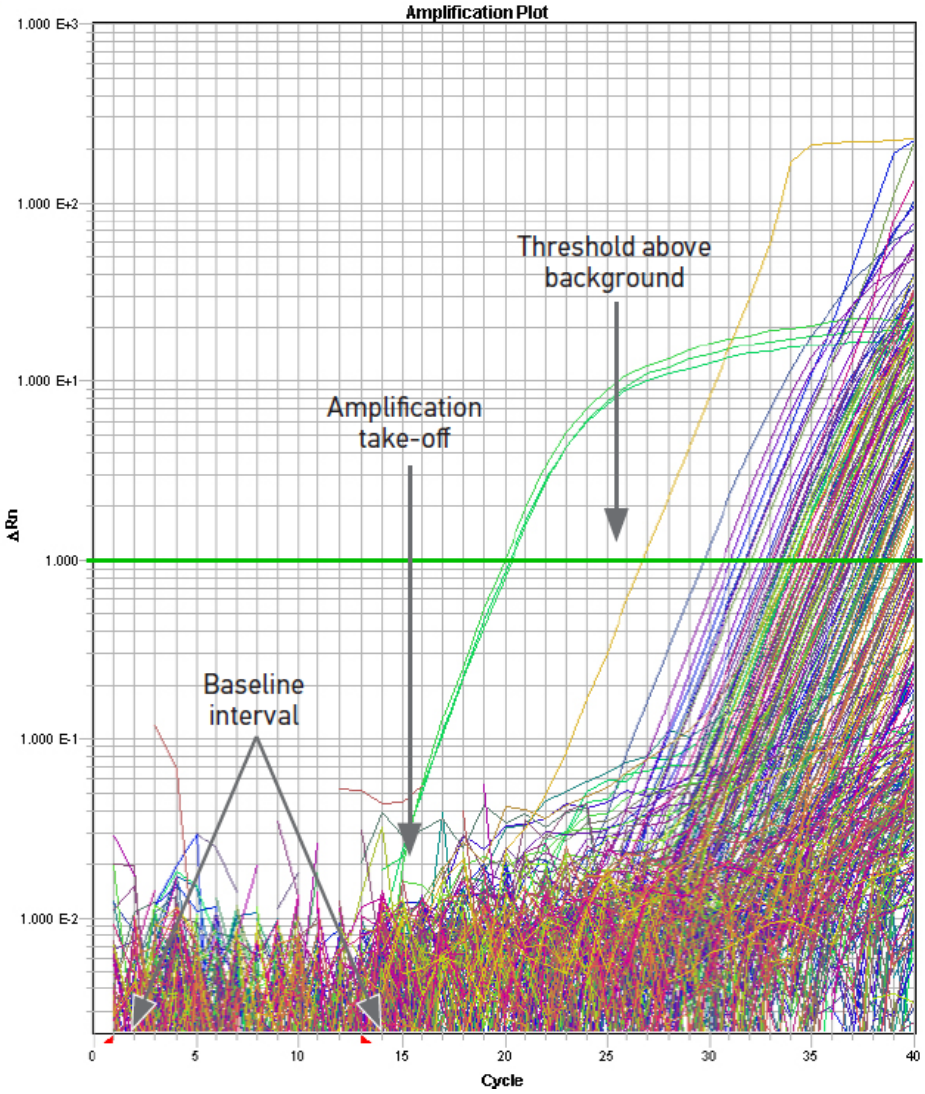

Figure 3. Manually setting the baseline and threshold.

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.

- RNA isolation kit
- miRCURY LNA SYBR Green PCR Kit (cat. nos. 339345, 339346 and 339347)
- Nuclease-free, low nucleic acid binding microcentrifuge tubes (e.g., Eppendorf DNA LoBind® tubes)
- Nuclease-free PCR tubes or plates for use with individual assays
- Pipettes
- Nuclease-free, aerosol-barrier pipette tips
- Ice
- PCR cycler, heating block or water bath (capable of reaching 95°C)
- Vortexer or multi-vial vortex shaker
- Microcentrifuge and plate centrifuge
- Sealing foils for PCR plates
- Real-time PCR instrument

Protocol: Quality Control of RNA Isolation and cDNA Synthesis

This protocol includes procedures for adding the QIAseq miRNA Library QC Spike-Ins to a biofluid sample during RNA isolation and conducting the first-strand cDNA synthesis and real-time PCR using the QIAseq miRNA Library QC PCR Panel, for assessing quality of biofluid samples before library preparation and NGS.

Important points before starting

- Liquid handling with pipettes or pipetting robots requires an excess volume of reagents due to loss during pipetting. The loss depends on the particular pipetting system, but losses in the range from 10–25% are not uncommon. This protocol indicates the required reaction volumes, so you may need to adjust the pipetting volumes to accommodate your pipetting system.
- ABI instruments: The use of manual background and threshold settings is necessary for obtaining correct PCR data.
- To ensure optimal performance, keep all reagents and reactions on ice during the protocol, except for reaction steps specifically involving raised temperatures.
- Store undiluted cDNA samples in nuclease-free, low nucleic acid binding microcentrifuge tubes. Storage of diluted cDNA is not recommended.

Things to do before starting

- Prior to first use, resuspend the QIAseq miRNA Library QC Spike-Ins in 500 µl nuclease-free water. Resuspend the UniSp6 RNA Spike-In in 80 µl nuclease-free water. Vortex for 5 s and leave the suspension on ice for 30 min to dissolve. Vortex for 5 s again and centrifuge to collect contents in the bottom of the tubes.

- Thaw previously resuspended QIAseq miRNA Library QC Spike-Ins on ice, briefly centrifuge and keep on ice until frozen again.
- Gently thaw the 5x Reaction Buffer and nuclease-free water and immediately place on ice. Mix by vortexing. Immediately before use, remove the Enzyme Mix from the freezer, mix by flicking the tube and place on ice. Briefly centrifuge all reagents.

Procedure: Adding QIAseq miRNA Library QC Spike-Ins to RNA isolations

To reduce the effect of inhibitors and nucleases, the starting material (biofluid) should be centrifuged at no more than 3000 *x g* for 5 min. This should be done prior to storage rather than immediately prior to isolation. Omitting this clearance step can affect PCR detection. Use excess sample to ensure sufficient supernatant volume for the next steps. If working with urine or CSF samples, an initial exosome isolation step is highly recommended.

1. Prepare a master mix of lysis buffer and spike-in. For each biofluid sample to be purified, add 1 µl QIAseq miRNA Library QC Spike-Ins solution per 400–500 µl serum/plasma volume to the volume of lysis buffer specified by the isolation kit in use. Mix well.
2. Add the spiked lysis buffer to each biofluid sample and mix well. Continue with the RNA isolation procedure according to the isolation kit instructions.

   Note: To avoid RNA degradation, make sure that the lysis buffer inhibits all RNase activity in the biofluid immediately, by thoroughly homogenizing the sample after addition of the lysis buffer.

Procedure: First-strand cDNA synthesis

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

1. Prepare the reverse transcription reactions on ice according to Table 3. Mix and then place on ice.

   Note: If you are setting up multiple reactions, prepare an RT reaction master mix with a volume 10% greater than required for the total number of reactions. Distribute the appropriate volume of master mix into individual tubes, followed by each RNA sample. Mix and then place on ice.

Table 3. Reverse transcription reaction setup.

Reagent	Volume per RT reaction
5x miRCURY Reaction Buffer	2 µl
Nuclease-free water	4.5 µl
10x miRCURY RT Enzyme Mix	1 µl
UniSp6 Spike-In	0.5 µl
Template RNA*	2 µl
Total volume	10 µl

* Optimal volumes may need to be determined depending on sample.

2. Incubate for 60 min at 42°C.
3. Heat inactivate the reverse transcriptase for 5 min at 95°C.
4. Immediately cool to 4 °C.
5. Store at 4 °C or freeze.

   Note: The protocol can be interrupted at this stage. The undiluted cDNA may be kept at –15 to –30°C for up to 5 weeks (or at 2–8°C for up to 4 days). We recommend storing synthesized cDNA in low nucleic acid binding tubes or plates.

Protocol: Quantitative, Real-Time PCR

This protocol describes performing quantitative, real-time PCR using the QIAseq miRNA Library QC PCR Panel (cat. no. 331541) and the miRCURY LNA SYBR Green PCR Kit (cat. nos. 339345, 339346 or 339347).

Important points before starting

- Keep reagents and reactions on ice (or at 4°C) at all times.
- We recommend using low nucleic acid binding tubes or plates.
- We do not recommend storing diluted cDNA.
- This protocol is optimized for detection of miRNA targets with any real-time cycler and conditions for fluorescence normalization. ROX dye is required at the following concentrations:

  No requirement for ROX dye: Rotor-Gene®, Bio-Rad® CFX, Roche® LightCycler® 480 and Agilent® Technologies Mx instruments.

  Low concentration of ROX dye: Applied Biosystems® 7500, ViiA™ 7 and QuantStudio™ Real-Time PCR Systems.

  High concentration of ROX dye: ABI PRISM® 7000, Applied Biosystems 7300, 7900 and StepOne™ Real-Time PCR Systems.
- The ROX Reference Dye should be used as a 20x concentrated solution for a 1x reaction when using an instrument requiring a high ROX dye concentration. For instruments requiring a low ROX dye concentration, use the dye as a 200x concentrate.

Things to do before starting

- Place the cDNA (from step 5 on page 21), nuclease-free water and miRCURY LNA SYBR Green Master Mix on ice and thaw for 15–20 min. Protect the Master Mix vials from

light. Immediately before use, mix the Master Mix by pipetting up and down. Vortex the rest of the reagents and briefly centrifuge.

- If using single assays, prepare the miRNA LNA PCR primer set: Spin down the tube before opening it the first time. Add 220 µl nuclease free water to the tube, let it sit for 20 min, vortex and spin down.

Procedure for quantitative PCR

1. Prepare the PCR reaction mix. If you are using the PCR Panel, refer to Table 4. If you are using individual PCR Assays, refer to Table 5. Mix the reaction mix gently by inverting the tube, and briefly centrifuge.

   Note: The amount of reaction mix described in Table 4 is sufficient for running the 8 assays of the PCR Panel with one cDNA template.

   Note: If replicates are needed, please calculate the required volumes accordingly.

Table 4. Reaction setup for QIAseq miRNA Library QC PCR Panel with cDNA.

Component	QC PCR Panel consisting of 1 x 8 assays
2x miRCURY SYBR Green Master Mix	50 µl
ROX Reference Dye (ABI instruments only)	5 µl/0.5 µl*
cDNA template (undiluted)	1 µl
RNase-free water	49 µl*
Total reaction volume	**100 µl**

* Use ROX Reference Dye as a 20x concentrate for cyclers requiring a high ROX dye concentration (i.e., ABI PRISM 7000, Applied Biosystems 7300, 7900 and StepOne Real-Time PCR Systems) and as a 200x concentrate for cyclers requiring a low ROX dye concentration (i.e., Applied Biosystems 7500, ViiA7 and QuantStudio Real-Time PCR Systems). Adjust the amount of RNase-free water accordingly.

Table 5. Reaction setup for QIAseq miRNA Library QC PCR Panels with cDNA.

Component	Primer sets*
2x miRCURY SYBR Green Master Mix	5 µl
ROX Reference Dye (ABI instruments only)	0.5 µl /0.05 µl†
PCR primer mix	1 µl
cDNA template (undiluted)	0.1 µl†
RNase-free water	3.9 µl‡
Total reaction volume	**10 µl**

* The volume shown is for a single reaction. Depending on plate layout, calculate the necessary reaction volume.

† Use ROX Reference Dye as a 20x concentrate for cyclers requiring a high ROX dye concentration (i.e., ABI PRISM 7000, Applied Biosystems 7300, 7900 and StepOne Real-Time PCR Systems) and as a 200x concentrate for cyclers requiring a low ROX dye concentration (i.e., Applied Biosystems 7500, ViiA7 and QuantStudio Real-Time PCR Systems). Adjust the amount of RNase-free water accordingly.

‡ The volume shown is for a single reaction. Depending on the experiment setup, you can pre-dilute the cDNA. Adjust the amount of RNase-free water accordingly. Diluted cDNA cannot the stored.

2. Mix the reaction mix thoroughly and dispense 10 µl per well into PCR tubes or plates.

   Note: The experiment can be paused at this point. Store the reactions protected from light at 4°C for up to 24 h.

3. Spin the tubes or plate briefly in a centrifuge. If you are using the PCR Panel, wait 5 min for the primers to dissolve.

4. Program the real-time cycler according to Table 6.

   Note: Data acquisition should be performed during the annealing/extension step.

Table 6. Cycling conditions.

Step	Time	Temperature	Ramp rate
PCR initial heat activation	2 min	95°C	Maximal/fast mode
2-step cycling			
Denaturation	10 s	95°C	Maximal/fast mode
Combined annealing/extension	60 s	56°C	Maximal/fast mode
Number of cycles	40*		
Melting curve analysis	60–95°C		

*If using a Roche LightCycler 480, use 45 cycles.

5. Place the PCR tubes or plates into the real-time cycler and start the cycling program.
6. Perform initial data analysis using the software supplied with the real-time PCR instrument to obtain raw C_q values (C_P or C_T, depending on PCR instrument). If you are using an ABI instrument, note that it is not recommended to use auto C_T settings.

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

   For interpretation of the miRCURY LNA miRNA QC PCR Panel data, refer to the guideline section on the next page.

Analysis and Interpretation of Data

The QIAseq miRNA Library QC Spike-In controls enable you to monitor the technical quality of the RNA isolation and cDNA synthesis and the 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 miRNAs. The concentration of UniSp101 Spike-In corresponds to highly abundant miRNA in a biofluid. C_q values will vary depending on experimental settings of the experiment and the method used as well as the elution volume and RNA input. The typical C_q value is in the range of 31–34 for UniSp100 and 25–28 for UniSp101. The ΔC_q for these two Spike-Ins should be around 5–7.

The UniSp100 and UniSp101 Spike-Ins were added at a fixed amount per RNA 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 UniSp101 give 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 C_q 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 in 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 C_q values for UniSp6 are usually below 20.

If the isolation controls (UniSp100 and UniSp101) and the 5 endogenous miRNAs 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 miRNAs are all affected by elevated C_qs in a few samples, this could suggest the 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 miRNAs are affected by high C_qs while none of the Spike-Ins are affected, this would indicate that the samples in question had a lowered miRNA content to begin with. In this case, consider excluding the samples from the study.

Refer to Figure 4 for a decision tree for identification of outlier samples.

A major source of variation in plasma and serum is potential cellular-derived miRNA 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 ΔC_q (miR-23a – miR-451a) lower than 5 in human serum or plasma represents non-hemolyzed samples. If the ΔC_q is close to or higher than 7, there is an increased risk of hemolysis. In case of high levels of hemolysis, miRNAs from red blood cells will make a significant contribution to the overall miRNA profile identified, and this may or may not disqualify the samples depending on the biological question, as detection of altered expression of red blood cell miRNAs may be relevant to the study.

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 ΔC_q (miR-23a – miR-451a) levels will be different.

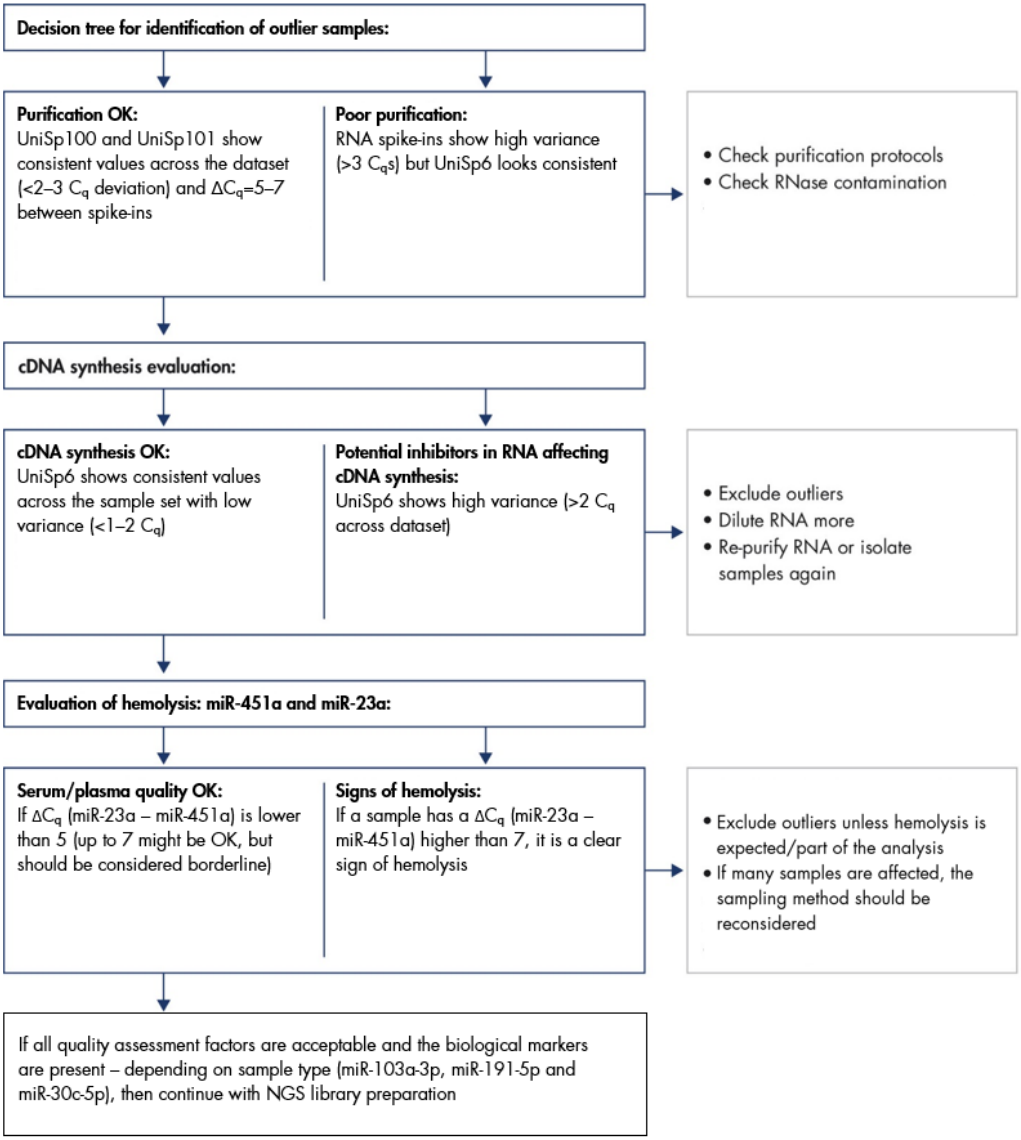

Figure 4. Decision tree for identification of outlier samples.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at 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 and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit **www.qiagen.com**).

	Comments and suggestions
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, even 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.
Generated signals are weak	On some real-time PCR cyclers, gain settings are adjustable. Make sure the gain settings of your real-time 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.
QIAseq miRNA Library QC Spike-Ins detected at very low (<1%) or very high (>10%) counts	Adjust the amount of QIAseq miRNA Library QC Spike-Ins added accordingly.

Appendix A: NGS Analysis

This protocol will provide sufficient reads derived from the QIAseq miRNA Library QC Spike-Ins for post-sequencing analysis using a standard small RNA NGS analysis pipeline. The sequences of the 52 QIAseq miRNA Library QC Spike-Ins can be downloaded as a single FASTA file at **www.qiagen.com**. Reads should be mapped to the QIAseq miRNA Library QC 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 QIAseq miRNA Library QC Spike-In reads in a dataset (FASTQ files). Following counting of the QIAseq miRNA Library QC 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 QIAseq miRNA Library QC Spike-In, calculate TPM based on each sample:

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

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 QIAseq miRNA Library QC 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 QIAseq miRNA Library QC Spike-Ins data lower than 1 TPM if read depth is approximately 10 million reads per sample.

Appendix B: General Remarks on Handling RNA

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate, and even minute amounts are sufficient to degrade RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. We recommend that you take care to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure. To create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and non-disposable vessels and solutions while working with RNA.

General handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible.

Disposable plasticware

The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

Non-disposable plasticware

Non-disposable plasticware should be treated before use to ensure that it is RNase-free. Plasticware should be thoroughly rinsed with 0.1 M NaOH*, 1 mM EDTA* followed by RNase-free water. Alternatively, chloroform-resistant plasticware can be rinsed with chloroform* to inactivate RNases.

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent*, thoroughly rinsed, and oven baked at 240°C for 4 hours or more (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC* (diethyl pyrocarbonate). Fill glassware with 0.1% DEPC (0.1% in water), allow to stand overnight (12 hours) at 37°C, and then autoclave or heat to 100°C for 15 minutes to eliminate residual DEPC.

Solutions

Solutions (water and other solutions) should be treated with 0.1% DEPC*. DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 ml DEPC to 100 ml of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37°C. Autoclave for 15 minutes to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris* buffers. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO_2. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer. Trace amounts of DEPC will modify purine residues in RNA by carbethoxylation. Carbethoxylated RNA is translated with very low efficiency in cell-free systems. However, its

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

ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected, unless a large fraction of the purine residues has been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

Appendix C: QIAseq miRNA Library QC Spike-In Sequences

All 52 QIAseq miRNA Library QC Spike-Ins are 5′ phosphorylated synthetic miRNAs with sequence length in the range 20–24 nucleotides. The sequences are of plant origin bear no significant homology to miRNAs from the following species: human (hsa), mouse (mmu), rat, (rno), rhesus monkey (mml), orangutan (ppy), chimpanzee (ptr) or pig (ssc).

Table 7. Spike-In sequences.

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	uaacuaaacauuggguguagua
UniSp116	uuugcuuccagcuuuugucuc	UniSp142	uaagauccggacuacaacaaag
UniSp117	uugguuacccauauggccauc	UniSp143	uaauccuaccaauaacuucagc
UniSp118	uucgaugucuagcagugcca	UniSp144	gauggauaugucuucaaggac

NGS Spike-In name	Sequence	NGS Spike-In name	Sequence
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

Ordering Information

Product	Contents	Cat. no.
QIAseq miRNA Library QC PCR Panel Kit	Enzyme mix, 5x Reaction Buffer, nuclease-free water, PCR assays in 384- or 96-well format, UniSp6 RNA Spike-In, QIAseq miRNA Library QC Spike-Ins	331541
QIAseq miRNA Library QC PCR Assay Kit	Enzyme mix, 5x Reaction Buffer, nuclease-free water, QIAseq miRNA Library QC Spike-ins, 8 PCR assays in tube format	331551
QIAseq miRNA Library QC Spike-Ins	52 5′ phosphorylated RNA molecules, dried-down in one tube, nuclease-free water	331535
Related products		
QIAseq miRNA Library Kit (12)	For 12 sequencing prep reactions: 3′ ligation, 5′ ligation, reverse transcription, cDNA cleanup, library amplification and library cleanup reagents; quality control primers	331502
QIAseq miRNA Library Kit (96)	For 96 sequencing prep reactions: 3′ ligation, 5′ ligation, reverse transcription, cDNA cleanup, library amplification and library cleanup reagents; quality control primers	331505
QIAseq miRNA 12 Index IL (12)	Sequencing adapters, primers and indexes compatible with Illumina® platforms. 12 indexes for 12 samples	331592
QIAseq miRNA 48 Index IL (96)	Sequencing adapters, primers and indexes compatible with Illumina platforms. Two 48 indexes for 96 samples	331595

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Notes

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