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miRCURY® LNA® miRNA PCR Assay Handbook for the QIAcuity® System

For highly sensitive detection of miRNA using EvaGreen®

Sample to Insight

QIAGEN

Contents

Kit Contents

Assay (1 single tube containing dried primer mix)	Catalog no. (167 reactions x 12 µl)* (50 reactions x 40 µl)*
miRCURY LNA miRNA PCR Assay†	339306
miRCURY LNA miRNA Custom PCR Assay†	339317

* Sufficient for 167 reaction using the QIAcuity Nanoplate 8.5k and 50 reactions using the QIAcuity Nanoplate 26k.
† Available at **geneglobe.qiagen.com**.

Product	Catalog no.
miRCURY LNA RT Kit (for 32 reactions of 20 µl)	339340
QIAcuity EG PCR Kit (for 250, 6250, and 125,000 reactions)*	250111, 250112, and 250113

* For using the QIAcuity Nanoplate 8.5k with a 12 µl dPCR reaction volume. Using the QIAcuity Nanoplate 26k with a 40 µl reaction results in 75, 375, and 1875 reactions, respectively.

Shipping and Storage

The miRCURY LNA miRNA PCR Assays are shipped lyophilized at room temperature (15–25°C). Upon receipt, store miRCURY LNA miRNA PCR Assays at 2–8°C or at –30 to –15°C. Under these conditions, all components are stable for at least 12 months without showing any reduction in performance and quality. After reconstitution, it is recommended to store LNA PCR assays in aliquots at –30 to –15°C to avoid repeated freeze–thaw cycles.

Intended Use

The miRCURY LNA miRNA PCR Assays 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 miRCURY LNA miRNA PCR Assays is tested against predetermined specifications to ensure consistent product quality.

Introduction

The miRCURY LNA miRNA PCR Assays provide highly sensitive and accurate locked nucleic acid (LNA)-enhanced digital PCR (dPCR) quantification assays for miRNA targets in an easy-to-handle format. They are designed for use with universal reverse transcription (RT), followed by PCR amplification. This handbook describes the detection in digital PCR on the QIAcuity using EvaGreen.

To obtain optimal results in dPCR, the use of miRCURY LNA PCR Assay products in combination with the miRCURY LNA RT Kit and the QIAcuity EG PCR Kit is recommended. The miRCURY LNA SYBR® Green PCR Kit cannot be used in a dPCR run on the QIAcuity.

The QIAcuity EG PCR Kit provides the highest specificity in dPCR because of a novel antibody-mediated hot-start mechanism. At low temperatures, the QuantiNova® DNA Polymerase is kept in an inactive state by the QuantiNova Antibody and QuantiNova Guard, a novel additive that stabilizes the complex. This improves the stringency of the hot-start mechanism and prevents extension of non-specifically annealed primers and formation of primer-dimers.

Table 1. Description of miRCURY LNA miRNA PCR System components for dPCR use

Component	Description
miRCURY LNA miRNA PCR Assays	Two mixed and lyophilized miRNA-specific primers designed for highly sensitive quantification of miRNA targets.
miRCURY LNA RT Kit	An optimized blend of Reverse Transcription Enzyme and Poly(A) polymerase developed for use in two-step PCR.
QIAcuity EG PCR Kit	A ready-to-use master mix for conducting dPCR runs on the QIAcuity instrument.

Principle and procedure

The miRCURY LNA miRNA PCR Assays and the QIAcuity instrument form a unique system for miRNA profiling that offers the best combination of performance and ease-of-use tools on the miRNA dPCR market.

- **cDNA synthesis using a universal RT**: One first-strand cDNA synthesis reaction provides the template for all miRNA PCR assays. This saves precious sample, reduces technical variation, consumes less reagents, and saves time in the laboratory. The same cDNA synthesis can be used across all assay formats.
- **LNA-enhanced dPCR amplification**: Both PCR amplification primers (forward and reverse) are miRNA specific with the LNAs placed intelligently in the primers to fully optimize the primer performance. The result is exceptional sensitivity and specificity with extremely low background, enabling reliable quantification of very low levels of miRNA. The highly specific assays allow discrimination between closely related miRNA sequences.

The miRCURY LNA miRNA PCR Assays and the outstanding performance of the QIAcuity EG PCR Kit offer solutions for both high-throughput miRNA expression profiling and for quantification of individual miRNAs.

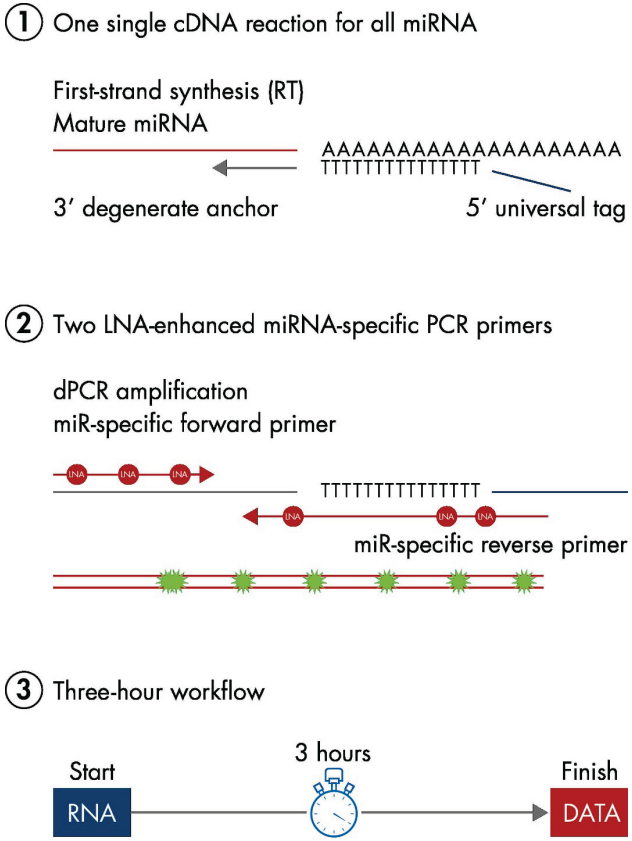

Figure 1. Schematic outline of the miRCURY LNA miRNA PCR System. In the cDNA synthesis, a poly(A) tail is added to the mature miRNA template, and cDNA is synthesized using a poly(T) primer with a 3′ degenerated anchor and a 5′ universal tag (1). The cDNA template is then amplified using miRNA-specific and LNA-enhanced forward and reverse primers, and EvaGreen is used for detection (2). The workflow only takes 3 hours to complete (3).

3x QIAcuity EG PCR Master Mix

The 3x QIAcuity EG PCR Master Mix includes QuantiNova DNA Polymerase. The dPCR-optimized master mix ensures ultrafast amplification in dPCR, with high specificity and sensitivity.

QuantiNova DNA Polymerase is provided in an inactive state and has no enzymatic activity at ambient or higher temperatures. The antibody-mediated hot-start mechanism prevents the formation and extension of nonspecific PCR products and primer–dimers during reaction setup and the first denaturation step. Therefore, this mechanism allows higher PCR specificity and accurate quantification. At low temperatures, the QuantiNova DNA Polymerase is kept in an inactive state by the QuantiNova Antibody and Guard, which stabilize the complex and improve the stringency of the hot-start.

Within 2 minutes of raising the temperature to 95°C, the QuantiNova Antibody and QuantiNova Guard are denatured, and the QuantiNova DNA Polymerase is activated, enabling PCR amplification (Figure 2). The hot-start enables rapid and convenient room temperature setup. After setup, the PCR can be stored for up to 2 hours at room temperature or up to 24 h at 2-8°C without impairing the performance of the subsequent reaction.

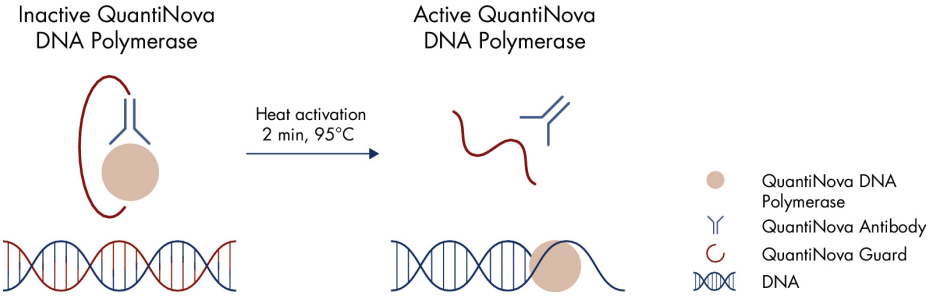

Figure 2. Principle of the novel QuantiNova hot-start mechanism. At ambient temperature, the QuantiNova DNA Polymerase is kept inactive by QuantiNova Antibody and QuantiNova Guard until the initial heat activation step.

LNA technology

LNAs are a class of high-affinity RNA analogs in which the ribose ring is "locked" in the ideal conformation for Watson–Crick binding. As a result, LNA oligonucleotides exhibit unprecedented thermal stability when hybridized to a complementary DNA or RNA strand. Since LNA oligonucleotides typically consist of a mixture of LNA and DNA or RNA, it is possible to optimize the sensitivity and specificity by varying the LNA content of the oligonucleotide. Incorporation of LNA into oligonucleotides has been shown to improve sensitivity and specificity for many hybridization-based technologies such as PCR, microarray, and in situ hybridization.

For each incorporated LNA monomer, the melting temperature (T_m) of the duplex increases by 2–8°C. In addition, LNA oligonucleotides can be made shorter than traditional DNA or RNA oligonucleotides and still retain a high T_m. This is important when the oligonucleotides are used to detect small or highly similar targets. Furthermore, optimal placement of LNA within the primers can increase the T_m between perfect match and mismatch targets, enabling better discrimination between closely related sequences even with single nucleotide differences.

Robust detection of all miRNA sequences, regardless of GC content

The small sizes and widely varying GC content (5–95%) of miRNAs make them challenging to analyze using traditional methods. DNA- or RNA-based methods for miRNA analysis can introduce high uncertainty and low robustness because the T_m of the oligonucleotide/miRNA duplex will vary greatly depending on the GC content of the sequences. This is especially problematic in applications such as microarray profiling and high-throughput experiments in which many miRNA targets are analyzed under the same experimental conditions.

Use of LNA-enhanced oligonucleotides overcomes these challenges. By simply varying the LNA content, oligonucleotides with specific duplex melting temperatures can be designed, regardless of the GC content of the miRNA. T_m-normalized primers perform well under the same experimental conditions.

Specific discrimination of highly similar targets

Another challenge of studying miRNAs is the high degree of similarity between the sequences. Some miRNA family members vary by only a single nucleotide. LNA can be used to enhance the discriminatory power of primers to allow excellent discrimination of closely related miRNA sequences. LNA offers significant improvement in sensitivity and specificity and ensures optimal performance for all miRNA targets.

An LNA oligonucleotide offers substantially increased affinity for its complementary strand, compared to traditional DNA or RNA oligonucleotides. This results in unprecedented sensitivity and specificity and makes LNA oligonucleotides ideal for the detection of small or highly similar DNA or RNA targets.

Description of protocols

The following protocols for the quantification of miRNAs using the QIAcuity dPCR system are described in this handbook:

Protocol: dPCR using miRCURY LNA miRNA PCR Assays

Description of miRNA analysis by dPCR.

Protocol: dPCR using miRCURY LNA miRNA PCR Assays in serum/plasma samples

Description of miRNA analysis by dPCR from serum, plasma, and other biofluids.

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.

- miRCURY LNA RT Kit (cat. no. 339340) and QIAcuity EG PCR Kit (cat. nos. 250111, 250112, or 250113)
- QIAcuity Nanoplates (cat. nos. 250001, 250011, or 250021)
- Sealing foils for dPCR plates (included with the nanoplate kits, or sold separately as cat. no. 250099)
- High-quality nuclease-free water (do not use DEPC-treated water)
- Nuclease-free plastic tubes for 10 µl reactions
- Nuclease-free PCR tubes or plates for use with individual assays
- Nuclease-free, aerosol-barrier pipette tips
- Single-channel or multichannel pipettor
- PCR cycler, heating block or water bath (capable of reaching 95°C)
- Vortexer
- Microcentrifuge and plate centrifuge

Important Notes

Poly(A) tailing and reverse transcription

Mature miRNAs are naturally occurring, 22-nucleotide, noncoding RNAs that mediate post-transcriptional gene regulation. Unlike mRNAs, miRNAs are not polyadenylated in nature. Mature miRNAs can be polyadenylated by poly(A) polymerase and reverse transcribed into cDNA using oligo-dT primers. Polyadenylation and reverse transcription are performed in parallel in the same tube. The reaction takes place at 42°C for 60 minutes and is then inactivated at 95°C.

RNA input amount

The miRCURY LNA miRNA PCR protocol is optimized for use of 10 ng total RNA per 10 µl cDNA synthesis reaction. The exact amount of total RNA needed may vary depending on the miRNA expression levels in the cells or tissue to be analyzed. For highly expressed miRNAs, it is possible to use down to 10 pg total RNA. For weakly expressed miRNAs, up to 200 ng total RNA may be used provided that the dilution of the cDNA is kept as described in the corresponding handbook. However, in samples with high amounts of PCR inhibitors such as FFPE tissue samples, this may not be feasible. Furthermore, inhibitors may be present in the RNA preparations from certain samples such as serum and plasma. Prior to conducting a larger miRNA profiling study, it is recommended to optimize the amount of input RNA to the RT reaction, to avoid conducting a larger study in which inhibition occurs sporadically throughout the dataset.

Template RNA requirements

Total RNA containing miRNA is the starting material required for the miRCURY LNA miRNA PCR System. It is not necessary to enrich for small RNAs. QIAGEN provides a range of solutions for purification of total RNA containing miRNA. For more information on miRNA purification, visit **www.qiagen.com/miRNA**.

Blood serum and plasma are particular sample types that require special RNA purification procedures, and usually, the amount of RNA present in the samples cannot be accurately determined. Due to low levels of miRNA and potentially high levels of inhibitors in samples derived from serum and plasma, specific aspects need to be considered for setting up experiments using these types of samples. Refer to Protocol: dPCR using miRCURY LNA miRNA PCR Assays in serum/plasma samples for details.

Control assays

Different types of control assays are available for usage with the miRCURY LNA miRNA PCR System:

- Reference assays and reference candidates
- RNA spike-in assays

Reference assays detect small noncoding RNAs – small nuclear RNA, small nucleolar RNA, or miRNA – which are frequently found to be stably expressed across different cells or tissues. Reference assays may therefore be candidate assays for normalization in a profiling study with several samples. Although this is a good and recommended approach, great caution should be taken in the selection of reference genes. The danger of using endogenous reference genes lies in the assumption that a specific gene is expressed at the exact same level in all sample types. This is rarely true. The selection of reference genes should therefore be made with care and should be specific to the sample set of interest. The actual selection of reference genes to be used for normalization should always be based on a determination of the most stably expressed gene(s).

Whenever applicable, it is recommended to use miRNA rather than small nuclear or nucleolar RNA for normalization. Firstly, those RNAs are longer than miRNA and may purify differently from miRNA. Moreover, small nuclear and nucleolar RNA have entirely different functions and subcellular locations. Finally, certain samples, such as blood plasma, do not contain small nuclear and nucleolar RNAs.

RNA spike-ins and the matching primer pairs enable to control quality of the RNA isolation, the cDNA synthesis reaction, and the PCR amplification. RNA isolations may vary in yield, purity, and integrity. Some sample types may contain compounds that inhibit the cDNA synthesis or the PCR amplification, even if the RNA was purified using the best standard procedures. This might result in different efficiencies of the reverse transcription or PCR between compared samples. One way to control for differences in efficiencies at each experimental level (isolation, cDNA synthesis, and PCR) is by adding known RNA spike-ins to the sample prior to isolation and cDNA synthesis. Use of the RNA spike-ins may also reveal if nucleases are present. After conducting the PCR, but before initiating the data analysis, wells detecting RNA spike-ins are compared and outlier samples identified and considered for exclusion from further data analysis.

Please refer to the *miRCURY LNA miRNA SYBR Green PCR Handbook* for additional information.

Protocol: dPCR Using miRCURY LNA miRNA PCR Assays

This protocol describes how to analyze miRNAs using miRCURY LNA miRNA PCR Assays (cat. nos. 339306 and 339217) with the QIAcuity EG PCR Kit (cat. nos. 250111, 250112, and 250113) on a QIAcuity dPCR System.

Important points before starting

- For cDNA synthesis of miRNA, the use of the miRCURY LNA RT Kit (cat. no. 339340) is critical with miRCURY LNA miRNA PCR assays. Additional instructions are provided in the *miRCURY LNA miRNA SYBR Green PCR Handbook.*
- For reverse transcription of miRNA from serum, plasma, and other biofluids, refer to the specific recommendations in the *miRCURY LNA miRNA SYBR Green PCR – Exosomes, Serum/Plasma, and Other Biofluid Samples Handbook*.
- For quantitative dPCR using the QIAcuity dPCR platform, the use of the QIAcuity EG PCR Kit is critical for obtaining optimal results with miRCURY LNA miRNA PCR Assays.
- The 3x QIAcuity EG PCR Master Mix contains the QuantiNova DNA Polymerase, which is inactive at room temperature. The PCR protocol must start with an initial incubation step of 2 min at 95°C to activate the QuantiNova DNA Polymerase.
- A fluorescent reference dye is provided as a component of the QIAcuity EG PCR Master Mix, for reliable detection of proper partition filling in the dPCR plates.
- Always start with the cycling conditions and primer concentrations specified in the corresponding protocols.

Things to do before starting (RT reaction)

- Thaw template RNA and 5x miRCURY RT SYBR Green Reaction Buffer on ice. Thaw RNase-free water at room temperature. Mix each solution by flicking the tubes. Centrifuge briefly to collect residual liquid from the sides of the tubes and then keep on ice.

Important: Be sure to use the correct 5x miRCURY RT SYBR Green Reaction Buffer and not the corresponding reaction buffer for the Probe reaction.

- Resuspend the UniSp6 RNA spike-in by adding 80 µl nuclease-free water to the tube. Mix by vortexing and briefly spin down. Leave for 20–30 min on ice to fully dissolve the RNA spike-in. Mix by vortexing and briefly spin down. Store in aliquots at –30 to –15°C.
- Immediately before use, remove the 10x miRCURY RT Enzyme from the freezer, mix by flicking the tube, and then place on ice. Briefly spin down to collect residual liquid from the sides of the tubes, and then keep on ice.

Procedure (RT reaction)

1. Dilute each template RNA sample to 5 ng/µl using nuclease-free water.

2. Prepare the reverse transcription reactions on ice according to Table 2. Mix and 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.

3. Incubate for 60 min at 42°C.

Table 2. Reverse transcription reaction setup per sample

Component	miRCURY PCR Assay (µl)
5x miRCURY SYBR Green RT Reaction Buffer	2
RNase-free water	4.5
10x miRCURY RT Enzyme Mix	1
UniSp6 RNA spike-in (optional)	0.5
Template RNA (5 ng/µl)	2
Total reaction volume	**10**

4. Incubate for 5 min at 95°C to heat inactivate the reverse transcriptase.

5. Immediately cool to 4°C.

6. Place the reverse transcription reactions on ice and proceed directly with PCR. Follow the provided recommendations for proper cDNA dilution.

   Note: If you do not plan to use the cDNA immediately, store it undiluted at 2–8°C for up to 4 days of at –30 to –15°C for up to 5 weeks. We recommend storing synthesized cDNA in low-nucleic acid binding tubes or plates.

Table 3. Reverse transcription reaction temperature cycling protocol

Step	Time	Temperature (°C)
Reverse transcription step	60 min	42
Inactivation of reaction	5 min	95
Storage	Forever	4

Things to do before starting (dPCR)

- Resuspend the miRCURY LNA miRNA PCR Assays: Spin down the tube before opening it for the first time. Add 220 µl nuclease-free water to the tube to obtain a 10x stock and leave at room temperature for 20 min. Vortex and spin down briefly.
- Thaw 3x QIAcuity EG PCR Master Mix, template cDNA, LNA PCR assays, and RNase-free water. Vortex and spin down briefly.

Procedure (dPCR)

1. Dilute the cDNA 1:60 by adding 590 µl RNase-free water to the 10 µl RT reaction immediately before use. Storing this 1:60 dilution of cDNA is not recommended.

2. Prepare a reaction mix according to Table 4. Due to the hot-start of the PCR reactions, it is not necessary to keep samples on ice during reaction setup or while programming the QIAcuity dPCR instrument.

3. Vortex the reaction mix.

Table 4. Reaction mix setup for miRCURY LNA miRNA PCR Assays

Component	Nanoplate 8.5k (24-well, 96-well) (µl)	Nanoplate 26k (24-well) (µl)
3x EvaGreen PCR Master Mix (green channel)	4	13.3
miRCURY LNA PCR Assay (10x)	1.2	4
Template cDNA	3*	10*
RNase-free water	3.8*	12.7*
Total reaction volume	**12**	**40**

* Appropriate template amount depends on various parameters. For detailed information, please refer to the *QIAcuity User Manual Extension: Application Guide* as well as to the *miRCURY LNA SYBR Green PCR Handbook*.

4. Dispense appropriate volumes of the reaction mix into the wells of a standard PCR plate.

5. Transfer the contents of each well of the standard PCR plate to the wells of the QIAcuity Nanoplate.

6. Seal the QIAcuity Nanoplate properly using the QIAcuity Nanoplate Seal provided in the QIAcuity Nanoplate Kits.

   Note: For exact sealing procedure, please see the *QIAcuity User Manual* (**www.qiagen.com/HB-2717**).

Thermal cycling and imaging conditions

1. In the QIAcuity Software Suite or on the QIAcuity instrument, under the dPCR parameters, set the cycling conditions according to Table 5.

2. Still under the dPCR parameters in the QIAcuity Software Suite or on the QIAcuity instrument, activate the green channel and deactivate the other channels in **Imaging**.

3. Place the QIAcuity Nanoplate into the QIAcuity instrument and start the dPCR run.

Table 5. Cycling conditions

Step	Time	Temperature (°C)
PCR initial heat activation	2 min	95
2-step cycling (40 cycles)		
Denaturation	15 s	95
Annealing/Extension	1 min	60
Cooling down	5 min	40

Data analysis

1. To set up a plate layout according to the experimental design, open the QIAcuity Software Suite and define the reaction mixes, samples, and controls. Plate layout can be defined before or after the nanoplate run.

   Note: Refer to the *QIAcuity User Manual* for details on setting up the plate layout.

2. After the run is completed, the raw data are automatically sent to the QIAcuity Software Suite.

3. For data analysis, open the QIAcuity Software Suite and select the individual nanoplate for the analysis in **Plate Overview** of the QIAcuity Software Suite.

   Note: See the *QIAcuity User Manual Extension: Application Guide* and *QIAcuity User Manual* for details on how to analyze absolute quantification data.

Protocol: dPCR Using miRCURY LNA miRNA PCR Assays in Serum/Plasma Samples

This protocol describes how to analyze miRNAs in serum/plasma samples using miRCURY LNA miRNA PCR Assays (cat. nos. 339306 and 339217) with the QIAcuity EG PCR Kit (cat. nos. 250111, 250112, and 250113) on a QIAcuity dPCR System.

Important points before starting

- The levels of total RNA found in serum and plasma are very low, so we recommend using carrier RNA in the purification procedure to ensure robust RNA isolation. When using carrier RNA, it is not possible to determine the concentration of RNA in a sample after purification. Therefore, we recommend basing the volume of input RNA for the RT reaction on the original volume of the starting sample material.
- Inhibitors may be present in RNA preparations. We recommend optimizing the amount of input RNA to the RT reaction. This can be done by running a few individual assays with different volumes of input RNA. For example, use 2 µl, 4 µl, and 6 µl of RNA per 20 µl cDNA synthesis reaction, corresponding to 8 µl, 16 µl, and 24 µl of original serum/plasma sample, respectively.
- For reverse transcription of miRNA from serum, plasma, and other biofluids, the use of the miRCURY LNA RT Kit (cat. no. 339340) is critical with miRCURY LNA miRNA PCR assays. Additional instructions are provided in the *miRCURY LNA miRNA SYBR Green PCR – Exosomes, Serum/Plasma, and Other Biofluid Samples Handbook*.
- For quantitative dPCR using the QIAcuity dPCR platform, the use of the QIAcuity EG PCR Kit is critical for obtaining optimal results with miRCURY LNA miRNA PCR Assays.
- The 3x QIAcuity EG PCR Master Mix contains the QuantiNova DNA Polymerase, which is inactive at room temperature. The PCR protocol must start with an initial incubation step of 2 min at 95°C to activate the QuantiNova DNA Polymerase.
- A fluorescent reference dye is provided as a component of the QIAcuity EG PCR Master Mix, for reliable detection of proper partition filling in the dPCR plates.

- Always start with the cycling conditions and primer concentrations specified in the corresponding protocols.

Things to do before starting (RT reaction)

- Thaw the template RNA and 5x miRCURY RT SYBR Green Reaction Buffer on ice. Thaw RNase-free water at room temperature. Mix each solution by flicking the tubes. Centrifuge briefly to collect residual liquid from the sides of the tubes and then keep on ice.

  Important: Be sure to use the correct 5x miRCURY RT SYBR Green Reaction Buffer and not the corresponding reaction buffer for the Probe reaction.
- Resuspend the UniSp6 RNA spike-in by adding 80 µl nuclease-free water to the tube. Mix by vortexing and briefly spin down. Leave for 20–30 min on ice to fully dissolve the RNA spike-in. Mix by vortexing and briefly spin down. Store in aliquots at –30 to –15°C.
- Immediately before use, remove the 10x miRCURY RT Enzyme from the freezer, mix by flicking the tube, and then place on ice. Briefly spin down to collect residual liquid from the sides of the tubes, and then keep on ice.

Procedure (RT reaction)

1. Calculate the volume of RNA corresponding to 16 µl of original serum/plasma sample for each 20 µl RT reaction (e.g., for RNA isolated from 200 µl plasma and eluted in 50 µl, use 4 µl eluate in each RT reaction or RNA isolated from 200 µl plasma and eluted in 14 µl, use 1.12 µl eluate in each 20 µl RT reaction [14 µl/200 µl * 16 µl]).
   Template RNA [µl] = Elution volume [µl] / Original sample volume [µl] * 16 [µl]

2. Prepare the reverse transcription reactions on ice according to Table 6. Mix and 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.

3. Incubate for 60 min at 42°C.

Table 6. Reverse transcription reaction setup per sample

Component	miRCURY PCR Assay (µl)
5x miRCURY SYBR Green RT Reaction Buffer	2
RNase-free water	4.5
10x miRCURY RT Enzyme Mix	1
UniSp6 RNA spike-in (optional)	0.5
Template RNA	2*
Total reaction volume	**10**

* Use a template RNA volume equivalent to 16 µl original serum/plasma for each 20 µl reverse transcription reaction. Use a template RNA volume equivalent to 8 µl original serum/plasma for each 10 µl reverse transcription reaction. The volumes here correspond to RNA isolated from 200 µl plasma and eluted in 50 µl.

4. Incubate for 5 min at 95°C to heat inactivate the reverse transcriptase.

5. Immediately cool to 4°C.

6. Place the reverse transcription reactions on ice and proceed directly with PCR. Follow the provided recommendations for proper cDNA dilution.

   Note: If you do not plan to use the cDNA immediately, store it undiluted at 2–8°C for up to 4 days of at –30 to –15°C for up to 5 weeks. We recommend storing synthesized cDNA in low-nucleic acid binding tubes or plates.

Table 7. Reverse transcription reaction temperature cycling protocol

Step	Time	Temperature (°C)
Reverse transcription step	60 min	42
Inactivation of reaction	5 min	95
Storage	Forever	4

Things to do before starting (dPCR)

- Resuspend the miRCURY LNA miRNA PCR Assays: Spin down the tube before opening it for the first time. Add 220 µl nuclease-free water to the tube to obtain a 10x stock and leave at room temperature for 20 min. Vortex and spin down briefly.
- Thaw 3x QIAcuity EG PCR Master Mix, template cDNA, LNA PCR assays, and RNase-free water. Vortex and spin down briefly.

Procedure (dPCR)

1. Dilute the cDNA 1:30 by adding 290 µl RNase-free water to the 10 µl RT reaction immediately before use. Storing this 1:30 dilution of cDNA is not recommended.

2. Prepare a reaction mix according to Table 8. Due to the hot-start of the PCR reactions, it is not necessary to keep samples on ice during reaction setup or while programming the QIAcuity dPCR instrument.

3. Vortex the reaction mix.

Table 8. Reaction mix setup for miRCURY LNA miRNA PCR Assays with serum/plasma samples

Component	Nanoplate 8.5k (24-well, 96-well) (µl)	Nanoplate 26k (24-well) (µl)
3x EvaGreen PCR Master Mix (green channel)	4	13.3
miRCURY LNA PCR Assay (10x)	1.2	4
Template cDNA	3*	10*
RNase-free water	3.8*	12.7*
Total reaction volume	**12**	**40**

* Appropriate template amount depends on various parameters. For detailed information, please refer to the *QIAcuity User Manual Extension: Application Guide* as well as to the *miRCURY LNA SYBR Green PCR Biofluid Samples Handbook*.

4. Dispense appropriate volumes of the reaction mix into the wells of a standard PCR plate.

5. Transfer the contents of each well of the standard PCR plate to the wells of the QIAcuity Nanoplate.

6. Seal the QIAcuity Nanoplate properly using the QIAcuity Nanoplate Seal provided in the QIAcuity Nanoplate Kits.

   Note: For exact sealing procedure, please see the *QIAcuity User Manual* (**www.qiagen.com/HB-2717**).

Thermal cycling and imaging conditions

1. In the QIAcuity Software Suite or on the QIAcuity instrument, under the dPCR parameters, set the cycling conditions according to Table 9.

2. Still under the dPCR parameters in the QIAcuity Software Suite or on the QIAcuity instrument, activate the green channel and deactivate the other channels in **Imaging**.

3. Place the QIAcuity Nanoplate into the QIAcuity instrument and start the dPCR run.

Table 9. Cycling conditions

Step	Time	Temperature (°C)
PCR initial heat activation	2 min	95
2-step cycling (40 cycles)		
Denaturation	15 s	95
Annealing/Extension	1 min	60
Cooling down	5 min	40

Data analysis

1. To set up a plate layout according to the experimental design, open the QIAcuity Software Suite and define the reaction mixes, samples, and controls. Plate layout can be defined before or after the nanoplate run.

   Note: Refer to the *QIAcuity User Manual* for details on setting up the plate layout.

2. After the run is completed, the raw data are automatically sent to the QIAcuity Software Suite.

3. For data analysis, open the QIAcuity Software Suite and select the individual nanoplate for the analysis in **Plate Overview** of the QIAcuity Software Suite.

   Note: See the *QIAcuity User Manual Extension: Application Guide* and *QIAcuity User Manual* for details on how to analyze absolute quantification data.

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 in 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**).

		Comments and suggestions
No or low signal (low signal-to-noise)		
a)	Incorrect cycling conditions	Always start with the optimized cycling conditions specified in the protocols. Ensure that the PCR cycling conditions include the initial step for activation of QuantiNova DNA Polymerase (95°C for 2 min) and the specified times for denaturation and annealing/extension.
b)	QuantiNova DNA Polymerase not activated	Ensure that the PCR cycling program includes the QuantiNova DNA Polymerase activation step (95°C for 2 min) as described in the protocols.
c)	Pipetting error or missing reagent	Check the concentrations and storage conditions of the reagents, including primers and template nucleic acid. Repeat the PCR.
d)	Problems with starting template	Check the concentration, storage conditions, and quality of the starting template. If necessary, make new serial dilutions of template nucleic acid from the stock solutions. Repeat the PCR using the new dilutions.
e)	Insufficient amount of starting template	Increase the amount of template if possible. Ensure that sufficient copies of the target nucleic acids are present in your samples.
f)	Insufficient number of cycles	Increase the number of cycles.
g)	Generated signals are weak	RNA samples may contain PCR inhibitors. Further purification of an alternative RNA extraction method may be necessary. Check positive controls.
h)	Incorrect temperature for RT reaction	RT reaction at 42°C is recommended.

		Comments and suggestions
High concentration values detected for no-template control (NTC)		
a)	Contamination of reagents	Discard all the components of the assay (e.g., master mix and primers) and repeat PCR with new components.
b)	Contamination during reaction setup	Take appropriate precautions during reaction setup, such as using aerosol-barrier pipette tips.
Nucleic acid recovery		
a)	Poor DNA/RNA recovery	It is recommended to perform dilutions in DNA/RNA LoBind tubes to obtain optimal nucleic acid recovery.

Appendix: 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 minimal amounts are sufficient to degrade RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. We recommend 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 the pretreatment and usage of both disposable and non-disposable vessels and solutions while working with RNA.

General handling

Proper microbiological, aseptic techniques 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 pre-treatment to inactivate RNases.

Nondisposable plasticware

Nondisposable 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

* 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.

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 diethyl pyrocarbonate (DEPC). * 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 then 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, treat water with DEPC first, and then dissolve the 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 ability to from 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.

* 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.

Ordering Information

Product	Contents	Cat. no.
miRCURY LNA miRNA PCR Assay	LNA-optimized PCR assay for miRNA quantification.	339306
miRCURY LNA miRNA Custom PCR Assay	Custom-designed and LNA-optimized PCR assay for miRNA quantification.	339317
miRCURY LNA RT Kit	5x miRCURY RT SYBR Green Reaction Buffer, 5x miRCURY RT Probe Reaction Buffer, 10x miRCURY RT Enzyme Mix, UniSp6 RNA Spike-in template, and RNase-free water; for 8-64 reactions.	339340
QIAcuity EG PCR Kit (1 ml)	1 ml 3x concentrated QIAcuity EvaGreen Master Mix, 2x 1.9 ml RNase-free water.	250111
QIAcuity EG PCR Kit (5 ml)	5x 1 ml 3x concentrated QIAcuity EvaGreen Master Mix, 8x 1.9 ml RNase-free water.	250112
QIAcuity EG PCR Kit (25 ml)	5x 5 ml 3x concentrated QIAcuity EvaGreen Master Mix, 4x 20 ml RNase-free water.	250113
QIAcuity Nanoplate 26k 24-well (10)	10 QIAcuity Nanoplate 26k 24-well, 11 Nanoplate Seals	250001
QIAcuity Nanoplate 8.5k 24-well (10)	10 QIAcuity Nanoplate 8.5k 24-well, 11 Nanoplate Seals	250011
QIAcuity Nanoplate 8.5k 96-well (10)	10 QIAcuity Nanoplate 8.5k 96-well, 11 Nanoplate Seals	250021
Nanoplate Seals (11)	11x Nanoplate Seals	250099

Product	Contents	Cat. no.
Related products		
miRNeasy Mini Kit (50)	50 RNeasy Mini Spin Columns, collection tubes (1.5 ml and 2 ml), QIAzol Lysis Reagent, RNase-free reagents and buffers.	217004
miRNeasy Serum/Plasma Advanced Kit (50)	50 RNeasy UCP MinElute Spin Columns, collection tubes (1.5 ml and 2 ml), QIAzol Lysis Reagent, RNase-free reagents and buffers.	217204
miRNeasy Micro Kit (50)	50 RNeasy UCP MinElute spin columns, collection tubes (1.5 ml and 2 ml), QIAzol Lysis Reagent, RNase-free reagents and buffers.	217084
miRNeasy FFPE Kit	50 RNeasy MinElute Spin Columns, collection tubes, proteinase K, RNase-free DNase I, DNase Booster Buffer, RNase-free buffers, RNase-free water.	217504
PAXgene® Tissue RNA/miRNA Kit (50)	PAXgene RNA MinElute Spin Columns, PAXgene Shredder Spin Columns, processing tubes, microcentrifuge tubes, carrier RNA, RNase-free DNase and RNase-free buffers, to be used with PAXgene Tissue Containers.	766134
PAXgene Tissue Container (10)	For collection, fixation and stabilization of 10 samples: 10 prefilled reagent containers containing PAXgene Tissue Fix and PAXgene Tissue Fix and PAXgene Tissue Stabilizer.	765112

Product	Contents	Cat. no.
PAXgene Blood miRNA Kit (50)	PAXgene Spin Columns, PAXgene Shredder Spin Columns, processing tubes, microcentrifuge tubes, RNase-free DNase, RNase-free reagents and buffers; to be used with PAXgene Blood RNA Tubes (available from BD, cat. no. 762165)	763134

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Document Revision History

Date	Changes
09/2021	Initial revision

Notes

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