
September 2019

miRCURY[®] LNA[®]

miRNA Probe PCR –

Exosomes, Serum/Plasma, and Other
Biofluid Samples Handbook

For highly sensitive, ultrafast real-time RT-PCR
detection of miRNAs from exosomes,
serum/plasma, and other biofluids

Contents

Kit Contents.....	4
Storage	7
Intended Use	8
Safety Information	8
Quality Control.....	8
Product Information	9
Introduction.....	10
Principle and workflow	11
LNA technology	12
Important Notes.....	14
Poly(A) tailing and reverse transcription	14
RNA input amount	14
Template RNA requirements	14
Mature miRNA expression profiling	15
2x QuantiNova Probe PCR Master Mix	16
Built-in visual control for correct pipetting	17
Control assays	17
Reference assays and reference candidates	18
Inter-plate calibrators	18
RNA spike-ins (synthetic control templates)	19
GeneGlobe® analysis tool	20
Passive reference dye	20

Adding ROX dye to the PCR master mix	21
Equipment and Reagents to Be Supplied by User	22
Protocol overview	23
Protocol: First-Strand cDNA Synthesis	24
Protocol: Quantitative, Real-Time PCR Using Individual miRCURY LNA miRNA Probe PCR Assays	28
Protocol: Quantitative, Real-Time PCR Using miRCURY LNA miRNome PCR Panels or Serum/Plasma Focus Probe PCR Panels	31
Protocol: Quantitative, Real-Time PCR Using miRCURY LNA miRNA Custom Probe PCR Panels	35
Analysis and Interpretation of Inter-Plate Calibrators	40
Normalization of miRNA Quantitative PCR Experiments	41
Troubleshooting Guide	43
Appendix A: Reaction Setup Using Master Mix Containing a High Concentration of ROX ...	45
Appendix B: General Remarks on Handling RNA	46
Ordering Information	48
Document Revision History	51

Kit Contents

miRCURY LNA RT Kit	(8–64)*
Catalog no.	339340
Number of standard 20 µl reactions	32†
5x miRCURY RT SYBR Green® Reaction Buffer†, including Mg ²⁺ , SYBR Green® RT primer and dNTPs	128 µl
5x miRCURY RT Probe Reaction Buffer†, including Mg ²⁺ , Probe RT primer and dNTPs	128 µl
10x miRCURY RT Enzyme Mix	64 µl
UniSp6 RNA Spike-in Template, dried down	12 fmol
RNase-free water	1.5 ml

* Depending on the reaction volumes used (10–80 µl), there are sufficient reagents in the kit for 8–64 reactions.

† The number of standard reactions is based on a reaction volume of 20 µl, containing 10 pg to 5 µg total RNA. The 5x miRCURY RT Reaction Buffers are not interchangeable between the SYBR Green® and the Probe-based workflows.

miRCURY LNA Probe PCR Kit	(200)	(800)	(4000)
Catalog no.	339371	339372	339373
Number of standard 10 µl reactions	200	800	4000
2x QuantiNova® Probe PCR Master Mix containing the following: QuantiNova Probe PCR Buffer, dNTP mix (dATP, dCTP, dGTP, dTTP), and QuantiNova DNA Polymerase composed of: Taq DNA Polymerase, QuantiNova Antibody, QuantiNova Guard	1 ml	4 x 1 ml	2 x 10 ml
miRCURY Probe Universal Reverse Primer	200 µl	800 µl	5 x 800 µl
ROX™ Reference Dye	250 µl	1 ml	2 x 1 ml
RNase-free Water	2 x 1.9ml	2 x 1.9 ml	9 x 1.9 ml

miRCURY LNA miRNA Probe PCR Assay	(200)
Catalog no.(specific additional assays cat. no varies)	339350
Number of 10 µl reactions	200
miRCURY LNA miRNA Probe PCR Primer Mix, dried down	1 vial

miRCURY LNA Probe miRNome PCR Panel I and II (339361*)	
96-well PCR plates containing dried-down LNA Probe PCR Assays for one 10 µl reaction per well	
Panel I	Panel II
372 LNA Probe PCR assays for the amplification of human miRNAs (distributed on 4 x 96-well plates)	380 LNA Probe PCR assays for the amplification of human miRNAs (distributed on 4 x 96-well plates)
4 x 1 inter-plate calibrators	4 x 1 inter-plate calibrators
3 assays for reference genes [†] (on plate 4)	
5 RNA spike-in control assays [†] (on plate 4)	

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

[†] Human Panels: three snRNAs (U6snRNA, SNORD38B, SNORD49A).

[†] The RNA spike-in control assay targets the UniSp6 RNA spike-in supplied in the miRCURY LNA RT Kit and the four RNA spike-ins contained in the RNA Spike-in Kit (UniSp2, UniSp4, UniSp5, and cel-miR-39-3p).

miRCURY LNA Probe miRNome PCR Panel I and II (339361 – specific cat. no. varies)	
384-well PCR plates containing dried-down LNA Probe PCR Assays for one 10 µl reaction per well	
Panel I	Panel II
372 LNA Probe PCR assays for the amplification of human miRNAs	380 LNA Probe PCR assays for the amplification of human miRNAs
4 inter-plate calibrators	4 inter-plate calibrators
3 assays for reference genes*	
5 RNA spike-in control assays [†]	

* Human Panels: three snRNAs (U6snRNA, SNORD38B, SNORD49A).

[†] The RNA spike-in control assay targets the UniSp6 RNA spike-in supplied in the miRCURY LNA RT Kit and the four RNA spike-ins contained in the RNA Spike-in Kit (UniSp2, UniSp4, UniSp5, and cel-miR-39-3p).

miRCURY LNA Probe miRNome PCR Panel I and II (339361 – specific cat. no. varies)**Rotor-Disc 100 containing dried-down LNA Probe PCR Assays for one 20 µl reaction per well on the Rotor-Gene Q****Panel I**

372 LNA Probe PCR assays for the amplification of human miRNAs (distributed on 4 x Rotor-Disc 100)

4 x 1 inter-plate calibrators

3 assays for reference genes* (on Disc 4)

5 RNA spike-in control assays† (on Disc 4)

Panel II

380 LNA Probe PCR assays for the amplification of human miRNAs (distributed on 4 x Rotor-Disc 100)

4 x 1 inter-plate calibrators

* Human Panels: three snRNAs (U6snRNA, SNORD38B, SNORD49A).

† The RNA spike-in control assay targets the UniSp6 RNA spike-in supplied in the miRCURY LNA RT Kit and the four RNA spike-ins contained in the RNA Spike-in Kit (UniSp2, UniSp4, UniSp5, and cel-miR-39-3p).

miRCURY LNA miRNA Serum/Plasma Focus Probe PCR Panel (339362 – specific cat. no. varies)**PCR plates compatible with various real-time PCR instruments containing dried-down LNA Probe PCR Assays for one 10/20 µl reaction per well**

96-well format or Rotor-Disc 100 (2 plates / Discs)

384-well format (2 panels per plate)

181 LNA Probe PCR Assays for the amplification of human miRNAs*

2 x 181 LNA Probe PCR Assays for the amplification of human miRNAs*

2 x 3 inter-plate calibrators

2 x 6 inter-plate calibrators

5 RNA spike-in control assays†

2 x 5 RNA spike-in control assays†

4 blank wells on each Rotor-Disc 100

* Serum/Plasma Focus Panel: miR-103a-3p, miR-191-5p, miR-423-5p, miR-16-5p, miR-425-5p, miR-93-5p and miR-451a are regarded reference gene candidates.

† The RNA spike-in control assay targets the UniSp6 RNA spike-in supplied in the miRCURY LNA RT Kit and the four RNA spike-ins contained in the RNA Spike-in Kit (UniSp2, UniSp4, UniSp5, and cel-miR-39-3p).

For more details on the plate layouts and targets, please see www.qiagen.com and download the plate layout files on the corresponding product webpage.

Storage

The miRCURY LNA RT Kit is shipped on dry ice. The kit, including all reagents and buffers, should be stored immediately upon receipt at -30 to -15°C in a constant-temperature freezer. miRCURY LNA Probe PCR Kits are shipped on dry ice. The kits should be stored immediately upon receipt at -30 to -15°C in a constant-temperature freezer and protected from light.

When the kits are stored under these conditions and handled correctly, performance is guaranteed until the expiration date. QuantiNova Probe PCR Master Mix and ROX Reference Dye can also be stored protected from light at $2-8^{\circ}\text{C}$ for up to 12 months, depending on the expiration date.

If desired, ROX Reference Dye can be added to 2x QuantiNova Probe PCR Master Mix for long-term storage. For details, see “Adding ROX dye to the PCR master mix” on page 21.

The PCR panels and assays are shipped dried down at room temperature. The primers can be stored at $2-8^{\circ}\text{C}$ or -30 to -15°C . Under these conditions, all components are stable for at least 12 months. After resuspension, it is recommended to store LNA PCR assays and reference gene assays in aliquots at -30 to -15°C to avoid repeated freeze-thaw cycles.

Intended Use

The miRCURY LNA RT Kit, the miRCURY LNA Probe PCR Kit, and miRCURY LNA miRNA Probe PCR Assays and Panels 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 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 miRCURY LNA RT Kit, miRCURY LNA Probe PCR Kit, and miRCURY LNA Probe PCR Assays is tested against predetermined specifications to ensure consistent product quality.

Product Information

Table 1. Descriptions of miRCURY LNA RT Kit components

Component	Description
10x miRCURY Reverse Transcription Enzyme	An optimized blend of Reverse Transcription Enzyme and Poly(A) polymerase developed for use in real-time, two-step PCR: <ul style="list-style-type: none"> • HoiStarRT-Script Reverse Transcriptase (HoiStarRT-Script Reverse Transcriptase is a modified form of a recombinant 77 kDa reverse transcriptase.) • <i>E. coli</i> Poly(A) Polymerase
5x miRCURY RT SYBR Green® Reaction Buffer	Buffer optimized for Poly(A) polymerization and reverse transcription; contains a universal reverse transcription primer for the SYBR® Green-based workflow, Mg ²⁺ and dNTPs.
5x miRCURY RT Probe Reaction Buffer	Buffer optimized for Poly(A) polymerization and reverse transcription; contains universal reverse transcription primer for the probe-based workflow, Mg ²⁺ and dNTPs.
UniSp6 RNA Spike-in Template	Synthetic transcript for monitoring successful reverse transcription
Nuclease-free water	Ultrapure quality, PCR-grade water

Table 2. Descriptions of miRCURY LNA Probe PCR Kit components

Component	Description
2x QuantiNova Probe PCR Master Mix, containing:	
QuantiNova DNA Polymerase	QuantiNova DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from <i>Thermus aquaticus</i> . QuantiNova DNA Polymerase is provided in an inactive state and has no enzymatic activity at ambient temperature. The enzyme is activated by a 2 minute, 95°C incubation step.
QuantiNova Probe PCR Buffer	Contains Tris-HCl, KCl, NH ₄ Cl, MgCl ₂ , and additives enabling fast cycling, including Q-Bond®.
dNTP mix	Contains dATP, dCTP, dGTP, and dTTP of ultrapure quality.
Other kit components:	
miRCURY Probe Universal Primer	Universal reverse primer for the miRCURY Probe PCR (10x concentrated)
ROX Reference Dye	Optimized concentration of fluorescent dye for normalization of fluorescent signals on all instruments from Applied Biosystems®.
RNase-Free Water	Ultrapure quality, PCR-grade water

Table 3. Descriptions of miRCURY LNA miRNA Probe PCR Assay components

Component	Description
10x miRCURY Probe Assay	One miRNA-specific LNA-primer and one miRNA-specific LNA-Probe (to be used together with the miRCURY Probe Universal Primer included in Probe PCR Kit)

Introduction

The miRCURY LNA miRNA Probe PCR System is an miRNA-specific, LNA-based system designed for sensitive and accurate detection of miRNA by quantitative, real-time PCR using dual labelled probes. The method is based on universal reverse transcription (RT), followed by real-time PCR amplification with LNA-enhanced primers and probe. The miRCURY LNA miRNA PCR portfolio is comprised of four types of reagent kits, including:

- miRCURY LNA RT Kit (cat. no. 339340)
- RNA Spike-in Kit, for RT (cat. no. 339390)
- miRCURY LNA Probe PCR Kit (cat. nos. 339371, 339372, and 339373)
- miRCURY LNA miRNA Probe PCR primers, available as individual assays and reference genes and pre-defined or customized PCR panels:
 - miRCURY LNA miRNA Probe PCR Assay (cat. no. 339350*)
 - miRCURY LNA miRNA Custom Probe PCR Assay (cat. no. 339351*)
 - miRCURY LNA miRNA Custom Probe PCR Panel (cat. no. 339360*)
 - miRCURY LNA miRNA miRNome Probe PCR Panels (cat. no. 339361*)
 - miRCURY LNA miRNA Focus Probe PCR Panels: Serum/Plasma (cat. no. 339362*)

All PCR panels are ready-to-use and include primers for one 10 µl PCR reaction per well. The PCR panels for the Rotor-Gene Q are ready-to-use and include primers for one 20 µl PCR reaction per well.

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

Principle and workflow

The miRCURY LNA miRNA Probe PCR System is a unique system for miRNA profiling, offering the best combination of performance and ease-of-use available on the miRNA real-time PCR market, because it unites two important features (Figure 1):

- **Universal RT:** One first-strand cDNA synthesis reaction provides the template for all probe-based real-time miRNA PCR assays. This saves precious sample, reduces technical variation, consumes less reagents, and saves time in the laboratory.
- **LNA-enhanced PCR amplification:** The forward PCR amplification primer is LNA enhanced and fully complement to the miRNA sequence. The result is exceptional sensitivity and extremely low background, enabling accurate quantification of very low levels of miRNA.
- **LNA-enhanced Probe Detection:** The hydrolysis probe is also miRNA specific and optimized with LNA. It allows reliable detection at the lowest quantities to get true positive signals.

The highly specific Probe Assays allow discrimination between closely related miRNA sequences. The miRCURY LNA miRNA Probe PCR System and the outstanding performance of the miRCURY LNA Probe PCR Kit offer solutions for both high-throughput miRNA expression profiling and for quantification of individual miRNAs.

High specificity and sensitivity in real-time PCR are achieved by a hot-start procedure. This allows room-temperature setup of the PCR reaction without the risk of primer–dimer formation. The hot start is achieved using QuantiNova DNA Polymerase, a novel, hot-start enzyme, and the additive QuantiNova Guard. These unique components further improve the stringency of the antibody-mediated hot start.

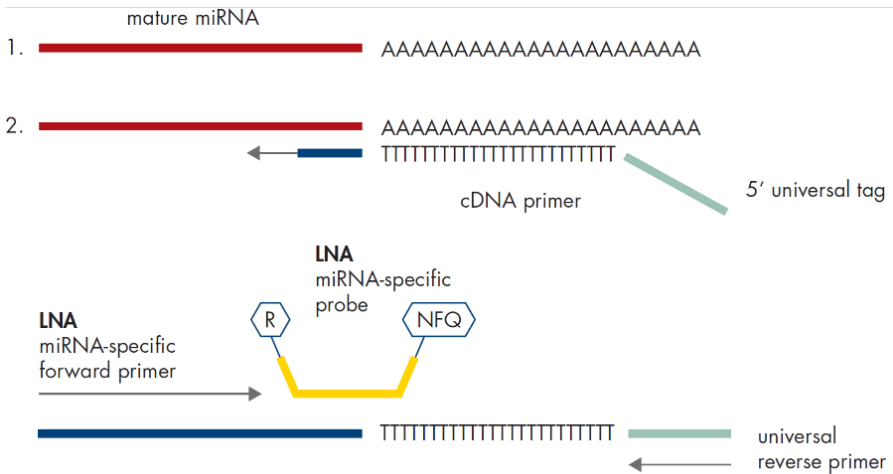


Figure 1. . Schematic outline of the miRCURY LNA miRNA Probe PCR System. In the cDNA synthesis, a poly(A) tail is added (step 1.) to the mature miRNA template, and cDNA is synthesized using a poly(T) primer with a 3' degenerate anchor and a 5' universal tag (step 2.). The cDNA template is then amplified using a miRNA-specific and LNA-enhanced forward and a universal reverse primer (step 2). The dual-labeled probe is then cleaved by the DNA polymerase during PCR, thus generating the fluorescence signal. The workflow only takes less than 3 hours to complete.

The miRCURY LNA Probe PCR Kit also features a built-in control for visual identification of correct template addition as well as Q-Bond, an additive in the PCR buffer that enables short cycling steps without loss of PCR sensitivity and efficiency. The kit has been optimized for use with any real-time cycler. ROX Reference Dye is provided in a separate tube and can be added if your cycler requires ROX as a passive reference dye.

LNA technology

Locked nucleic acids (LNA) 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. 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 oligonucleotide is used to detect small or highly similar targets.

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 including PCR, microarray, and *in situ* hybridization.

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, probes and inhibitors all 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 and probes 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.

Important Notes

Poly(A) tailing and reverse transcription

Mature miRNAs are naturally occurring, 22-nucleotide, non-coding 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 oligo-dT primers have a 3' degenerate anchor that allows amplification of mature miRNA in the real-time PCR step. The reaction takes place at 40°C for 60 minutes and is then inactivated at 95°C.

RNA input amount

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.

Template RNA requirements

Serum, plasma, and other biofluids are particular sample types that require special RNA purification procedures, and the amount of RNA present in the samples can usually not be accurately determined. Inhibitors may be present in RNA preparations from certain samples (e.g., serum and plasma). The presence of such inhibitors prohibits increasing the amount of sample input to obtain better sensitivity. The amount of inhibitors remaining after purification can vary greatly between different extraction methods and from sample to sample.

Prior to conducting a larger miRNA profiling study, we highly recommend that you optimize the amount of input RNA to the RT reaction, to avoid conducting a larger study in which inhibition occurs sporadically throughout the dataset. We recommend that you test varying volumes of total input RNA in the cDNA synthesis reaction (e.g., 2 μ l, 4 μ l, and 6 μ l per 20 μ l cDNA synthesis reaction) on a number of individual assays, or alternatively, on a set of full panels. This will provide an indication of the miRNA level and possible presence of inhibitors in your particular serum/plasma sample. QIAGEN provides a range of solutions for purification of total RNA containing miRNA (Table 4). For more information on miRNA purification, visit www.qiagen.com/miRNA.

Table 4. Kits for purification of RNA including miRNA

Kit	Cat. no.	Starting material
miRNeasy Serum/Plasma Advanced Kit*	217204	Serum/plasma samples
miRNeasy 96 Advanced QIAcube HT Kit (5x96)	217261	Serum/plasma samples
exoRNeasy Serum/Plasma Midi Kit*	77044	Serum/plasma samples
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.

Mature miRNA expression profiling

cDNA prepared in a reverse transcription reaction using the miRCURY LNA RT Kit serves as the template for real-time PCR analysis using miRCURY LNA miRNA Probe PCR Panels and the miRCURY LNA Probe PCR Kit. To profile mature miRNA expression, a premix of cDNA, QuantiNova Probe PCR Master Mix, miRCURY Probe Universal Primer, and RNase-free water is added to the miRCURY LNA miRNA Probe PCR Panel. There is no need to set up the reactions on ice. Furthermore, the whole reaction can be left for up to 2 hours at room temperature (15–25°C) or up to 24 hours at 2–8°C without any loss of performance.

2x QuantiNova Probe PCR Master Mix

The components of 2x QuantiNova Probe PCR Master Mix include QuantiNova DNA Polymerase and QuantiNova Probe PCR Buffer. The optimized Master Mix ensures ultrafast real-time PCR amplification with high specificity and sensitivity.

QuantiNova Probe PCR Buffer is specifically designed for ultrafast, real-time PCR using sequence-specific probes. The buffer additive, Q-Bond, allows short cycling times on any real-time cycler. Q-Bond increases the affinity of the QuantiNova DNA Polymerase for short single-stranded DNA, reducing the time required for primer/probe annealing to a few seconds. In addition, the unique composition of the buffer supports the melting behavior of DNA, enabling short denaturation and annealing/extension times.

QuantiNova DNA Polymerase is provided in an inactive state and has no enzymatic activity at ambient or higher temperatures. The enzyme remains completely inactive during the reverse transcription reaction and does not interfere with it. 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.

After the reverse transcription and within 2 minutes of raising the temperature to 95°C, the QuantiNova Antibody and 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 (15–25°C) or up to 24 hours at 2–8°C without impairing the performance of the subsequent reaction.

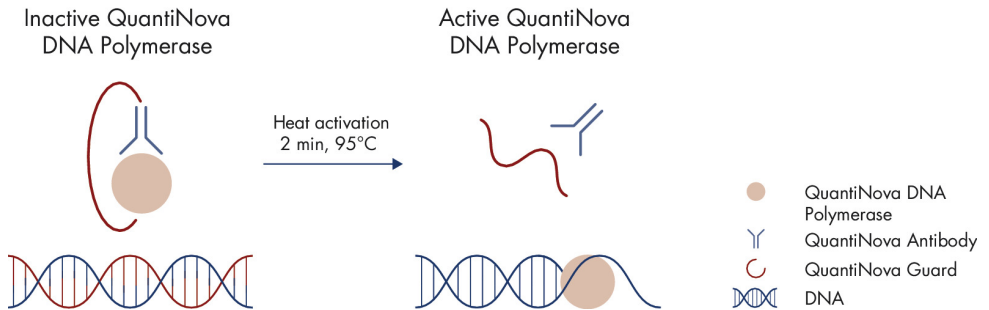


Figure 2. Principle of the novel QuantiNova hot-start mechanism. At ambient temperature, the QuantiNova DNA Polymerase is kept inactive by QuantiNova Antibody and QuantiNova Guard until the initial heat activation step.

Built-in visual control for correct pipetting

The 2x QuantiNova Probe PCR Master Mix included in the miRCURY LNA Probe PCR Kit contains an inert blue dye that increases visibility in the tube or well, without interfering with the PCR.

Control assays

There are three different types of control assays available in the miRCURY LNA miRNA Probe PCR System:

- Reference assays and reference candidates
- Inter-plate calibrators
- RNA spike-in assays

All of these control assays are available in the miRCURY LNA miRNA Probe PCR Panels. One RNA spike-in template is provided with the miRCURY LNA RT Kit. In addition, RNA spike-in templates are available in the RNA Spike-in Kit, for RT (cat. no. 339390). The assays for detecting these four templates, as well as the reference assays, are available individually.

Reference assays and reference candidates

These assays detect small non-coding RNAs – either small nuclear RNA, small nucleolar RNA or miRNA – which frequently are 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. Though 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 you are working with. 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).

When applicable, we recommend using miRNA, rather than small nuclear RNA or small nucleolar RNA for normalization. Firstly, small nuclear and nucleolar RNAs are longer RNA species than miRNA and may purify differently from miRNA. Moreover, small nuclear and nucleolar RNAs have entirely different functions and sub-cellular locations. Finally, certain samples, such as blood plasma, do not contain the small nuclear and nucleolar RNAs. Global mean normalization is a preferred alternative to using reference genes for normalization when working with panels and samples in which many miRNAs are screened per sample and many miRNAs are detected in all samples.

Inter-plate calibrators

Three wells within the pre-defined miRNome PCR Panels and the Focus Probe PCR Panels contain the inter-plate calibrator assay, which is annotated as UniSp9 IPC in the plate layout files. Depending on the plate layout, the Custom Probe PCR Panels contain at least three inter-plate calibrators. Each of these wells contains a pre-aliquoted primer pair and a DNA template, so the variation of these assays is very minimal from well-to-well and from plate-to-plate. The inter-plate calibrators are used for calibration between PCR plate runs, a very helpful feature when using instruments that apply the cycle threshold method for C_q determination

(e.g., ABI7900 PCR cycler). The inter-plate calibrators give a signal that is independent of cDNA quality, but it may be affected by PCR inhibitors in the sample, so they may be used to perform quality control of each plate run.

The UniSp6 RNA spike-in can also be used as an inter-plate calibrator. This application is only relevant when using individual LNA PCR assays and reference gene assays in a multi-plate setup, as the Probe PCR Panels already contain an inter-plate calibrator, as described above. To use the UniSp6 RNA spike-in as an inter-plate calibrator, add 1 μ l synthetic spike-in (10^8 copies/ μ l) to 20 ng of a complex RNA sample (e.g., total RNA from MS2, yeast or a cell line; not provided with the kit). Proceed with first-strand cDNA synthesis and subsequent real-time PCR as described in the protocols of this handbook. At least one spike-in amplification reaction per PCR plate is used for inter-plate calibration.

RNA spike-ins (synthetic control templates)

The primary purpose of the RNA spike-ins and the matching primer pairs for their detection is to provide controls for the 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 may 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 may be identified and considered for exclusion from further data analysis.

We have designed a collection of RNA spike-ins for this purpose. The UniSp6 RNA spike-in template is provided with the miRCURY LNA RT Kit. Additionally, four RNA spike-in templates are available separately in the RNA Spike-in Kit, for RT (cat. no. 339390). This kit includes a vial of three RNA spike-in templates, UniSp2, UniSp4, and UniSp5, mixed at different

concentrations, for use during RNA isolation. The cel-miR-39-3p RNA template is provided in a separate vial in the RNA Spike-in Kit; it can be mixed with the UniSp6 template from the miRCURY LNA RT Kit to obtain two different template concentrations. This combination can be added during the cDNA synthesis. Five wells in the pre-defined Probe PCR Panel plates contain the matching PCR assays for these spike-ins. For Custom Probe Panels, the choice of RNA spike-in control assay can be customized to your specific need. The RNA spike-ins are shipped dried down and must be resuspended before use. When using the RNA Spike-in Kit, for RT follow the protocol in the *RNA Spike-in Kit, for RT Handbook*.

GeneGlobe® analysis tool

miRCURY LNA miRNA Probe PCR Array and Panel data can be analyzed using the free miRNA PCR Array Data Analysis tool, which is available in GeneGlobe at: <https://geneglobe.qiagen.com/analyze>. This tool provides a summary of the data from the PCR Array and interprets the miRNA reverse transcription control and positive PCR control.

Passive reference dye

For certain real-time cyclers, the presence of ROX passive reference dye in real-time PCR compensates for non-PCR-related variations in fluorescence detection. Fluorescence from ROX dye does not change during the course of real-time PCR, but provides a stable baseline to which PCR-related fluorescent signals are normalized. Thus, ROX dye compensates for differences in fluorescence detection between wells due to slight variations in reaction volume or to differences in well position. ROX dye does not interfere with real-time PCR, since it is not involved in the reaction and has an emission spectrum different from the FAM dye.

The use of ROX dye is necessary for instruments from Applied Biosystems. The miRCURY LNA Probe PCR Kit includes a separate tube of ROX Reference Dye, which can be added to the real-time PCR if you are using a real-time cycler that uses ROX as a passive reference dye. For instruments requiring a high concentration of ROX dye, use the ROX Reference Dye as a 20x concentrate. For instruments requiring a low concentration of ROX dye, use the dye as a 200x

concentrate. Refer to Table 5 for details on real-time cyclers that require low or high ROX concentrations. If desired, ROX Reference Dye can be diluted with 2x QuantiNova Probe PCR Master Mix for long-term storage (Table 6). For details, see “Adding ROX dye to the PCR master mix” on page 21.

Table 5. Real-time cyclers requiring high/low concentrations of ROX

High ROX concentration (ROX Reference Dye to be used at a 20x dilution)	Low ROX concentration (ROX Reference Dye to be used at a 200x dilution)
ABI PRISM® 7000	Applied Biosystems 7500
Applied Biosystems 7300	Applied Biosystems ViiA™ 7
Applied Biosystems 7900	Applied Biosystems QuantStudio® Systems
Applied Biosystems StepOne™	
Applied Biosystems StepOne Plus	

Adding ROX dye to the PCR master mix

If you only use cyclers from Applied Biosystems with the miRCURY LNA Probe PCR Kit, you can add ROX Reference Dye to the 2x QuantiNova Probe PCR Master Mix for long-term storage, if desired (Table 6). For information on the concentration of ROX required for Applied Biosystems instruments, refer to Table 5. For setting up reactions with master mix that already contains a high concentration of added ROX Reference Dye, refer to Appendix A: Reaction Setup Using Master Mix Containing a High Concentration of ROX on page 45.

Table 6. Addition of ROX Reference Dye to master mix

Volume of 2x QuantiNova Probe PCR Master Mix (without ROX Reference Dye)	Volume of ROX Reference Dye for high ROX concentration/low ROX concentration
1 ml	100 µl / 10 µl
10 ml	1 ml / 100 µl

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 reverse transcription and real-time PCR:

- Nuclease-free plastic tubes (for 20 μ l reactions)
- Nuclease-free PCR tubes or plates for use with individual assays
- Nuclease-free, aerosol-barrier pipette tips
- Ice
- PCR cycler, heating block or water bath (capable of reaching 95°C)
- Vortexer
- Microcentrifuge and plate centrifuge
- Sealing foils for PCR plates
- Real-time PCR instrument

Protocol overview

The following protocols for the first-strand cDNA synthesis and real-time PCR amplification are described in this handbook:

- Protocol: First-Strand cDNA Synthesis, page 24
- Protocol: Quantitative, Real-Time PCR Using Individual miRCURY LNA miRNA Probe PCR Assays, page 28
- Protocol: Quantitative, Real-Time PCR Using miRCURY LNA miRNome PCR Panels or Serum/Plasma Focus Probe PCR Panels, page 31
- Protocol: Quantitative, Real-Time PCR Using miRCURY LNA miRNA Custom Probe PCR Panels, page 35

Table 7. Protocol overview for analyzing miRNA from exosomes, serum/plasma and other biofluid samples

PCR assays or panel	miRNA Probe PCR Assays	miRNome PCR Panels		Focus Probe PCR Panels, Custom Probe PCR Panels		
		Panel I	Panel I+II	1–96 miRNAs	97–192 miRNAs	193–384 miRNAs
RT reaction volume	10 µl	40 µl	80 µl	10 µl	20 µl	40 µl
Number of reactions possible with the miRCURY LNA RT Kit	64	16	8	64	32	16

These protocols are for analysis of miRNA from serum, plasma and other biofluids. For other sample types, refer to the *miRCURY LNA miRNA Probe PCR Handbook*.

Protocol: First-Strand cDNA Synthesis

This protocol describes performing first-strand cDNA synthesis reactions using the miRCURY LNA RT Kit (cat. no. 339340).

Important points before starting

- This protocol is for reverse transcription of miRNA from serum, plasma and other biofluids. For other sample types, refer to the *miRCURY LNA miRNA PCR Handbook*.
- 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 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. Increasing the RNA input volume reduces the C_q values at which inhibition sets in.
- Set up all reactions on ice to minimize the risk of RNA degradation.
- dNTPs are already included in the kit components. Do not add additional dNTPs.
- The RNA Spike-in Kit, for RT (cat. no. 339390) is an internal extraction and amplification control for assessing RNA isolation, reverse transcription, and PCR amplification. The kit is designed to indicate instrument or chemistry failures, errors in assay setup, and the presence of inhibitors. Refer to the *RNA Spike-in Kit, for RT Handbook* for details.
- The RT primer is included in the 5x miRCURY RT Reaction Buffer.
- The 10x miRCURY RT Enzyme Mix contains both the Poly(A) polymerase and the Reverse Transcriptase.

- We recommend setting up the reactions in 200 µl PCR tubes and using a PCR cycler for the incubation steps.
- After reverse transcription, the reaction must be inactivated by incubation at 95°C for 5 minutes.
- The temperature steps can be conveniently set up using the cycling protocol described in Table 9.
- If working with RNA for the first time, read Appendix B: General Remarks on Handling RNA, on page 46.

Things to do before starting

- Thaw template RNA and 5x miRCURY RT Probe Reaction Buffer on ice. Thaw RNase-free water at room temperature (15–25°C). 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 note: Be sure to use the correct 5x miRCURY RT Probe Reaction Buffer and not the corresponding Reaction Buffer for the SYBR Green® reaction.

- Resuspend the UniSp6 RNA spike-in by adding 80 µl nuclease-free water to the tube. Mix by vortexing and briefly centrifuge. Leave for 20–30 min on ice to fully dissolve the RNA spike-in. Mix by vortexing and briefly centrifuge. 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 place on ice. Briefly centrifuge to collect residual liquid from the sides of the tubes and then keep on ice.

Procedure

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 [$14 \mu\text{l} / 200 \mu\text{l} * 16 \mu\text{l}$]).

Template RNA [µl] = Elution volume [µl] / Original sample volume [µl] * 16 [µl]

2. Prepare the reverse transcription reactions on ice according to Table 8. 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 8. Reverse transcription reaction setup per sample

Component	miRNA Probe PCR Assay	miRNome PCR Panels: Focus Probe PCR Panel: Serum/Plasma			Other Focus PCR Panels or Custom Probe PCR Panel: 1-96 miRNAs analyzed per sample	Custom Probe PCR Panel: 97-192 miRNAs analyzed per sample	Custom Probe PCR Panel: 193-384 miRNAs analyzed per sample
		2x 96-well Focus Serum/Plasma	384-well Focus Serum/Plasma or miRNome Panel I	2x 384-well miRNome Panel I+II			
5x miRCURY RT Probe Reaction Buffer	2 µl	4 µl	8 µl	16 µl	2 µl	4 µl	8 µl
RNase-free water	4.5 µl	9 µl	18 µl	36 µl	4.5 µl	9 µl	18 µl
10x miRCURY RT Enzyme Mix	1 µl	2 µl	4 µl	8 µl	1 µl	2 µl	4 µl
UniSp6 RNA spike-in (optional)	0.5 µl	1 µl	2 µl	4 µl	0.5 µl	1 µl	2 µl
Template RNA	2 µl*	4 µl*	8 µl*	16 µl*	2 µl*	4 µl*	8 µl*
Total reaction volume	10 µl†	20 µl†	40 µl†	80 µl†	10 µl†	20 µl†	40 µl†

* 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 listed here correspond to RNA isolated from 200 µl plasma and eluted in 50 µl.
 Template RNA [µl] = Elution volume [µl] / Original sample volume[µl] * 16 [µl]

† All volumes refer to corresponding PCR reaction volumes of 10 µl. For the Rotor-Disc 100, a reaction volume of 20 µl is recommended, double the amount of all reagents in the Reverse Transcription setup.

3. Incubate for 60 min at 40°C.
4. Incubate for 5 min at 95°C to heat inactivate the reverse transcriptase.
5. Immediately cool to 4°C.

Table 9. Reverse transcription reaction temperature cycling protocol

Step	Time	Temperature
Reverse transcription step	60 min	40°C
Inactivation of reaction	5 min	95°C
Storage	forever	4°C

6. Place the reverse transcription reactions on ice and proceed directly with real-time PCR. Follow the recommendations for proper cDNA dilution provided in the protocol for the Probe PCR Assay or Panel to be used.

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

Note: We recommend using the miRCURY LNA Probe PCR Kit for real-time PCR. For detailed information on use of the RNA Spike-in Kit, for RT and interpretation of real-time PCR results, refer to the *RNA Spike-in Kit, for RT Handbook*.

Protocol: Quantitative, Real-Time PCR Using Individual miRCURY LNA miRNA Probe PCR Assays

This protocol is for use with the miRCURY LNA Probe PCR Kit (cat. nos. 339371, 339372, and 339373) and miRCURY LNA miRNA Probe PCR Assays (cat. no. 339350), and miRCURY LNA miRNA Custom Probe PCR Assay (cat. no. 339351 *) on any real-time cycler.

Important points before starting

- This protocol is for analysis of miRNA from serum, plasma, and other biofluids. For other sample types, refer to the *miRCURY LNA miRNA PCR Handbook*.
- 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 and 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.
- The 2x QuantiNova Probe 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.
- Always start with the cycling conditions and primer concentrations specified in this protocol.

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

Things to do before starting

- If using single miRCURY LNA miRNA Probe PCR Assays, resuspend the PCR assays: centrifuge the tube before opening it for the first time. Add 220 μ l nuclease-free water to the tube and leave at room temperature (15–25°C) for 20 min. Vortex and briefly centrifuge.
- Thaw the 2x QuantiNova Probe PCR Master Mix, 10x miRCURY Probe Universal Primer, template cDNA, LNA Probe PCR assay, ROX Reference Dye (if required), and RNase-free water. Vortex and briefly centrifuge.

Procedure

1. Dilute the cDNA 1:40 by adding 390 μ l RNase-free water to the 10 μ l RT reaction immediately before use. We do not recommend storing this 1:20 dilution of cDNA.
2. Prepare a reaction mix according to Table 10. 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 real-time cyclers.

Table 10. Reaction mix setup for miRCURY LNA miRNA Probe PCR Assays

Component	Volume	Volume Rotor-Disc 100
2x QuantiNova Probe Master Mix	5 μ l	10 μ l
10x miRCURY Probe Universal Primer	1 μ l	2 μ l
ROX Reference Dye (ABI instruments only)	0.5 μ l/0.05 μ l*	
Resuspended PCR assay	1 μ l	2 μ l
cDNA template	2 μ l (Diluted 1:40)	4 μ l (diluted 1:40)
RNase-free water	1 μ l*	2 μ l
Total reaction volume	10 μl	20 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 and 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.

- Mix the reactions thoroughly and dispense 10 μ l per well into PCR tubes or PCR plates or 20 μ l into the Rotor-Disc 100.

Note: The experiment can be paused at this point. Store the reactions protected from light at 2–8°C for up to 24 hours.

- Briefly centrifuge the tubes or plate at room temperature (15–25°C).
- Program the real-time cycler according to Table 11.

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

- Place the PCR tubes or plates in the real-time cycler and start the cycling program.

Table 11. PCR cycling conditions

Step	Time	Temperature	Ramp rate	Additional comments
PCR initial heat activation	2 min	95°C	Maximal/fast mode	
2-step cycling:				
Denaturation	5 s	95°C	Maximal/fast mode	
Combined annealing/extension	30 s	56°C	Maximal/fast mode	Perform fluorescence data collection (FAM-Dark Quencher)
Number of cycles	40*			

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

- Perform the initial data analysis using the software supplied with your real-time PCR instrument to obtain raw C_q values (C_p or C_T , depending on PCR instrument).

Protocol: Quantitative, Real-Time PCR Using miRCURY LNA miRNome PCR Panels or Serum/Plasma Focus Probe PCR Panels

This protocol is for use with the miRCURY LNA Probe PCR Kit (cat. nos. 339371, 339372, and 339373) on any real-time PCR cycler. This protocol is used for conducting the first-strand cDNA synthesis and real-time PCR using the following PCR panels:

- miRCURY LNA miRNome Probe PCR Panels (cat. no. 339361 *)
Panel I + II (ZAHS-312Z-...)
Panel I (ZAHS-301Z-...)
Full product number varies with plate format for specific PCR instrument model
- miRCURY LNA Serum/Plasma Focus Probe PCR Panels (ZAHS-106Z-...)
Full product number varies with plate format for specific PCR instrument model

Important points before starting

- This protocol is for analysis of miRNA from serum, plasma, and other biofluids. For other sample types, refer to the *miRCURY LNA miRNA PCR Handbook*.
- 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, ViiA7, and QuantStudio Real-Time PCR Systems.
High concentration of ROX dye: ABI PRISM 7000, Applied Biosystems 7300 and 7900, and StepOne Real-Time PCR Systems.

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

- 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.
- The 2x QuantiNova Probe 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.
- Always start with the cycling conditions specified in this protocol.

Things to do before starting

- Thaw the 2x QuantiNova Probe PCR Master Mix, 10x miRCURY Probe Universal Primer, template cDNA, miRNA Probe PCR panel(s), ROX Reference Dye (if required), and RNase-free water. Vortex and briefly centrifuge.

Procedure

1. Prepare a reaction mix according to Table 12 or Table 13. 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 real-time cyclers.

Table 12. Reaction setup for miRCURY LNA and Serum/Plasma Focus Probe PCR Panels

Component	Serum/Plasma Focus Probe PCR Panel	
	192 assays (in 96-well or 384-well plate format)	192 assays (in Rotor-Disc 100 format)
2x QuantiNova Probe Master Mix	1000 µl	2000 µl
10x miRCURY Probe Universal Primer	200 µl	400 µl
ROX Reference Dye (ABI instruments only)	100 µl/10 µl*	
cDNA template (undiluted)	20 µl	40 µl
RNase-free water	780 µl*	1560 µl
Total reaction volume	2000 µl	4000 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 13. Reaction setup for miRCURY LNA miRNome Probe PCR Panels

Component	miRNome Probe PCR Panels (in 96-wells or 384-wells plate format)		miRNome Probe PCR Panels (in Rotor-Disc 100 format)	
	Panel I	Panels I+II	Panel I	Panels I+II
2x QuantiNova Probe Master Mix	2000 µl	4000 µl	4000 µl	8000 µl
10x miRCURY Probe Universal Primer	400 µl	800 µl	800 µl	1600 µl
ROX Reference Dye (ABI instruments only)	200 µl/20 µl*	400 µl/40 µl*		
cDNA template (undiluted)	40 µl	80 µl	80 µl	160 µl
RNase-free water	1560 µl*	3120 µl*	3120 µl	6240 µl
Total reaction volume	4000 µl	8000 µl	8000 µl	16000 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 and 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.

- Mix the reaction mix thoroughly, and dispense 10 µl per well into the plates containing the miRNome PCR Panels or 20 µl per well into the Rotor-Disc 100.

Note: The experiment can be paused at this point. Store the reactions protected from light at 2–8°C for up to 24 hours.

- Seal the plate. Carefully vortex it to dissolve the primers (optional). Briefly centrifuge the plate(s) at room temperature (15–25°C). Wait 5 min while the primers dissolve in the reaction mix.
- Program the real-time cycler according to Table 14.

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

- Place the plates into the real-time cycler and start the cycling program.

Table 14. PCR cycling conditions.

Step	Time	Temperature	Ramp rate	Additional comments
PCR initial heat activation	2 min	95°C	Maximal/fast mode	
2-step cycling:				
Denaturation	5 s	95°C	Maximal/fast mode	
Combined annealing/ extension	30 s	56°C	Maximal/fast mode	Perform fluorescence data collection (FAM- Dark Quencher)
Number of cycles	40*			

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

6. Perform the initial data analysis using the software supplied with your real-time PCR instrument to obtain raw C_q values (C_p or C_T , depending on PCR instrument).

Protocol: Quantitative, Real-Time PCR Using miRCURY LNA miRNA Custom Probe PCR Panels

This protocol is for use with the miRCURY LNA Probe PCR Kit (cat. nos. 339371, 339372, and 339373) on any real-time PCR cycler. This protocol is used for conducting real-time PCR using the following PCR panels in 96- or 384-well format:

- miRCURY LNA miRNA Custom Probe PCR Panels (cat. nos. 339360 *)

Important points before starting

- This protocol is for analysis of miRNA from serum, plasma, and other biofluids. For other sample types, refer to the *miRCURY LNA miRNA PCR Handbook*.
- 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, ViiA7, and QuantStudio Real-Time PCR Systems.

High concentration of ROX dye: ABI PRISM 7000, Applied Biosystems 7300 and 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.

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

-
- The 2x QuantiNova Probe 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.
 - Always start with the cycling conditions specified in this protocol.

Things to do before starting

- Thaw the 2x QuantiNova Probe PCR Master Mix, 10x miRCURY Probe Universal Primer, template cDNA, Custom Probe PCR panels, ROX Reference Dye (if required), and RNase-free water. Vortex and briefly centrifuge.

Procedure

1. Dilute the cDNA 1:30 according to Table 15 immediately before use. We do not recommend storing this 1:30 dilution of cDNA.

Table 15 refers to a 10 µl PCR reaction volume. For the Rotor-Disc 100, double the amounts.

Table 15. cDNA dilution for miRCURY LNA miRNA Custom Probe PCR Panels. The numbers in parentheses indicate the total number of assays per sample, including controls and inter-plate calibrators. Loss from pipetting is not included in the “Volumes of diluted cDNA needed” column.

	Custom Probe PCR Panel configuration	Suggested cDNA dilution per sample; 1:30 (cDNA + nuclease-free water, in μl)	Volume of 1:30 diluted cDNA needed for each sample on a Custom Probe PCR Panel plate
96	12 x 8 (12 samples/8 Assays)	2 + 58	24 μ l
96	8 x 12 (8 samples/12 Assays)	2 + 58	36 μ l
96	6 x 16 (6 samples/16 Assays)	2 + 58	48 μ l
96	4 x 24 (4 samples/24 Assays)	3 + 87	72 μ l
96	3 x 32 (3 samples/32 Assays)	4 + 116	96 μ l
96	2 x 48 (2 samples/48 Assays)	5 + 145	144 μ l
96	1 x 96 (1 sample/96 Assays)	10 + 290	288 μ l
384	48 x 8 (48 samples/8 Assays)	2 + 58	24 μ l
384	32 x 12 (32 samples/12 Assays)	2 + 58	36 μ l
384	24 x 16 (24 samples/16 Assays)	2 + 58	48 μ l
384	16 x 24 (16 samples/24 Assays)	3 + 87	72 μ l
384	12 x 32 (12 samples/32 Assays)	4 + 116	96 μ l
384	8 x 48 (8 samples/48 Assays)	5 + 145	144 μ l
384	6 x 64 (6 samples/64 Assays)	7 + 203	192 μ l
384	4 x 96 (4 samples/96 Assays)	10 + 290	288 μ l
384	3 x 128 (3 samples/128 Assays)	15 + 435	384 μ l
384	2 x 192 (2 samples/192 Assays)	20 + 580	576 μ l
384	1 x 384 (1 sample/384 Assays)	40 + 1160	1152 μ l

2. Prepare a reaction mix according to Table 16. 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 real-time cycler.

Table 16. Reaction setup per sample for miRCURY LNA miRNA Custom Probe PCR Panels

Component	Volume	Volume
	96 wells or 384 wells	Rotor-Disc 100
2x QuantiNova Probe Master Mix	5 µl	10 µl
10x miRCURY Probe Universal Primer	1 µl	2 µl
ROX Reference Dye (ABI instruments only)	0.5 µl/0.05 µl*	
cDNA template	3 µl (Diluted 1:30)	6 µl (Diluted 1:30)
RNase-free water	1 µl†	2 µl
Total reaction volume	10 µl	20 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 and 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. Calculate the total volume needed for your particular plate layout.

- Mix the reaction mix thoroughly and dispense 10 µl per well into the Probe PCR Panel plate(s) or 20 µl per well into the Rotor-Disc 100.

Note: The experiment can be paused at this point. Store the reactions protected from light at 2–8°C for up to 24 hours.

- Seal the plate. Carefully vortex it to dissolve the primers (optional). Briefly centrifuge the plate(s) at room temperature (15–25°C). Wait 5 min while the primers dissolve in the reaction mix.

- Program the real-time cycler according to Table 17.

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

- Place the plates into the real-time cycler and start the cycling program.

Table 17. PCR cycling conditions

Step	Time	Temperature	Ramp rate	Additional comments
PCR initial heat activation	2 min	95°C	Maximal/fast mode	
2-step cycling:				
Denaturation	5 s	95°C	Maximal/fast mode	
Combined annealing/ extension	30 s	56°C	Maximal/fast mode	Perform fluorescence data collection (FAM- Dark Quencher)
Number of cycles	40*			

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

7. Perform the initial data analysis using the software supplied with your real-time PCR instrument to obtain raw C_q values (C_p or C_T , depending on PCR instrument).

Analysis and Interpretation of Inter-Plate Calibrators

Three wells within the pre-defined miRNome and Focus Probe PCR Panels contain the inter-plate calibrator assay, annotated as UniSp9 IPC in the plate layout files. Depending on the particular plate layout, the Custom Probe PCR Panels contain at least three inter-plate calibrators. Each of these wells contain a pre-aliquoted primer pair and a DNA template, so the variation of these assays is minimal from well-to-well and from plate-to-plate. The inter-plate calibrators are used for calibration between PCR plate runs, a very helpful feature when using instruments that apply the cycle threshold method for C_q determination. Since the inter-plate calibrators give a signal that is independent of cDNA quality but may be affected by PCR inhibitors in the sample, they can be used for quality control of each plate run.

Inter-plate calibration (IPC) may be performed using the IPC assay replicates as described below. For each plate, verify that the replicates have a C_q standard deviation within 0.5. If not, eliminate the outlier, if it can be identified. Calculate the average of the replicates for each plate, the overall average (average of IPC values from all plates). The calibration factor is calculated as the difference between plate average and overall average for each plate (calibration factor = IPC plate – IPC overall). An example is shown in Table 18. Calibrate each plate by subtracting the calibration factor from all C_q values in the plate.

Table 18. Example of inter-plate calibration

	Plate 1	Plate 2	Plate 3
let-7c	21.12	20.93	21.34
IPC plate average	19.72	19.70	20.00
IPC overall average	19.81	19.81	19.81
Calibration factor	-0.09	-0.11	0.19
let-7c calibrated	21.21	21.04	21.15

Normalization of miRNA Quantitative PCR Experiments

The purpose of normalization is to remove technical and biological variation between samples that is not related to the biological changes under investigation. Proper normalization is critical for the correct analysis and interpretation of results from real-time PCR experiments. Most commonly, stably expressed reference genes are used for normalization.

In general, it is recommended to test several endogenous control candidates (reference genes) before setting up the actual miRNA expression analysis. These candidates should be chosen among genes that can be expected to be stably expressed over the whole range of samples being investigated. They could be stably expressed, small non-coding RNA or stably expressed miRNAs that are selected based on literature or pre-existing data (e.g., microarray analysis or qPCR panel screening).

QIAGEN offers LNA PCR assays for a number of different small RNAs that tend to be stably expressed, and are therefore often good candidates for reference genes. It is important to keep in mind that despite being small non-coding RNAs, most of these are significantly larger than miRNA and therefore may have different extraction efficiency and stability.

U6 is one reference gene that is often used. However, U6 is significantly larger than miRNAs and has a different sub-cellular distribution. The existence of several different isoforms also makes it a suboptimal reference gene. Another popular option is 5s ribosomal RNA, but this RNA has a much higher expression level than most miRNAs, and is often found as a PCR contaminant.

If working with samples from blood serum or plasma, please note that only circulating RNA is present. In this case, the small non-coding RNAs (5S, U6, SNORs, etc.) are not good candidates for reference genes, since they are most probably not present in the sample.

Using stably expressed miRNAs as reference genes offers several advantages, such as equal size, extraction efficiency and stability, as well as having expression levels within a similar range of the target miRNAs. Several candidates can be found in the literature, including miR-191-5p, miR-103a-3p, let-7a-5p and miR-16-5p. Microarray or qPCR panel screening data may also be used to support selection of candidate reference genes.

All reference gene candidates should be empirically validated for each study. One option for normalizing data from PCR panels for profiling a large number of miRNAs is to normalize against the global mean – the average of all expressed miRNAs. This can be a good option in samples with a high call rate (expressed miRNAs), but should be used with caution in samples with low call rates. It is also not a good option in samples for which the general miRNA expression level is changed.

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/support/faqs/. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and/or protocols in this handbook or sample and assay technologies (for contact information, visit www.qiagen.com).

Comments and suggestions

No signal or one or more signals detected late in PCR

- | | |
|---|--|
| 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 (2 min at 95°C) 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) Wrong or no detection step | Ensure that fluorescence detection takes place during the combined annealing/extension step. |
| e) Problems with starting template | Check the concentration, storage conditions, and quality of the starting template (see Appendix B: General Remarks on Handling RNA on page 46). If necessary, make new serial dilutions of template nucleic acid from the stock solutions. Repeat the PCR using the new dilutions. |
| f) 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 sample. |
| g) Insufficient number of cycles | Increase the number of cycles. |
| h) Reaction volume too high | We recommend a final reaction volume of 10 µl. We recommend using 20 µl final reaction volume on the Rotor-Disc 100. |

Comments and suggestions

- | | |
|--|---|
| i) Generated signals are weak | RNA samples may contain PCR inhibitors. Further purification or an alternative RNA extraction method may be necessary. Check positive controls. |
| j) Wrong detection channel/filter chosen | Ensure that FAM detection channel is activated and that the correct filter set is chosen for the reporter dye. |
| k) No detection activated | Check that fluorescence detection was activated in the cycling program. |
| l) Incorrect temperature for RT reaction | We recommend performing the RT reaction at 40°C. |

Primer dimers and/or nonspecific PCR products

- | | |
|---|---|
| a) Mg ²⁺ concentration adjusted | Do not adjust the Mg ²⁺ concentration in 2x QuantiNova Probe PCR Master Mix. |
| b) Contamination of RNA sample with genomic DNA | Treat the RNA sample with DNase to digest the contaminating genomic DNA. |

Increased fluorescence or C_q value for no-template control (NTC)

- | | |
|--|--|
| a) Contamination of reagents | Discard all the components of the assay (e.g., master mix and primers). Repeat the assay using new components. |
| b) Contamination during reaction setup | Take appropriate precautions during reaction setup, such as using aerosol-barrier pipet tips. |

Varying fluorescence intensity

- | | |
|--|--|
| a) Contamination of real-time cyclers | Decontaminate the real-time cycler according to the manufacturer's instructions. |
| b) Real-time cycler no longer calibrated | Recalibrate the real-time cycler according to the manufacturer's instructions. |

All cycler systems

- | | |
|--|--|
| Wavy curve at high template amounts for highly expressed targets | In the analysis settings, reduce the number of cycles used for background calculation (if your real-time cycler allows you to do so) or reduce the amount of template. |
|--|--|

Applied Biosystems instruments only

- | | |
|---|--|
| ΔRn values unexpectedly too high or too low | In the analysis settings, reduce the number of cycles used for background calculation (if your real-time cycler allows you to do so) or reduce the amount of template. Check that the correct concentration of ROX was used. |
|---|--|

Appendix A: Reaction Setup Using Master Mix Containing a High Concentration of ROX

Note: This appendix and Table 19 are only relevant for setting up reactions using a master mix to which a high concentration of ROX has already been added according to Table 6 on page 21. When using a master mix containing a low concentration of ROX, the volume of ROX added is negligible, and the standard reaction setup as described in the protocols should be used.

Table 19. PCR setup using a master mix that contains a high concentration of ROX dye

Component	miRNA Probe PCR Assay	miRNome PCR Panels (384)	Focus Probe PCR Panel (192 assays in 96-wells or 384-wells plate format) Serum/Plasma	Custom Probe PCR Panel (per Assay)
2x QuantiNova Probe Master Mix with high ROX	5.5 µl*	2200 µl*	1100 µl*	5.5 µl*
10x miRCURY Probe Universal Primer	1 µl	400 µl	200 µl	1 µl
Resuspended PCR assay	1 µl	–	–	–
cDNA template	2 µl (diluted 1:20)	20 µl	20 µl	3 µl (diluted 1:30)
RNase-free water	0.5 µl	1360 µl	680 µl	0.5 µl
Total reaction volume	10 µl	4000 µl	2000 µl	10 µl

* Already contains a 1:20 dilution of ROX Reference Dye instruments requiring a high ROX concentration (i.e., ABI PRISM 7000, Applied Biosystems 7300 and 7900, and StepOne Real-Time PCR Systems).

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.

* 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.

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 CO₂. 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 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.

* 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.
Products for reverse transcription and PCR		
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
miRCURY LNA Probe PCR Kit (200)	2x QuantiNova Probe PCR Master Mix, miRCURY Probe Universal Reverse Primer, ROX Reference Dye, and RNase free Water; for 200 reactions	339371
miRCURY LNA Probe PCR Kit (800)	2x QuantiNova Probe PCR Master Mix, miRCURY Probe Universal Reverse Primer, ROX Reference Dye, and RNase free Water; for 800 reactions	339372
miRCURY LNA Probe PCR Kit (4000)	2x QuantiNova Probe PCR Master Mix, miRCURY Probe Universal Reverse Primer, ROX Reference Dye, and RNase free Water; for 4000 reactions	339373
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

Product	Contents	Cat. no.
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	Pre-designed 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	Pre-designed 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*

Product	Contents	Cat. no.
Related products for miRNA purification		
miRNeasy Serum/ Plasma Advanced Kit (50)	For 50 total RNA preps: 50 RNeasy UCP MinElute® Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	217204
miRNeasy 96 Advanced QIAcube HT Kit (5x96)	For 5x96 total RNA prep	217261
PAXgene Tissue Containers (10)	For collection, fixation, and stabilization of 10 samples: 10 Prefilled Reagent Containers, containing PAXgene Tissue Fix and PAXgene Tissue Stabilizer	765112
PAXgene Blood miRNA Kit (50)	For 50 RNA preps: 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

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

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Document Revision History

Date	Changes
09/2019	Initial release

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