October 2017

miRCURY® LNA® miRNA 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

QIAGEN

Contents

Kit Contents

miRCURY LNA RT Kit	**(8–64)***
Catalog no.	**339340**
Number of standard 20 µl reactions	**32†**
5x miRCURY RT Reaction Buffer†	128 µl
10x miRCURY RT Enzyme Mix, including Mg^{2+}, RT primers and dNTPs	64 µl
UniSp6 RNA Spike-in Template	12 fmol, dried down
Nuclease-free water	1.5 ml
Quick-Start Protocol	1

* 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.

miRCURY LNA SYBR® Green PCR Kit	**(200)**	**(600)**	**(4000)**
Catalog no.	**339345**	**339346**	**339347**
Number of standard 10 µl reactions	**200**	**600**	**4000**
2x miRCURY SYBR Green PCR Master Mix containing the following: miRCURY SYBR Green PCR Buffer, dNTP mix (dATP, dCTP, dGTP, dTTP), QuantiNova® DNA Polymerase composed of: Taq DNA Polymerase, QuantiNova Antibody, QuantiNova Guard	1 ml	3 x 1 ml	2 x 10 ml
ROX™ Reference Dye	250 µl	1 ml	2 x 1 ml
Nuclease-free Water	2 x 1.5ml	2 x 1.5 ml	2 x 1.5 ml
Quick-Start Protocol	1	1	1

miRCURY LNA miRNA PCR Assay	**(200)**
Catalog no.	**339306**
Number of 10 µl reactions	**200**
miRCURY LNA miRNA PCR Primer Mix, dried down	1 vial

miRCURY LNA miRNA PCR Starter Kit	(20)
Catalog no.	339320
Number of 10 µl reactions	20
5x miRCURY Reaction Buffer	128 µl
10x miRCURY RT Enzyme Mix	64 µl
UniSp6 RNA Spike-in Template	12 fmol, dried down
UniSp6 RNA Spike-in Control Assay, v2	200 reactions, dried down
hsa-miR-103a-3p Assay (also works for mmu+rno)	200 reactions, dried down
2 LNA PCR Assays of your choice from stocked primers	2 x 200 reactions
2x miRCURY SYBR Green Master Mix	1 ml
Nuclease-free Water	1 ml

miRCURY LNA miRNome Human PCR Panel I and II (product numbers vary YAHS-312Y-)	
384-well PCR plates containing dried-down LNA PCR Assays for one 10 µl reaction per well	
Panel I	**Panel II**
372 LNA PCR Assays for the amplification of human miRNAs	380 LNA PCR Assays for the amplification of human miRNAs
3 inter-plate calibrators	3 inter-plate calibrators
3 LNA PCR Assays for reference genes*	1 blank well
5 RNA spike-in control PCR assays†	
1 blank well	

* Human Panels and Cancer Focus Panel: three snRNAs (U6snRNA, SNORD38B, SNORD49A).
Mouse/Rat Panels: three snRNAs (U6snRNA, RNU5G, RNU1A1).
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).

miRCURY LNA miRNome Mouse/Rat PCR Panel I and II (product numbers vary YAMR-312Y-) 384-well PCR plates containing dried-down LNA PCR assays for one 10 µl reaction per well	
Panel I	**Panel II**
372 LNA PCR assays for the amplification of mouse and rat miRNAs	380 LNA assays for the amplification of mouse and rat miRNAs
3 inter-plate calibrators	3 inter-plate calibrators
3 LNA PCR assays for reference genes*	1 blank well
5 RNA spike-in control PCR assays†	
1 blank well	

miRCURY LNA miRNA Serum/Plasma Focus PCR Panel (product numbers vary YAHS-106Y-) PCR plates compatible with various real-time PCR instruments containing dried-down LNA PCR assays for one 10 µl reaction per well	
96-well format (2 plates)	**384-well format (2 panels per plate)**
179 LNA PCR assays for the amplification of human miRNAs*	2 x 179 LNA 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 PCR assays†	2 x 5 RNA spike-in control PCR assays†
2 blank wells, 1 in each plate	2 x 2 blank wells

* Human Panels and Cancer Focus Panel: three snRNAs (U6snRNA, SNORD38B, SNORD49A).
Mouse/Rat Panels: three snRNAs (U6snRNA, RNU5G, RNU1A1).
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).

miRCURY LNA miRNA Cancer Focus PCR Panel (product numbers vary YAHS-102Y-) PCR plates compatible with various real-time PCR instruments containing dried-down LNA PCR assays for one 10 µl reaction per well	
96-well format (1 plate)	**384-well format (4 panels per plate)**
84 LNA PCR assays for the amplification of human miRNAs	4 x 84 LNA PCR assays for the amplification of human miRNAs
3 LNA PCR assays for reference genes*	4 x 3 LNA PCR assays for potential reference genes*
3 inter-plate calibrators	4 x 3 inter-plate calibrators
5 RNA spike-in control PCR assays†	4 x 5 RNA spike-in control PCR assays†
1 blank well	4 blank wells

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.

* Human Panels and Cancer Focus Panel: three snRNAs (U6snRNA, SNORD38B, SNORD49A).
Mouse/Rat Panels: three snRNAs (U6snRNA, RNU5G, RNU1A1).
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).

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 SYBR Green PCR Kits are shipped on dry ice. The kits should be stored immediately upon receipt at –15 to –30ºC 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 (see the quality control label inside the kit box or on the kit envelope). miRCURY SYBR Green PCR Master Mix and ROX Reference Dye can also be stored protected from light at 2–8ºC for up to 12 months, depending on the expiration date.

If desired, ROX Reference Dye can be added to 2x miRCURY SYBR Green PCR Master Mix for long-term storage. For details, see "Adding ROX dye to the PCR master mix" on page 26.

The PCR panels and assays are shipped dried down at room temperature. The primers can be stored at 2–8°C or –15 to –30°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 –15 to –30ºC to avoid repeated freeze-thaw cycles.

Intended Use

The miRCURY LNA RT Kit, the miRCURY SYBR Green PCR Kit and miRCURY miRNA 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 SYBR Green PCR Kit and miRCURY LNA 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: HotStarRT-Script Reverse Transcriptase (HotStarRT-Script Reverse Transcriptase is a modified form of a recombinant 77 kDa reverse transcriptase.) *E. coli* Poly(A) Polymerase
5x miRCURY RT Reaction Buffer	Buffer optimized for Poly(A) polymerization and reverse transcription; contains universal reverse transcription primer, 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 SYBR Green PCR Kit components.

Component	Description
2x miRCURY SYBR Green PCR Master Mix, containing:	
QuantiNova DNA Polymerase	QuantiNova DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from *Thermus aquaticus*. 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, 95ºC incubation step.
miRCURY SYBR Green PCR Buffer	Contains Tris-HCl, KCl, NH_4SO_4, $MgCl_2$ and additives including Q-Bond® that enable fast cycling
dNTP mix	Contains dATP, dCTP, dGTP and dTTP of ultrapure quality.
Other kit components:	
ROX Reference Dye	Optimized concentration of fluorescent dye for normalization of fluorescent signals on all instruments from Applied Biosystems®.
Nuclease-free water	Ultrapure quality, PCR-grade water

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

Component	Description
10x miRCURY Primer Assay	Two miRNA-specific primers

Introduction

The miRCURY LNA miRNA PCR System is an miRNA-specific, LNA-based system designed for sensitive and accurate detection of miRNA by quantitative, real-time PCR using SYBR Green. The method is based on universal reverse transcription (RT), followed by real-time PCR amplification with LNA-enhanced primers. 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 SYBR Green PCR Kit (cat. nos. 339345, 339346, 339347)
- miRCURY LNA miRNA PCR primers, available as individual assays and reference genes and pre-defined or customized PCR panels:
  - miRCURY LNA miRNA PCR Assay (cat. no. 339306)
  - miRCURY LNA miRNA Custom PCR Assay (cat. no. 339317)
  - miRCURY LNA miRNA Custom Bulk Plate (cat. no. 339319)
  - miRCURY LNA miRNA Custom PCR Panel (cat. nos. 339330, 339332)
  - miRCURY LNA miRNA miRNome PCR Panels: Human, Mouse/Rat (cat. no. 339322)
  - miRCURY LNA miRNA Focus PCR Panels: Cancer, Serum/Plasma, Urine Exosome, CSF Exosome (cat. no. 339325)

All PCR panels are ready-to-use and include primers for one 10 µl PCR reaction per well.

Principle and workflow

The miRCURY LNA miRNA 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 real-time miRNA PCR assays. This saves precious sample, reduces technical variation, consumes less reagents and saves time in the laboratory.
- **LNA PCR amplification**: Both PCR amplification primers (forward and reverse) are miRNA specific and optimized with LNA. The result is exceptional sensitivity and extremely low background, enabling accurate quantification of very low levels of miRNA. The highly specific assays allow discrimination between closely related miRNA sequences. The miRCURY LNA miRNA PCR System and the outstanding performance of the miRCURY SYBR Green 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.

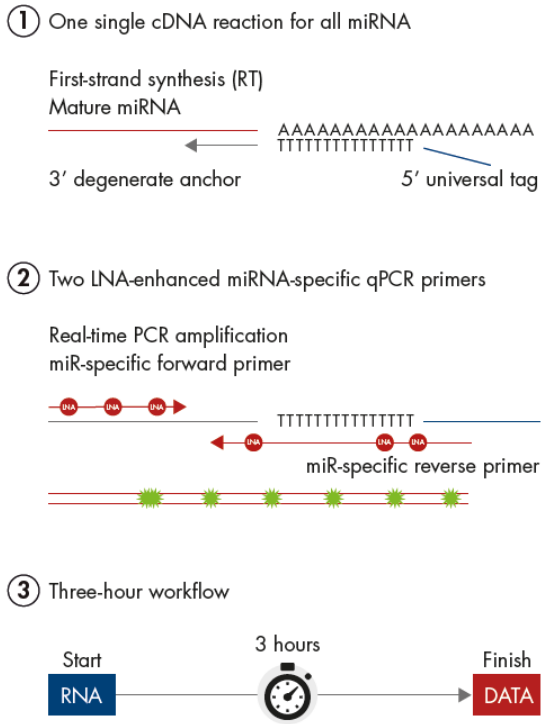

Figure 1. Schematic outline of the miRCURY LNA miRNA PCR System. A poly(A) tail is added to the mature miRNA template (step 1A). cDNA is synthesized using a poly(T) primer with a 3′ degenerate anchor and a 5′ universal tag (step 1B). The cDNA template is then amplified using miRNA-specific and LNA-enhanced forward and reverse primers (step 2A). SYBR Green is used for detection (step 2B). (figure rebranding 159-161)

The miRCURY SYBR Green 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 2–8ºC. 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 42°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. For this reason, we recommend adding carrier RNA during the purification procedure and subsequently using RNA amounts based on starting sample volume, rather than RNA concentration.

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 µl, 4 µl and 6 µl per 20 µL 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® Mini Kit*	217004	Animal/human tissues and cells
miRNeasy Serum/Plasma Advanced Kit	217204	Serum/plasma samples
miRNeasy FFPE Kit	217504	Formalin-fixed paraffin-embedded (FFPE) tissue samples
miRNeasy 96 Kit*	217061	Animal/human tissues and cells
PAXgene® Tissue miRNA Kit	766134	Animal/human tissues that have been fixed and stabilized in PAXgene Tissue Containers
PAXgene Blood miRNA Kit	763134	Human blood that has been stabilized in PAXgene Blood RNA Tubes

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

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 PCR Panels and the miRCURY SYBR Green PCR Kit. To profile mature miRNA expression, a premix of cDNA, SYBR Green PCR Master Mix and RNase-free water is added to the miRCURY LNA miRNA

PCR Panel. There is no need to set up the reactions on ice. Furthermore, the whole reaction can be left for up to 2 h at room temperature (15–25ºC) without any loss of performance.

2x miRCURY SYBR Green PCR Master Mix

The components of the 2x miRCURY SYBR Green PCR Master Mix include QuantiNova DNA Polymerase, 2x miRCURY SYBR Green PCR Buffer and SYBR Green I. This optimized master mix ensures fast, real-time PCR amplification with high specificity and sensitivity.

The 2x miRCURY SYBR Green PCR Buffer contains an optimized concentration of the fluorescent dye SYBR Green I, which binds all double-stranded DNA molecules and emits a fluorescent signal upon binding. The 2x miRCURY SYBR Green PCR Master Mix can be stored at 2–8°C or –15 to –30°C without loss of SYBR Green I fluorescence activity. The excitation and emission maxima of SYBR Green I are 494 nm and 521 nm, respectively, which makes the dye compatible with any real-time cycler.

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 H at room temperature (15–25ºC) without impairing the performance of the subsequent reaction.

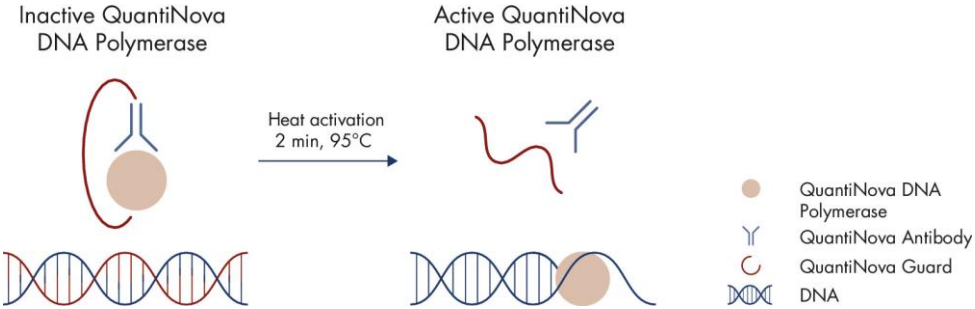

Figure 2. Principle of the novel QuantiNova hot-start mechanism. At ambient temperature, the QuantiNova DNA Polymerase is kept inactive by QuantiNova Antibody and QuantiNova Guard, until the initial heat activation step.

Built-in visual control for correct pipetting

The 2x miRCURY SYBR Green PCR Master Mix included in the miRCURY LNA SYBR Green 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 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 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 Human and Mouse/Rat miRNome PCR Panels and the Focus PCR Panels contain the inter-plate calibrator assay, which is annotated as UniSp3 IPC in the plate layout files. Depending on the plate layout, the Custom 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 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 µl synthetic spike-in (10^8 copies/µl) 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 PCR Panel plates contain the matching assays for these spike-ins. For Custom 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 PCR Array and Panel data can be analyzed using the free miRNA PCR Array Data Analysis tool, which is available in GeneGlobe at:
http://www.qiagen.com/shop/genes-and-pathways/data-analysis-center-overview-page.
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 SYBR Green dye.

The use of ROX dye is necessary for instruments from Applied Biosystems. The miRCURY SYBR Green 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 miRCURY SYBR Green PCR Master Mix for long-term storage (Table 6). For details, see "Adding ROX dye to the PCR master mix" on page 26.

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 SYBR Green PCR Kit, you can add ROX Reference Dye to the 2x miRCURY SYBR Green 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 53.

Table 6. Addition of ROX Reference Dye to master mix.

Volume of 2x miRCURY SYBR Green 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 µl 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 29
- Protocol: Quantitative, Real-Time PCR Using Individual miRCURY LNA miRNA PCR Assays, page 33
- Protocol: Quantitative, Real-Time PCR Using miRCURY LNA miRNome PCR Panels or Serum/Plasma Focus PCR Panels, page 36
- Protocol: Quantitative, Real-Time PCR Using Other miRCURY LNA miRNA Focus PCR Panels, page 40
- Protocol: Quantitative, Real-Time PCR Using miRCURY LNA miRNA Custom PCR Panels, page 44

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

PCR assays or panel	miRNA PCR Assays (<96)	miRNome PCR Panels		Custom PCR Panels, Focus PCR Panels	
		Panel I	Panel I+II	1–96 miRNAs	97–192 miRNAs
RT reaction volume	10 µl	40 µl	80 µl	10 µl	20 µl
Number of reactions possible with the miRCURY LNA RT Kit	64	16	8	64	32
Dilution of cDNA for qPCR	1:30	Use undiluted	Use undiluted	1:40	1:40

These protocols are for analysis of miRNA from serum, plasma and other biofluids. For other sample types, refer to the *miRCURY LNA miRNA 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 54.

Things to do before starting

- Thaw template RNA and 5x miRCURY RT 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.
- 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 –15 to –30ºC.
- 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).

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 PCR Assay	miRNome PCR Panels: Human or Mouse/Rat Focus PCR Panel: Serum/Plasma			Other Focus PCR Panels	Custom PCR Panel: <100 miRNAs analyzed per sample	Custom PCR Panel: >100 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 Reaction Buffer	2 µl	4 µl	8 µl	16 µl	2 µl	2 µl	4 µl
RNase-free water	4.5 µl	9 µl	18 µl	36 µl	4.5 µl	4.5 µl	9 µl
10x miRCURY RT Enzyme Mix	1 µl	2 µl	4 µl	8 µl	1 µl	1 µl	2 µl
UniSp6 RNA spike-in (optional)	0.5 µl	1 µl	2 µl	4 µl	0.5 µl	0.5 µl	1 µl
Template RNA	2 µl*	4 µl*	8 µl*	16 µl*	2 µl*	2 µl*	4 µl*
Total reaction volume	**10 µl**	**20 µl**	**40 µl**	**80 µl**	**10 µl**	**10 µl**	**20 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.

3. Incubate for 60 min at 42ºC.
4. Incubate for 5 min at 95ºC 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	42°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 PCR Assay or Panel to be used.

   Note: If you do not plan to use it immediately, store the cDNA undiluted at 2–8ºC for up to 4 days or at –15 to –30ºC 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 SYBR Green 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 PCR Assays

This protocol is for use with the miRCURY SYBR Green PCR Kit (cat. nos. 339345, 339346, 339347) and miRCURY LNA miRNA PCR Assays (cat. no. 339306) 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, 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 miRCURY SYBR Green 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.

Things to do before starting

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

Procedure

1. Dilute the cDNA 1:30 by adding 290 µl RNase-free water to the 10 µl RT reaction immediately before use. We do not recommend storing this 1:30 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 cycler.

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

Component	Volume
2x miRCURY SYBR Green Master Mix	5 µl
ROX Reference Dye (ABI instruments only)	0.5 µl / 0.05 µl*
PCR primer mix	1 µl
cDNA template	3 µl (Diluted 1:30)
RNase-free water	1 µl*
Total reaction volume	**10 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.

† The total reaction volume is for a single reaction. Calculate the volume required for multiple reactions, depending on your plate layout.

3. Mix the reactions thoroughly and dispense 10 µl into PCR tubes or PCR plate wells.

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

4. Briefly centrifuge the tubes or plate at room temperature (15–25ºC).

5. Program the real-time cycler according to Table 11.

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

6. 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
PCR initial heat activation	2 min	95°C	Maximal/fast mode
2-step cycling:			
Denaturation	10 s	95°C	Maximal/fast mode
Combined annealing/extension	60 s	56°C	Maximal/fast mode
Number of cycles	40*		
Melting curve analysis		60–95°C	

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

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). If you are using an ABI instrument, please note that it is not recommended to use auto C_T settings. If you are using a Roche LC480 instrument, we recommend analysis using the 2nd derivative method.

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

This protocol is for use with the miRCURY SYBR Green PCR Kit (cat. nos. 339345, 339346, 339347) 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 Human PCR Panels (product numbers vary YAHS-312Y-, YAHS-301Y-)
- miRCURY LNA miRNome Mouse/Rat PCR Panels (product numbers vary YAMR-312Y-. YAMR-301Y-)
- miRCURY LNA miRNA Serum/Plasma Focus PCR Panel (product numbers vary YAHS-106Y-)

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, 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 miRCURY SYBR Green 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 miRCURY SYBR Green Master Mix, template cDNA, miRNA PCR panel(s), ROX Reference Dye (if required) and RNase-free water. Vortex and briefly centrifuge.

Procedure

1. Before removing the seal from the PCR panel plate, briefly centrifuge the plate(s) to ensure that all material is in the bottom of the wells.
2. Prepare a reaction mix according to Table 12. 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 12. Reaction setup for miRCURY LNA miRNome PCR Panels and Serum/Plasma Focus PCR Panels.

Component	Serum/Plasma Focus PCR Panel		miRNome PCR Panels: Human and Mouse/Rat	
	2x 96 well	384 well	Panel I	Panels I+II
2x miRCURY SYBR Green Master Mix	1000 µl	2000 µl	2000 µl	4000 µl
ROX Reference Dye (ABI instruments only)	100 µl/10 µl*	200 µl/20 µl*	200 µl/20 µl*	400 µl/40 µl*
cDNA template (undiluted)	20 µl	40 µl	40 µl	80 µl
RNase-free water	980 µl*	1960 µl*	1960 µl*	3920 µl*
Total reaction volume	**2000 µl**	**4000 µl**	**4000 µl**	**8000 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.

3. Mix the reaction mix thoroughly and dispense 10 µl per well into the plates containing the miRNome PCR Panels.

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

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

5. Program the real-time cycler according to Table 13.

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

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

Table 13. PCR cycling conditions.

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

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

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). If you are using an ABI instrument, please note that it is not recommended to use auto C_T settings. If you are using a Roche LC480 instrument, we recommend analysis using the 2nd derivative method.

Protocol: Quantitative, Real-Time PCR Using Other miRCURY LNA miRNA Focus PCR Panels

This protocol is for use with the miRCURY SYBR Green PCR Kit 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 Cancer Focus PCR Panel (product numbers vary YAHS-102Y-)
- miRCURY LNA miRNA Urine Exosomes Focus PCR Panel (product numbers vary YAHS-123Y-)
- miRCURY LNA miRNA CSF Exosomes Focus PCR Panel (product numbers vary YAHS-124Y-)

If you are using the Serum/Plasma Focus PCR Panels, use Protocol: Quantitative, Real-Time PCR Using miRCURY LNA miRNome PCR Panels or Serum/Plasma Focus PCR Panels on page 36.

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, 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 miRCURY SYBR Green 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 miRCURY SYBR Green Master Mix, template cDNA, PCR panels, ROX Reference Dye (if required) and RNase-free water. Vortex and briefly centrifuge.

Procedure

1. Before removing the seal from the PCR panel plate, briefly centrifuge the plate(s) to ensure that all material is in the bottom of the wells.
2. Prepare a reaction mix according to Table 14. 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 14. Reaction setup for miRCURY LNA miRNA Focus PCR Panels other than Serum/Plasma Focus PCR Panels.

Component	Focus panel consisting of 1 x 96 assays	Focus panel consisting of 1 x 384 assays
2x miRCURY SYBR Green Master Mix	500 µl	2000 µl
ROX Reference Dye (ABI instruments only)	50 µl/5 µl*	200 µl/20 µl*
cDNA template (undiluted)	10 µl	40 µl
RNase-free water	490 µl*	1960 µl*
Total reaction volume	**1000 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.

3. Mix the reaction mix thoroughly and dispense 10 µl per well into the PCR Panel plate(s).

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

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

5. Program the real-time cycler according to Table 15.

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

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

Table 15. PCR cycling conditions.

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

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

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). If you are using an ABI instrument, please note that it is not recommended to use auto C_T settings. If you are using a Roche LC480 instrument, we recommend analysis using the 2nd derivative method.

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

This protocol is for use with the miRCURY SYBR Green PCR Kit (cat. nos. 339345, 339346, 339347) 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 PCR Panel (cat. nos. 339330, 339332)

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, 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 miRCURY SYBR Green 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 miRCURY SYBR Green Master Mix, template cDNA, PCR panels, ROX Reference Dye (if required) and RNase-free water. Vortex and briefly centrifuge.

Procedure

1. Before removing the seal from the PCR panel plate, briefly centrifuge the plate(s) to ensure that all material is in the bottom of the wells.
2. Prepare cDNA dilutions according to Table 16.

Table 16. cDNA dilution for miRCURY LNA miRNA Custom 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 PCR Panel configuration	Suggested cDNA dilution (cDNA + nuclease-free water, in µl)	Volume of diluted cDNA needed for each Custom PCR Panel plate
8 x 10 (12)	2 + 78	48 µl
4 x 22 (24)	3 + 117	96 µl
1 x 92 (96)	10 + 390	384 µl
16 x 22 (24)	3 + 117	96 µl
8 x 46 (48)	6 + 234	192 µl
4 x 94 (96)	10 + 390	384 µl
1 x 380 (384)	40 + 1560	1536 µl

3. Prepare a reaction mix according to Table 17. 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 17. Reaction setup per sample for miRCURY LNA miRNA Custom PCR Panels.

Component	Volume per reaction
2x miRCURY SYBR Green Master Mix	5 µl
ROX Reference Dye (ABI instruments only)	0.5 µl/0.05 µl*
cDNA template (diluted 1:40)	4 µl
RNase-free water	1 µl*
Total reaction volume	**10 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.

† The volume shown is for a single reaction. Calculate the total volume needed for your particular plate layout.

4. Mix the reaction mix thoroughly and dispense 10 µl per well into the PCR Panel plate(s).

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

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

6. Program the real-time cycler according to Table 18.

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

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

Table 18. PCR cycling conditions.

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

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

8. 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). If you are using an ABI instrument, please note that it is not recommended to use auto C_T settings. If you are using a Roche LC480 instrument, we recommend analysis using the 2nd derivative method.

Analysis and Interpretation of Inter-Plate Calibrators

Three wells within the pre-defined miRNome (Human and Mouse/Rat) and Focus (Cancer, Urine Exosome, CSF Exosome) PCR Panels contain the inter-plate calibrator assay, annotated as UniSp3 IPC in the plate layout files. Depending on the particular plate layout, the Custom 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 19. Calibrate each plate by subtracting the calibration factor from all C_q values in the plate.

Table 19. 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 such 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. 5S ribosomal RNA is another popular option, 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/FAQ/FAQList.aspx**. 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 (95ºC 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 95ºC) 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 54). 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.
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.

		Comments and suggestions
j)	Wrong detection channel/filter chosen	Ensure that SYBR Green 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 42°C.
Primer dimers and/or nonspecific PCR products		
a)	Mg^{2+} concentration adjusted	Do not adjust the Mg^{2+} concentration in 2x miRCURY SYBR Green 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.
High fluorescence in no-template control		
Contamination of RNA sample with genomic DNA		Design primers that span exon–exon boundaries, so that only cDNA targets can be amplified and detected. Treat the RNA sample with DNase to digest the contaminating genomic DNA.
Varying fluorescence intensity		
a)	Contamination of real-time cycler	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 20 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 26. 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 20. PCR setup using a master mix that contains a high concentration of ROX dye.

Component	miRNA PCR Assay	miRNome PCR Panels: Human, Mouse/Rat	Focus PCR Panel: Serum/ Plasma	Focus PCR Panel: Cancer	Custom PCR Panel
2x miRCURY SYBR Green Master Mix*	5.5 µl*	2200 µl *	1100 µl*	550 µl *	5.5 µl *
PCR primer mix	1 µl	–	–	–	–
cDNA template	3 µl (diluted 1:60)	20 µl	20 µl	5 µl	4 µl (diluted 1:80)
RNase-free water	0.5 µl	1780 µl	880 µl	445 µl	0.5 µl
Total reaction volume	**10 µl**	**4000 µl**	**2000 µl**	**1000 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, 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.

Glassware

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

Solutions

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

* 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.

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.

Ordering Information

Product	Contents	Cat. no.
Products for reverse transcription and PCR		
miRCURY LNA RT Kit	5x RT Reaction Buffer, 10x RT Enzyme Mix, UniSp6, RNA Spike-in template, RNase-free water; for 8–64 reactions	339340
miRCURY LNA miRNA PCR Starter Kit	2 miRCURY LNA PCR Assays of your choice, UniSp6 Spike-in control assay, miR-103-3p endogenous control assay, 5x RT Reaction Buffer, 10x RT Enzyme Mix, UniSp6 RNA Spike-in template, RNase-free water, 2x miRCURY SYBR Green Master Mix; for 20 RT reactions and 100 PCR amplifications	339320
miRCURY LNA SYBR Green PCR Kit (200)	2x miRCURY SYBR Green PCR Master Mix, miRCURY SYBR Green PCR Buffer and dNTP mix (dATP, dCTP, dGTP, dTTP), ROX Reference Dye, Nuclease-free Water; for 200 reactions	339345
miRCURY LNA SYBR Green PCR Kit (600)	2x miRCURY SYBR Green PCR Master Mix, miRCURY SYBR Green PCR Buffer and dNTP mix (dATP, dCTP, dGTP, dTTP), ROX Reference Dye, Nuclease-free Water; for 600 reactions	339346
miRCURY LNA SYBR Green PCR Kit (4000)	2x miRCURY SYBR Green PCR Master Mix, miRCURY SYBR Green PCR Buffer and dNTP mix (dATP, dCTP, dGTP, dTTP), ROX Reference Dye, Nuclease-free Water; for 4000 reactions	339347

Product	Contents	Cat. no.
RNA Spike-in Kit, for RT	UniSp2, UniSp4, UniSp5 RNA Spike-in template mix, cel-miR-39-3p RNA Spike-in template; for 50 reactions	339390
PCR assays and panels		
miRCURY LNA miRNA PCR Assay	LNA-optimized PCR assay for miRNA quantification; for 200 reactions	339306
miRCURY LNA miRNA Custom PCR Assay	Custom-designed and LNA-optimized PCR assay for miRNA quantification; for 200 reactions	339317
miRCURY LNA miRNA Custom Bulk Plate (200)	Custom-configured plate of LNA-optimized PCR assays for miRNA quantification; 96-well format; for 200 reactions per well	339319
miRCURY LNA miRNA Custom 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	339330
miRCURY LNA miRNA miRNome PCR Panels	Pre-made panels of LNA assays for miRNome profiling; for one 10 µl qPCR reaction per well; 96-well or 384-well format	339322
miRCURY LNA miRNA Focus PCR Panels	Pre-made panel of LNA assays focused on disease, pathway or sample type; for one 10 µl qPCR reaction per well; 96-well or 384-well format	339325
miRCURY LNA miRNA QC PCR Panel	Pre-made panel of LNA assays for miRNA quality control; for one 10 µl qPCR reaction per well; 96-well or 384-well format	339331

Product	Contents	Cat. no.
Related products for miRNA purification		
miRNeasy Mini Kit (50)	For 50 preps: 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)	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 Micro Kit (50)	For 50 total RNA preps: 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)	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 miRNA Kit (50)	For 50 RNA preps: 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 Containers (10)	For collection, fixation, and stabilization of 10 samples: 10 Prefilled Reagent Containers, containing PAXgene Tissue Fix and PAXgene Tissue Stabilizer	765112

Product	Contents	Cat. no.
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

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Notes

Notes

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