

October 2019

miRCURY[®] LNA[®] miRNA SYBR[®] Green PCR Handbook

For highly sensitive, real-time RT-PCR detection of
miRNAs using SYBR[®] Green*

* For biofluid samples, please see the *miRCURY LNA miRNA
SYBR[®] Green PCR – Exosomes, Serum/Plasma, and Other
Biofluid Samples Handbook*.

Contents

Kit Contents.....	4
Storage	8
Intended Use	8
Safety Information	9
Quality Control.....	9
Product Information	10
Introduction.....	11
Principle and workflow	12
LNA technology	14
Important Notes.....	16
Poly(A) tailing and reverse transcription	16
RNA input amount	16
Template RNA requirements	16
Mature miRNA expression profiling	17
2x miRCURY SYBR® Green PCR Master Mix	18
Built-in visual control for correct pipetting	19
Control assays	19
Reference assays and reference candidates	20
Interplate calibrators	20
RNA spike-ins (synthetic control templates)	21
GeneGlobe® analysis tool	22
Passive reference dye	22
Adding ROX dye to the PCR master mix	23

Equipment and Reagents to Be Supplied by User	24
Protocol Overview	25
Protocol: First-Strand cDNA Synthesis	26
Protocol: Quantitative, Real-Time PCR Using Individual miRCURY LNA miRNA PCR Assays .	30
Protocol: Quantitative, Real-Time PCR Using miRCURY LNA miRNome PCR Panels	33
Protocol: Quantitative, Real-Time PCR Using miRCURY LNA miRNA Focus PCR Panels	36
Protocol: Quantitative, Real-Time PCR Using miRCURY LNA miRNA Custom PCR Panels ..	39
Analysis and Interpretation of Interplate Calibrators	43
Normalization of miRNA Quantitative PCR Experiments	44
Troubleshooting Guide	46
Appendix A: Reaction Setup Using Master Mix Containing a High Concentration of ROX ..	48
Appendix B: General Remarks on Handling RNA	49
Ordering Information	51
Handbook Revision History	55

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
Nuclease-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 – 5 µg total RNA. The 5x miRCURY RT Reaction Buffers are not interchangeable between the SYBR® Green- and the Probe-based workflows.

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, and dTTP), and 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 1.5 ml	2 x 1.5 ml	2 x 1.5 ml

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	64
5x miRCURY RT SYBR® Green Reaction Buffer	128 µl
10x miRCURY RT Enzyme Mix	64 µl
UniSp6 RNA Spike-in Template, dried down	12 fmol
UniSp6 RNA Spike-in Control PCR Assay, v2, dried down	200 reactions
hsa-miR-103a-3p PCR Assay (also works for mmu+rno), dried down	200 reactions
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 miRNA miRNome PCR Panels Human panel I + II (cat. no. 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 (distributed on 384-well plates)	380 LNA PCR assays for the amplification of human miRNAs (distributed on 384-well plates)
3 interplate calibrators	3 interplate calibrators
3 LNA PCR assays for reference genes†	1 blank well
5 RNA spike-in control assays‡	
1 blank well	

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

† 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 as 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 miRNome Mouse & Rat PCR Panels I + II (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 PCR assays for the amplification of mouse and rat miRNAs
3 x 1 interplate calibrators	3 x 1 interplate calibrators
3 LNA PCR assays for reference genes†	1 blank well
5 RNA spike-in control PCR assays‡	
1 blank well	

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

† 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 as 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 Focus PCR Panel: Human Serum/Plasma (cat. no. 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 interplate calibrators	2 x 6 interplate calibrators
5 RNA spike-in control assays‡	2 x 5 RNA spike-in control assays‡
2 blank wells, 1 in each plate	2 x 2 blank wells

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

† Human Panels and Cancer Focus Panel: three snRNAs (U6snRNA, SNORD38B, SNORD49A).

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).

Refer to the *miRCURY LNA miRNA SYBR® Green PCR – Exosomes, Serum/Plasma and Other Biofluid Samples Handbook* for protocols specifically for use with the miRCURY LNA miRNA Serum/Plasma Focus PCR Panel.

miRCURY LNA miRNA Focus PCR Panel: Human Cancer (cat. no. 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 assays for reference genes [†]	4 x 3 assays for reference genes [†]
3 interplate calibrators	4 x 3 interplate calibrators
5 RNA spike-in control assays [‡]	4 x 5 RNA spike-in control assays [‡]
1 blank well	4 blank wells

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

[†] Human Panels and Cancer Focus Panel: three snRNAs (U6snRNA, SNORD38B, SNORD49A).

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 SYBR[®] Green 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. miRCURY SYBR[®] Green PCR Master Mix and ROX Reference Dye can also be stored protected from light at 2 – 8°C 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 23.

The PCR panels and assays are shipped dried down at room temperature (15 – 25°C). The primers can be stored at 2 – 8°C or at -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 SYBR[®] Green PCR Kit and miRCURY LNA 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 LNA 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 Mix	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"> ● HotStarRT-Script Reverse Transcriptase (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 a universal reverse transcription primer for the Probe-based workflow, Mg ²⁺ , and dNTPs.
UniSp6 RNA Spike-in Template, dried down	Synthetic transcript for monitoring successful reverse transcription
Nuclease-free water	Ultrapure quality, PCR-grade water

Table 2. Descriptions of miRCURY LNA SYBR® Green PCR Kit components

Component	Description
2x miRCURY SYBR® Green PCR Master Mix, containing:	
QuantiNova DNA Polymerase	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
miRCURY SYBR® Green PCR Buffer	Contains Tris-HCl, KCl, NH ₄ SO ₄ , MgCl ₂ and additives, including Q-Bond®.
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
miRCURY LNA miRNA PCR Primer Mix, dried down	Two miRNA-specific primers

Introduction

The miRCURY LNA miRNA SYBR® Green 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 SYBR® Green PCR portfolio is comprised of four types of reagent kits:

- miRCURY LNA RT Kit (cat. no. 339340)
- RNA Spike-in Kit, for RT (cat. no. 339390)
- miRCURY LNA SYBR® Green PCR Kit (cat. nos. 339345, 339346, and 339347)
- miRCURY LNA miRNA PCR primers, available as individual assays and reference genes and predefined 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. The recommended reaction volume for the Rotor-Gene Q is 20 µl PCR reaction per well.

In addition to the SYBR® Green detection system, an alternative Probe detection system is available. The miRCURY LNA RT Kit is the same for both, but the PCR kit, assays, and panels are specific for each system and cannot be interchanged.

Principle and workflow

The miRCURY LNA miRNA SYBR® Green 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, page 13):

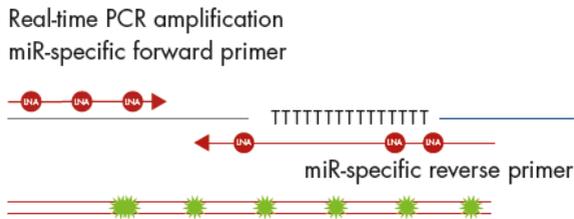
- **Universal RT:** One first-strand cDNA synthesis reaction provides the template for all SYBR® Green-based real-time miRNA PCR assays. This saves precious sample, reduces technical variation, consumes less reagents, and saves time in the laboratory.
- **LNA PCR amplification:** Both PCR amplification 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 SYBR® Green PCR System and the outstanding performance of the miRCURY LNA SYBR® Green PCR Kit offer solutions for both high-throughput miRNA expression profiling and for quantification of individual miRNAs.

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.

① One single cDNA reaction for all miRNA



② Two LNA-enhanced miRNA-specific qPCR primers



③ Three-hour workflow



Figure 1. Schematic outline of the miRCURY LNA miRNA SYBR® Green PCR System. In the cDNA synthesis, a poly(A) tail is added to the mature miRNA template, and cDNA is synthesized using a poly(T) primer with a 3' degenerate anchor and a 5' universal tag (step 1). The cDNA template is then amplified using miRNA-specific and LNA-enhanced forward and reverse primers, and SYBR® Green is used for detection (step 2). The workflow only takes 3 hours to complete.

The miRCURY LNA 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 2–8°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, noncoding RNAs that mediate post-transcriptional gene regulation. Unlike mRNAs, miRNAs are not polyadenylated in nature. Mature miRNAs can be polyadenylated by poly(A) polymerase and reverse transcribed into cDNA using oligo-dT primers. Polyadenylation and reverse transcription are performed in parallel in the same tube. The 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

The miRCURY LNA miRNA PCR protocol is optimized for use of 20 ng total RNA per 20 µl cDNA synthesis reaction. The exact amount of total RNA needed depends on whether the downstream application uses individual assays or panels. The amount of total RNA to be used may also vary depending on the miRNA expression levels in the cells or tissue to be analyzed. For highly expressed miRNAs, it is possible to use down to 10 pg total RNA as starting material. For weakly expressed miRNAs, it may be possible to use up to 200 ng of total RNA; however, in samples with high amounts of PCR inhibitors (e.g., FFPE tissue samples), this may not be feasible. Furthermore, inhibitors may be present in RNA preparations from certain samples (e.g., serum and plasma). Prior to conducting a larger miRNA profiling study, we 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.

Template RNA requirements

Total RNA containing miRNA is the starting material required for the miRCURY LNA miRNA SYBR® Green PCR System. It is not necessary to enrich for small RNAs. 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® Micro Kit*	217084	Animal/human tissues and cells
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
exoRNeasy Serum/Plasma Midi Kit*	77044	Serum/plasma samples
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 recommend 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.

Blood serum and plasma are particular sample types that require special RNA purification procedures, and usually, the amount of RNA present in the samples cannot be accurately determined. Due to the low levels of miRNAs and potentially high levels of inhibitors in samples derived from serum and plasma, we make specific recommendations for setting up experiments using these types of samples. Refer to the *miRCURY LNA miRNA SYBR® Green PCR – Exosomes, Serum/Plasma, and Other Biofluid Samples Handbook* for details.

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 LNA 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 hours at room temperature 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 at –30 to –15°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 hours at room temperature without impairing the performance of the subsequent reaction.

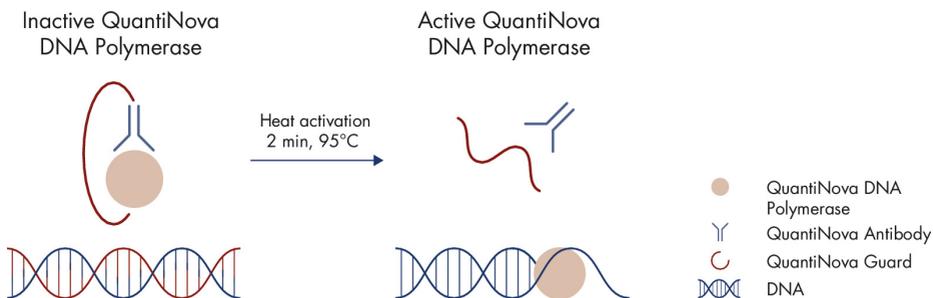


Figure 2. Principle of the novel QuantiNova hot-start mechanism. At ambient temperature, the QuantiNova DNA Polymerase is kept inactive by QuantiNova Antibody and QuantiNova Guard until the initial heat activation step.

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
- Interplate 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 are frequently found to be stably expressed across different cells or tissues. Reference assays may therefore be candidate assays for normalization in a profiling study with several samples. 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 subcellular locations. Finally, certain samples, such as blood plasma, do not contain 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.

Interplate calibrators

Three wells within the predefined Human and Mouse & Rat miRNome PCR Panels and the Focus PCR Panels contain the interplate 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 interplate calibrators. Each of these wells contains a prealiquoted 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 interplate 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 interplate 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 interplate 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 interplate calibrator, as described above. To use the UniSp6 RNA spike-in as an interplate 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 interplate 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 predefined PCR panel plates contain the matching PCR 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 www.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, because 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 LNA 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 23.

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 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, see Table 5. To set up reactions with master mix that already contains a high concentration of added ROX Reference Dye, see Appendix A (page 48).

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 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 26
- Protocol: Quantitative, Real-Time PCR Using Individual miRCURY LNA miRNA PCR Assays, page 30
- Protocol: Quantitative, Real-Time PCR Using miRCURY LNA miRNome PCR Panels, page 33
- Protocol: Quantitative, Real-Time PCR Using miRCURY LNA miRNA Focus PCR Panels, page 36
- Protocol: Quantitative, Real-Time PCR Using miRCURY LNA miRNA Custom PCR Panels, page 39

Table 7. Protocol overview for analyzing miRNA from tissue and cell samples

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

For analysis of miRNA from serum, plasma, and other biofluids, or for instructions for the miRCURY LNA miRNA Serum/Plasma Focus PCR Panels, refer to the specific recommendations in the *miRCURY LNA miRNA SYBR® Green PCR – Exosomes, Serum/Plasma and Other Biofluid Samples Handbook*.

Protocol: First-Strand cDNA Synthesis

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

Important points before starting

- For reverse transcription of miRNA from serum, plasma and other biofluids, refer to the specific recommendations in the *miRCURY LNA miRNA SYBR® Green PCR – Exosomes, Serum/Plasma and Other Biofluid Samples Handbook*.
- This protocol is optimized for use with 20 ng RNA in a 20 µl reverse-transcription reaction. The exact amount of total RNA needed depends on whether the downstream application uses individual assays or panels. Furthermore, the amount of total RNA to be used may also vary depending on the miRNA expression levels in the cells or tissue to be analyzed. For highly expressed miRNAs, it is possible to use down to 10 pg total RNA as starting material. For weakly expressed miRNAs, it may be possible to use up to 200 ng total RNA; however, in samples with high amounts of PCR inhibitors (e.g., FFPE tissue samples), this may not be feasible. Inhibitors may be present in RNA preparations from certain samples (e.g., serum and plasma). We recommend that you optimize the amount of input RNA for the RT reaction.
- 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 SYBR® Green 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 μ l 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 min.
- The temperature steps can be conveniently set up using the cycling protocol described in Table 9, page 28.
- If working with RNA for the first time, read Appendix B, page 49.

Things to do before starting

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

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

- Resuspend the UniSp6 RNA spike-in by adding 80 μ l 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 then place on ice. Briefly centrifuge to collect residual liquid from the sides of the tubes, and then keep on ice.

Procedure

1. Dilute each template RNA sample to 5 ng/ μ l using nuclease-free water.
2. Prepare the reverse transcription reactions on ice according to Table 8 (page 28). 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, Mouse & Rat (Panel I)	miRNome PCR Panels : Human, Mouse & Rat (Panel I+II)	Focus PCR Panel: Cancer (1 x 96 assays)	Custom PCR Panel: ≤192 miRNAs analyzed per sample	Custom PCR Panel: 193–384 miRNAs analyzed per sample
5x miRCURY SYBR® Green RT Reaction Buffer	2 µl	4 µl	8 µl	2 µl	2 µl	4 µl
RNase-free water	4.5 µl	9 µl	18 µl	4.5 µl	4.5 µl	9 µl
10x miRCURY RT Enzyme Mix	1 µl	2 µl	4 µl	1 µl	1 µl	2 µl
UniSp6 RNA spike-in (optional)	0.5 µl	1 µl	2 µl	0.5 µl	0.5 µl	1 µl
Template RNA (5 ng/µl)	2 µl	4 µl	8 µl	2 µl	2 µl	4 µl
Total reaction volume	10 µl*	20 µl*	40 µl*	10 µl*	10 µl*	20 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 42°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	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 the cDNA immediately, store it 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 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 LNA SYBR® Green PCR Kit (cat. nos. 339345, 339346, and 339347) and the miRCURY LNA miRNA PCR Assay (cat. no. 339306) on any real-time cycler.

Important points before starting

- For analysis of miRNA from serum, plasma, and other biofluids, refer to the specific recommendations in the *miRCURY LNA miRNA SYBR® Green PCR – Exosomes, Serum/Plasma and Other Biofluid Samples Handbook*.
- This protocol is optimized for detection of miRNA targets with any real-time cycler and conditions for fluorescence normalization. The amount of required ROX dye varies, depending on the instrument used:
 - **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 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 PCR assays: centrifuge the tube before opening it for the first time. Add 220 μl nuclease-free water to the tube and leave at room temperature for 20 min. Vortex and briefly centrifuge.
- Thaw the 2x miRCURY SYBR[®] Green Master Mix, template cDNA, LNA PCR assays, ROX Reference Dye (if required), and RNase-free water. Vortex and briefly centrifuge.

Procedure

1. Dilute the cDNA 1:60 by adding 590 μl RNase-free water to the 10 μl RT reaction immediately before use. We do not recommend storing this 1:60 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	Volume Rotor-Disc 100
2x miRCURY SYBR [®] Green Master Mix	5 μl	10 μl
ROX Reference Dye (ABI instruments only)	0.5 μl /0.05 μl *	
Resuspended PCR primer mix	1 μl	2 μl
cDNA template	3 μl (diluted 1:60)	6 μl (diluted 1:60)
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, ViiA 7 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.

- Mix the reactions thoroughly and dispense 10 µl into PCR tubes or PCR plate wells or 20 µl 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.
- 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 for miRCURY LNA miRNA PCR Assays

Step	Time	Temperature	Ramp rate	Additional comments
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	Perform fluorescence data collection (SYBR® Green)
Number of cycles	40*			
Melting curve analysis		60–95°C		

* 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

This protocol is for use with the miRCURY LNA SYBR® Green PCR Kit (cat. nos. 339345, 339346, and 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 (cat. nos. YAHS-312Y and YAHS-301Y*)
- miRCURY LNA miRNome Mouse & Rat PCR Panels (cat. nos. YAMR-312Y and YAMR-301Y)*

Important points before starting

- For analysis of miRNA from serum, plasma and other biofluids, refer to the specific recommendations in the *miRCURY LNA miRNA SYBR® Green PCR – Exosomes, Serum/Plasma and Other Biofluid Samples Handbook*.
- This protocol is optimized for detection of miRNA targets with any real-time cycler and conditions for fluorescence normalization. The amount of required ROX dye varies, depending on the instrument used:
 - **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.

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

- 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 Nuclease-free water. Vortex and briefly centrifuge.

Procedure

1. 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

miRNome PCR Panels (in 384-wells plate format)		
Component	Human and Mouse & Rat PCR Panel I	Human and Mouse & Rat PCR Panels I+II
2x miRCURY SYBR® Green Master Mix	2000 µl	4000 µl
ROX Reference Dye (ABI instruments only)	200 µl/20 µl*	400 µl/40 µl*
cDNA template (undiluted)	20 µl	40 µl
RNase-free water	1980 µl*	3960 µl*
Total reaction volume	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 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, ViiA 7, and QuantStudio Real-Time PCR Systems). Adjust the amount of RNase-free water accordingly.

2. Vortex 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 h.

3. Seal the plate. Carefully vortex it to dissolve the primers (optional). Briefly centrifuge the plate at room temperature. Wait 5 min for the primers to completely dissolve in the reaction mix.
4. Program the real-time cycler according to Table 13.

Note: Data acquisition should be performed during the annealing/extension step.
5. 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.

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 Focus PCR Panels

This protocol is for use with the miRCURY LNA SYBR® Green PCR Kit on any real-time PCR cyclers. 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, cat. no. YAHS-102Y)

If you are using the Serum/Plasma, Urine Exosomes or CSF Exosomes Focus PCR Panels, refer to the protocols in the *miRCURY LNA miRNA SYBR® Green PCR – Exosomes, Serum/Plasma and Other Biofluid Samples Handbook*.

Important points before starting

- For analysis of miRNA from serum, plasma and other biofluids, refer to the specific recommendations in the *miRCURY LNA miRNA SYBR® Green PCR – Exosomes, Serum/Plasma and Other Biofluid Samples Handbook*.
- This protocol is optimized for detection of miRNA targets with any real-time cyclers and conditions for fluorescence normalization. The amount of required ROX dye varies, depending on the instrument used:
 - **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 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. 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

Component	Cancer Focus PCR Panel consisting of 1 x 96 assays Volume 96-well or 384-well format
2x miRCURY SYBR® Green Master Mix	500 µl
ROX Reference Dye (ABI instruments only)	50 µl/5 µl*
cDNA template (undiluted)	5 µl
RNase-free water	495 µl*
Total reaction volume	

* Use ROX Reference Dye as a 20x concentrate for cyclers requiring a high ROX dye concentration (i.e., ABI PRISM 7000, Applied Biosystems 7300, 7900 and StepOne Real-Time PCR Systems) and as a 200x concentrate for cyclers requiring a low ROX dye concentration (i.e., Applied Biosystems 7500, ViiA 7 and QuantStudio Real-Time PCR Systems). Adjust the amount of RNase-free water accordingly.

2. Vortex the reaction mix, thoroughly and dispense 10 μ l 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 h.

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

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

5. 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.

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 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)

If you are using a biofluid as a template, refer to the protocols in the *miRCURY LNA miRNA SYBR® Green PCR – Exosomes, Serum/Plasma and Other Biofluid Samples Handbook*.

Important points before starting

- For analysis of miRNA from serum, plasma and other biofluids, refer to the specific recommendations in the *miRCURY LNA miRNA SYBR® Green PCR – Exosomes, Serum/Plasma and Other Biofluid Samples Handbook*.
- This protocol is optimized for detection of miRNA targets with any real-time cycler and conditions for fluorescence normalization. The amount of required ROX dye varies, depending on the instrument used:
 - **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 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. Dilute the cDNA 1:80 according to Table 16 immediately before use. We do not recommend storing this 1:80 dilution of cDNA.

Table 16 refers to a 10 µl PCR reaction volume. For the Rotor-Disc 100, double the amounts.

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 interplate calibrators. Loss from pipetting is not included in the “Volumes of diluted cDNA needed” column.

	Custom PCR panel configuration	Suggested cDNA dilution for each sample, 1:80 (cDNA + nuclease-free water, µl)	Volume of 1:80 diluted cDNA needed for each sample on a custom PCR panel plate
96	12 x 8 (12 samples)	2 + 158	32 µl
96	8 x 12 (8 samples)	2 + 158	48 µl
96	6 x 16 (6 samples)	2 + 158	64 µl
96	4 x 24 (4 samples)	2 + 158	96 µl
96	3 x 32 (3 samples)	2 + 158	128 µl
96	2 x 48 (2 samples)	3 + 237	192 µl
96	1 x 96 (1 sample)	5 + 395	384 µl
384	48 x 8 (48 samples)	2 + 158	32 µl
384	32 x 12 (32 samples)	2 + 158	48 µl

Table continues on next page

Table continued from previous page

	Custom PCR panel configuration	Suggested cDNA dilution for each sample, 1:80 (cDNA + nuclease-free water, μl)	Volume of 1:80 diluted cDNA needed for each sample on a custom PCR panel plate
384	24 x 16 (24 samples)	2 + 158	64 μ l
384	16 x 24 (16 samples)	2 + 158	96 μ l
384	12 x 32 (12 samples)	2 + 158	128 μ l
384	8 x 48 (8 samples)	3 + 237	192 μ l
384	6 x 64 (6 samples)	4 + 316	256 μ l
384	4 x 96 (4 samples)	5 + 395	384 μ l
384	3 x 128 (3 samples)	7 + 553	512 μ l
384	2 x 192 (2 samples)	10 + 790	768 μ l
384	1 x 384 (1 sample)	20 + 1580	1536 μ l

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 cyclers.

Table 17. Reaction setup per sample for miRCURY LNA miRNA Custom PCR Panels

Component	Volume per reaction	Volume Rotor-Disc 100
2x miRCURY SYBR [®] Green Master Mix	5 μ l	10 μ l
ROX Reference Dye (ABI instruments only)	0.5 μ l/0.05 μ l*	
cDNA template (diluted 1:80)	4 μ l (diluted 1:80)	8 μ l (diluted 1:80)
RNase-free water	1 μ l*	2 μ l
Total reaction volume	10 μl[†]	20 μ l

* 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, ViiA 7 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.

- Vortex the reaction mix thoroughly and dispense 10 μ l 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 h.

- Seal the plate. Carefully vortex it to dissolve the primers (optional). Briefly centrifuge the plate(s) at room temperature. Wait 5 min while the primers dissolve in the reaction mix.
- Program the real-time cycler according to Table 18.

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 18. PCR cycling conditions for miRCURY LNA miRNA Custom PCR Panels

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.

- 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 Interplate Calibrators

Three wells within the predefined miRNome (Human and Mouse & Rat) and Focus (Cancer, Urine Exosome, CSF Exosome) PCR panels contain the interplate 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 interplate calibrators. Each of these wells contain a prealiquoted primer pair and a DNA template, so the variation of these assays is minimal from well to well and from plate to plate. The interplate 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 interplate 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.

Interplate 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 IPC replicates for each single plate and the overall average, i.e., the average of IPC values from all plates. The calibration factor for each plate is calculated by subtracting the overall average from the average of replicates for that plate ($IPC_{\text{plate}} - IPC_{\text{overall}} = \text{calibration factor}$). 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 interplate 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 intersample variations that are 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 either be stably expressed, small noncoding RNA or stably expressed miRNAs that are selected based on literature or preexisting 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 noncoding 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 subcellular distribution. The fact that U6 has 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 noncoding RNAs (5S, U6, SNORs, etc.) are not good candidates for reference genes, because 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 has 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 (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, page 49).
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. |
| 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 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) Mg²⁺ concentration adjusted Do not adjust the Mg²⁺ 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 NTC

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 23. 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 (384): Human, Mouse & Rat	Focus PCR Panel (96): Cancer	Custom PCR Panel
2x miRCURY SYBR® Green Master Mix*	5.5 µl*	2200 µl*	550 µl*	5.5 µl*
Resuspended PCR primer mix	1 µl	–	–	–
cDNA template	3 µl (diluted 1:60)	20 µl	5 µl	4 µl (diluted 1:80)
RNase-free water	0.5 µl	1780 µl	445 µl	0.5 µl
Total reaction volume	10 µl	4000 µl	1000 µl	10 µl

* Already contains a 1:20 dilution of ROX Reference Dye; suitable for 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 the pretreatment and usage of both disposable and nondisposable 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.

Nondisposable plasticware

Nondisposable plasticware should be treated before use to ensure that it is RNase-free. Plasticware should be thoroughly rinsed with 0.1 M NaOH,* 1 mM EDTA* followed by RNase-free water. Alternatively, chloroform-resistant plasticware can be rinsed with 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 four hours or more (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with diethyl pyrocarbonate (DEPC).* Fill glassware with 0.1% DEPC (0.1% in water), allow to stand overnight (12 hours) at 37°C, and then autoclave or heat to 100°C for 15 minutes to eliminate residual DEPC.

Solutions

Solutions (water and other solutions) should be treated with 0.1% DEPC.* DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 ml DEPC to 100 ml of the solution to be treated, and then shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37°C. Autoclave for 15 minutes to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris* buffers. DEPC is highly unstable in the presence of Tris buffers 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 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

Product	Contents	Cat. no.
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
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 (8)	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	339330
miRCURY LNA miRNA miRNome PCR Panels	Pre-designed panels of LNA PCR assays for miRNome profiling; for one 10 µl qPCR reaction per well; 96-well or 384-well format	339322
miRCURY LNA miRNA Focus PCR Panels	Pre-designed panel of LNA PCR assays focused on disease, pathway or sample type; for one 10 µl qPCR reaction per well; 96-well or 384-well format	339325

Product	Contents	Cat. no.
miRCURY LNA miRNA QC PCR Panel	Pre-designed panel of LNA PCR assays for miRNA quality control; for one 10 µl qPCR reaction per well; 96-well or 384-well format	339331
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 RNA/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

Product	Contents	Cat. no.
PAXgene Tissue Container (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

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

Visit www.qiagen.com/geneXpression to find out more about standardized solutions for gene expression analysis – from RNA preparation to real-time RT-PCR.

Handbook Revision History

Date	Changes
10/2019	<ul style="list-style-type: none"> • Added “SYBR® Green” to handbook title and various product names. • Added cross-reference to separate handbook for RT-PCR detection of miRNAs <i>using biofluid samples</i>. • Made protocols and product descriptions specific for SYBR® Green-based usage. • Removed printed quick-start protocols from kit contents. • Added note on availability of an alternative Probe detection system, in addition to the SYBR® Green detection system. • In Table 4, added cat. nos. 217084, 217061, 77044 and 766134; deleted cat. no. 217061. • Modified Table 7 column headers and deleted “Dilution of cDNA for qPCR” row. • Modified Table 8 headers for columns 5–7. • Replaced contents of Table 16. • URL to GeneGlobe Data Analysis Center replaced with shortlink. • Added options to use Rotor Gene.

Limited License Agreement for the miRCURY® LNA® miRNA SYBR® Green PCR System

Use of this product signifies the agreement of any purchaser or user of the product to the following terms:

1. The product may be used solely in accordance with the protocols provided with the product and this handbook and for use with components contained in the kit only. QIAGEN grants no license under any of its intellectual property to use or incorporate the enclosed components of this kit with any components not included within this kit except as described in the protocols provided with the product, this handbook, and additional protocols available at www.qiagen.com. Some of these additional protocols have been provided by QIAGEN users for QIAGEN users. These protocols have not been thoroughly tested or optimized by QIAGEN. QIAGEN neither guarantees them nor warrants that they do not infringe the rights of third-parties.
2. Other than expressly stated licenses, QIAGEN makes no warranty that this kit and/or its use(s) do not infringe the rights of third-parties.
3. This kit and its components are licensed for one-time use and may not be reused, refurbished, or resold.
4. QIAGEN specifically disclaims any other licenses, expressed or implied other than those expressly stated.
5. The purchaser and user of the kit agree not to take or permit anyone else to take any steps that could lead to or facilitate any acts prohibited above. QIAGEN may enforce the prohibitions of this Limited License Agreement in any Court, and shall recover all its investigative and Court costs, including attorney fees, in any action to enforce this Limited License Agreement or any of its intellectual property rights relating to the kit and/or its components.

For updated license terms, see www.qiagen.com.

Notice to purchaser

Locked Nucleic Acids (LNAs) are protected by US Pat No. 6,268,490, US Pat No. 6,770,748, US Pat No. 6,639,059, US Pat No. 6,734,291 and other applications and patents owned or licensed by Exiqon A/S. Products are provided to buyers for research use only. The products in their original or any modified form may be used only for the buyer's internal research purposes and not for commercial, diagnostic, therapeutic, or other use, including contract research. The buyer may not provide products to third parties in their original or any modified form. The purchase of products does not include or carry an implied right or license for the buyer to use such products in their original or any modified form in the provision of services to third parties, and a license must be obtained directly from Exiqon A/S for such uses.

Limited license

Use of this product is covered by one or more of the following US patents and corresponding patent claims outside the US: 5,994,056 and 6,171,785. The purchase of this product includes a limited, nontransferable immunity from suit under the foregoing patent claims for using only this amount of product solely in Contract Research, including reporting results of purchaser's activities for a fee or other commercial consideration, and also for the purchaser's own internal research. No right under any other patent claim is conveyed expressly, by implication, or by estoppel. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA. Furthermore, this product is provided under an agreement between Molecular Probes, Inc., a wholly owned subsidiary of Invitrogen Corporation, and EXIQON and the manufacture, use, sale or import of this product is subject to one or more U.S. Patents and corresponding international equivalents. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer, where such research does not include testing, analysis or screening services for any third party in return for compensation on a per test basis. The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. For information on purchasing a license to this product for purposes other than research, contact Molecular Probes, Inc., Business Development, 29851 Willow Creek Road, Eugene, OR 97402. Tel: (541) 465-8300, Fax: (541) 335-0354.

Trademarks: QIAGEN®, Sample to Insight®, QIAzol®, GeneGlobe®, LNA®, MinElute®, miRCURY®, miRNeasy®, Q-Bond®, QuantiNova®, Rotor-Gene® (QIAGEN Group); PAXgene® (PreAnalytix GmbH); Agilent® (Agilent Technologies, Inc.); ABI PRISM®, Applied Biosystems®, QuantStudio®, ROX®, StepOne®, SYBR®, ViA® (Thermo Fisher Scientific or its subsidiaries); Bio-Rad® (Bio-Rad Laboratories, Inc.); LightCycler®, Roche® (Roche Group). Registered names, trademarks, etc. used in this document, even when not specifically marked as such, are not to be considered unprotected by law.

10/2019 HB-2431-002 © 2019 QIAGEN, all rights reserved.

Ordering www.qiagen.com/shop | Technical Support support.qiagen.com | Website www.qiagen.com