miRCURY LNA™ microRNA PCR System

Instruction manual

Version 3.0 (April 2009)

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Literature citations

Please refer to miRCURY LNA™ microRNA PCR System when describing a procedure for publication using this product.

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Table of contents

Product summary

miRCURY LNA™ microRNA PCR System content

The miRCURY LNA™ microRNA PCR System is a microRNA-specific, LNA™-based three-component system designed for sensitive and accurate detection of microRNA by quantitative PCR using SYBR® Green. The miRCURY LNA™ microRNA PCR System is comprised of the following components:

miRCURY LNA™ microRNA PCR System, First-strand cDNA synthesis kit (product # 201100)

This kit contains all reagents required for first-strand cDNA synthesis, 200 reactions*:

Reagent		Amount supplied
Reverse transcriptase, Transcriptor	20 U/µL	2× 50 µL
5× RT Reaction buffer	5× conc.	2× 1 mL
RNase inhibitor	40 U/µL	2× 100 µL
dNTP mix	10 mM, each	2× 200 µL
Random hexamer primer **	600 µM	2× 200 µL
Nuclease-free water		2× 1 mL

*200 reactions is based on a standard reaction volume of 10 µL.
** Used exclusively for the positive control assay included in the miRCURY LNA™ SYBR® Green master mix kit

miRCURY LNA™ microRNA PCR System, SYBR® Green master mix (product# 201000)

This kit contains all required reagents for PCR amplification of microRNAs including the system-specific universal PCR primer. In addition, a 5S-specific positive control assay is provided with this kit. All reagents are supplied at volumes for 200 reactions*:

Reagent		Amount supplied
SYBR® Green master mix	2× conc.	2x 1 mL
Universal PCR primer	Vial C - Blue	200 reactions
Reference primer mix, 5S rRNA		200 reactions
Nuclease-free water		2× 1 mL

*200 reactions is based on a standard reaction volume of 20 µL.

miRCURY LNA™ microRNA PCR System, microRNA primer set (product# varies**)

Each primer set is designed specifically for use in combination with the miRCURY LNA™ First-strand cDNA synthesis kit and SYBR® Green master mix:

Reagent		Amount supplied
miR-specific RT primer	Vial A – Red	50 reactions
LNA™ PCR primer	Vial B – Blue	100 reactions

** Please visit **www.exiqon.com/microrna-real-time-pcr** to find listings of specific microRNA primer sets

miRCURY LNA™ microRNA PCR System
Endogenous control primer set (product # varies***)

Reagent		Amount supplied
Control RT primer	Vial A – Red	50 reactions
Control PCR primers	Vial B – Blue	100 reactions

*** Please visit **www.exiqon.com/microrna-real-time-pcr** to find listings of Endogenous control primer sets.

Storage

miRCURY LNA™ microRNA and Endogenous Control primer sets

The miRCURY LNA™ PCR primer sets are shipped at room temperature. For long-term storage, it is recommended to store the reagents at -20°C and to avoid repeated freeze-thaw cycles. In order to avoid repeated freeze-thaw cycles, it is recommended to aliquot the primers after the first thawing cycle and store aliquots at -20°C. Under these conditions, the primers are stable until the expiry date listed on the vial.

miRCURY LNA™ First-strand cDNA synthesis kit and miRCURY LNA™ SYBR® Green master mix

These kits are shipped on dry ice in polystyrene containers and should be stored at -15°C to -25°C. Do not store in a frost-free freezer. All reagents are stable for at least 6 months under these conditions.

Note

Information on the miRCURY LNA™ microRNA PCR System and technical data is available at www.exiqon.com. For technical assistance, please e-mail support@exiqon.com. Alternatively call +45 45 650 929 (outside North America) or +1 781 376 4150 (in North America).

Additional required materials

Reagents not supplied
- ROX passive reference dye (optional)
- Uracil-DNA Glycosylase (optional)

Materials and Equipment not supplied
- Nuclease-free PCR tubes
- Nuclease-free filter barrier pipette tips
- Heating block, thermal cycler or other incubators
- Real-time PCR instrument
- Nuclease-free low nucleic acid binding (siliconized) microcentrifuge tubes

Recommended accompanying products

Exiqon recommends the miRCURY™ microRNA Isolation kits for purification of total RNA that includes the small RNA fraction. RNA purified using the miRCURY™ Isolation kits is fully compatible with the miRCURY LNA™ microRNA PCR System.
The following kits are available:

miRCURY™ RNA Isolation Kit – Cell & Plant
Provides a rapid method for purification of total RNA from cultured animal cells, small tissue samples, blood, bacteria, yeast, fungi and plants.

miRCURY™ RNA Isolation Kit – Tissue
Specifically designed for purification of total RNA from animal tissue samples.

Related products

Exiqon offers a broad variety of tools enabling new discoveries concerning the expression, function and spatial distribution of microRNAs:

miRCURY LNA™ microRNA Array, microarray kit

Pre-printed miRCURY LNA™ microRNA Array microarray slides, available in pack sizes of 3, 6 and 24 (product # 208000-A, 208001-A, 208002-A). Kit includes hybridization and wash buffers as well as synthetic spike-in microRNAs.

miRCURY LNA™ microRNA Power labeling kit

For fluorescent labeling of microRNAs from total RNA samples ready for array hybridization (product # 208030-A, 208031-A, 208032-A).

miRCURY LNA™ microRNA Array, ready-to-spot probe set

Ready-to-spot oligos for direct printing of arrays, or coupling in bead-based applications (product # 208010-A).

miRCURY LNA™ microRNA Detection

For in situ hybridization and northern blotting of all annotated microRNAs.

miRCURY LNA™ microRNA Knockdown

For direct inhibition of microRNA expression: determine or confirm microRNA function.

MicroRNAs and Locked Nucleic Acid (LNA™)

microRNAs have rapidly emerged as an important class of short endogenous RNAs that act as post-transcriptional regulators of gene expression by base-pairing with their target messenger RNAs (mRNAs), thereby causing mRNA cleavage or translational repression. microRNAs may regulate as much as 30% of all genes in the genome, thus comprising a new and very important level of gene regulation. In the cytoplasm of the cell, microRNAs are typically found in the mature form as 19-25 nucleotide RNAs that have been processed sequentially from longer hairpin transcripts (pri-microRNAs and pre-microRNAs) by the RNAse III ribonucleases Drosha and Dicer.

Analyses of microRNA expression have been impeded by the short nature of the target sequence, and specificity has been difficult to achieve with standard technologies. The miRCURY LNA™ microRNA PCR System takes advantage of the LNA™ technology enabling design of shorter yet high-affinity annealing primers to overcome these difficulties. Incorporation of LNA™ in the microRNA-specific PCR primer markedly increases the binding affinity between primer and target, thereby adding unique specificity to the assay.

Note

Locked Nucleic Acid (LNA™) is a conformationally restricted nucleic acid analogue in which the ribose ring is "locked" with a methylene bridge connecting the 2'-*O* atom with the 4'-*C* atom. Incorporation of LNA™ in one strand of a nucleic acid duplex increases the melting temperature of the duplex by 2-8°C/LNA™ monomer.

Product description

The miRCURY LNA™ microRNA PCR System is a three-component system developed for rapid, accurate, and sensitive quantification of microRNA by gene-specific real-time PCR based on SYBR® Green detection. The system consists of the miRCURY LNA™ First-strand cDNA synthesis kit, the miRCURY LNA™ SYBR® Green master mix and pre-designed validated miRCURY LNA™ microRNA and control primer sets. A variety of miRCURY LNA™ microRNA primer sets and Endogenous control primer sets are available. The miRCURY LNA™ microRNA primer set contains two primers; a microRNA specific reverse transcription (RT) primer for the initial conversion of RNA to cDNA and a microRNA-specific Locked Nucleic Acid (LNA™)-enhanced PCR primer for quantitative real-time PCR amplification.

The full advantage of the LNA™ technology and SYBR® Green detection is obtained when using the miRCURY LNA™ microRNA PCR System in combination as it enables both the initial conversion of microRNA to cDNA and the subsequent real-time PCR step that specifically targets the mature microRNA of interest allowing a more accurate microRNA detection. Hence, an initial general polyadenylation of the RNA is not necessary.

Key features of the miRCURY LNA™ microRNA PCR System:

- The use of an LNA™-enhanced microRNA-specific PCR primer adds unique specificity to the assay and enables excellent mismatch discrimination between closely related microRNA even for templates differing by a single nucleotide.
- 1 - 10 ng of total RNA can be used as starting material, but as little as 10 pg total RNA can be used for detection of highly expressed microRNAs.
- Enrichment for small RNAs is not necessary.
- A fast two-step protocol taking less than 3 hours.
- Accurate quantification with a dynamic range of at least 8 logs.

Important

The miRCURY LNA™ primer sets have been developed for optimal performance when used in combination with the miRCURY LNA™ First-strand cDNA synthesis kit and the miRCURY LNA™ SYBR® Green master mix from Exiqon. These two kits contain all necessary universal reagents for the real-time PCR experiment. In addition, the miRCURY LNA™ SYBR® Green master mix contains the Universal PCR primer required for the microRNA real-time PCR amplification step and the 5S rRNA positive control assay for use with random hexamer priming.

Figure 1

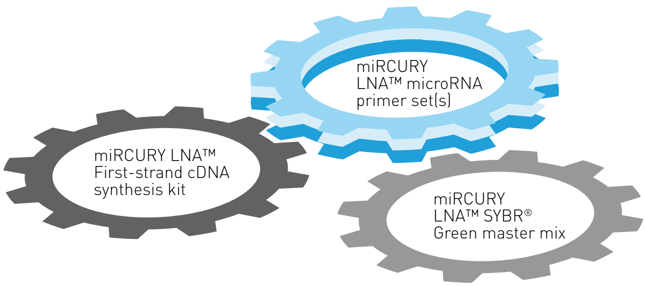


Figure 1. How the miRCURY LNA™ microRNA PCR System interrelate. The miRCURY LNA™ First-strand cDNA synthesis kit and miRCURY LNA™ SYBR® Green master mix can be used with several miRCURY LNA™ microRNA primer sets detecting microRNAs or control RNAs (e.g. small nuclear RNAs, 5S, U6).

Endogenous Control assays

A number of miRCURY LNA™ Endogenous Control primer sets are available to detect and quantify different small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs). Since these molecules are ubiquitously expressed, measurement of one or more of these small RNAs can be used as a reference gene for the miRCURY LNA™ microRNA PCR System in order to normalize microRNA expression across different samples.

To view a list of available endogenous control primer sets for the miRCURY LNA™ microRNA PCR System, please visit www.exiqon.com/microrna-real-time-pcr.

It is also possible to use microRNAs as reference genes as long as their expression levels are stable across all the samples in the study.

Note: It is important to validate the stability of any selected endogenous control or microRNA gene in all samples in order to confirm their suitability for use as reference genes.

For more information regarding normalization using endogenous controls, please see page 25 and visit www.exiqon.com/microrna-real-time-pcr

Principle and procedure

The miRCURY LNA™ microRNA PCR System is a microRNA-specific, LNA™-based, real-time RT-PCR assay using SYBR® Green detection, performed on total RNA according to a two-step protocol:

1. **microRNA-specific first-strand cDNA synthesis.**
   In this first step the microRNA is converted by reverse transcription (RT) into a cDNA template using a miR-specific RT primer for the microRNA of interest. Together, the use of a miR-specific primer and the very thermostable and sensitive Transcriptor Reverse Transcriptase ensures a highly specific cDNA synthesis reaction. The cDNA template can be generated directly from a total RNA sample. Prior microRNA enrichment is not needed.

Note: The method used for purification of total RNA must conserve small RNA species.

2. **microRNA-specific real-time PCR reaction.**
   The cDNA template from the first-strand cDNA synthesis reaction is used in a microRNA-specific real-time PCR amplification. The microRNA is amplified using the LNA™ PCR primer together with the Universal PCR primer. The short design of the LNA™-enhanced primer enables a microRNA-sequence specific amplification by direct annealing to microRNA primer binding sites. Combined with the use of SYBR® Green detection, where the assay is followed by monitoring fluorescence at 520 nm, a highly sensitive and accurate microRNA real-time PCR reaction is achieved.

Figure 2

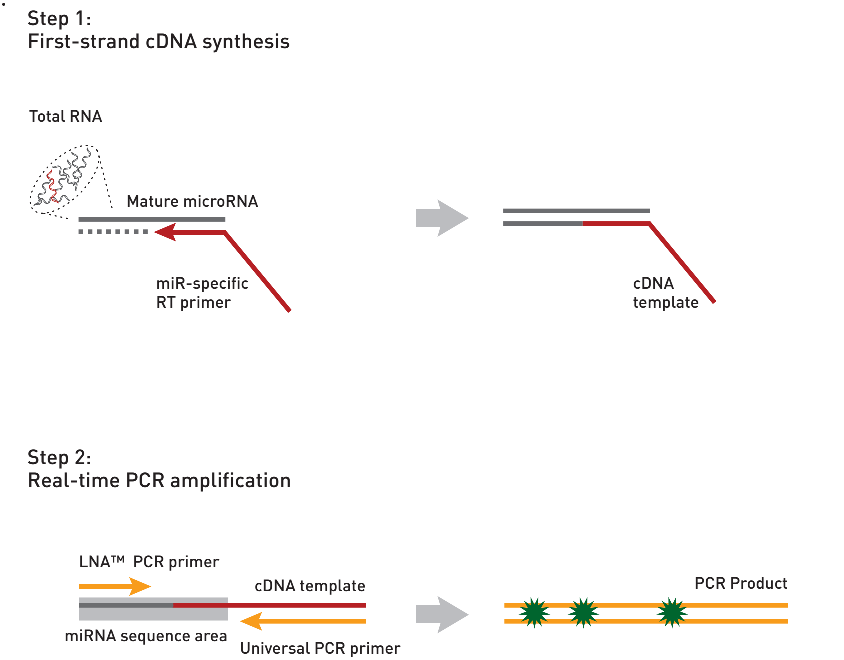


Figure 2. miRCURY LNA™ microRNA PCR System overview. A total RNA sample containing the microRNA of interest is suitable for the miRCURY LNA™ microRNA PCR System. (Step 1) A microRNA-specific reverse primer converts the microRNA to cDNA by a first-strand synthesis using Transcriptor Reverse Transcriptase, and finally, (Step 2) the cDNA is used as a template for the real-time PCR amplification using the LNA™ PCR primer and the Universal PCR primer.

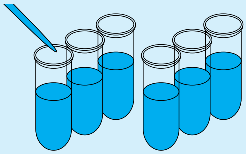
Protocol

Overview of work-flow

Step 1

Always wear gloves when working with RNA. Adjust the RNA template concentrations in nuclease-free water.

Note: Keep the RNA and required reagents (e.g. RT and PCR master mixes) on ice (or at 4°C) at all times!



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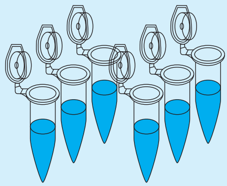
Step 2

Prepare a reverse transcription (RT) master mix for each microRNA/control gene to be quantified. Each mix should contain enough reagents for two RT-reactions per RNA sample. Similar master mixes without the RT enzyme should be prepared for the "no reverse transcription" controls.



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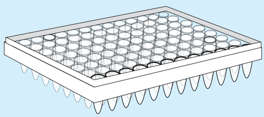
Step 3

Combine the RT master mixes with the RNA templates in nuclease-free tubes or a 96-well plate.



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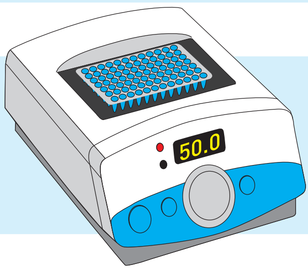
Step 4

Perform the first strand cDNA synthesis followed by RT enzyme heat inactivation.



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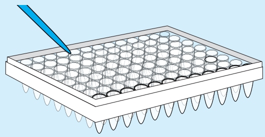
Step 5

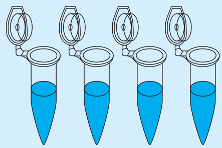
Dilute the cDNA 1:10 in nuclease-free water.

Note: If very few microRNA copies are present the cDNA reaction may be used undiluted.



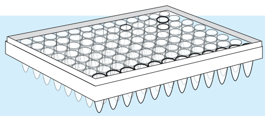
Step 6

Prepare master mixes for the real-time PCR reactions. Prepare one mix per microRNA/control gene to be quantified. The mix should contain enough reagents for two PCR reactions per RT performed as well as one "no template control" reaction.



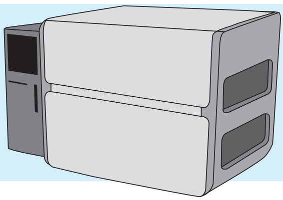
Step 7

Combine the PCR master mixes and cDNA templates in a 96-well/384-well PCR plate.



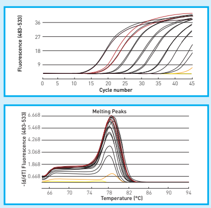
Step 8

Perform real-time PCR on your preferred instrument using the suggested cycling conditions, followed by melting curve analysis.



Step 9

Perform preliminary data extraction and check the melting curves using the instrument software. Export quantification cycle (Cq) values for further data analysis and normalization.



Before starting the experiment

The protocol is optimized for an input of 1 - 10 ng total RNA. The amount of total RNA to be used may vary depending on the microRNA expression levels in the cells or tissue to be analyzed. For highly expressed microRNAs it is possible to use as little as to 10 pg total RNA as starting material. If a larger amount is used, and the microRNA of interest is highly expressed, we recommend diluting the cDNA a 100-fold or more.

Total RNA should be prepared using a method that preserves small RNA species. The miRCURY™ RNA Isolation kits are recommended for use with the miRCURY LNA™ microRNA PCR System. DNAse treatment may be recommended, please see Tip 1.

See tip 1

RNA work requires specific handling and precautions should be taken to prevent RNase contamination and degradation of the RNA sample and reagents. Find information on how to handle RNA in the tips section (p. 24). The tips section also lists simple guidelines for good laboratory practice to ensure optimal performance of PCR experiments.

See tip 2 & 3

The miRCURY LNA™ microRNA PCR System protocol is a two-step protocol consisting of:

1. First-strand cDNA synthesis
2. Real-time PCR amplification

It is critical for the success of the experiment to follow the protocol setup below. The protocol should be carried out in a continuous workflow from first-strand cDNA synthesis to real-time PCR amplification (around 3 hours). However, if necessary the assay can be paused immediately after the first-strand cDNA synthesis. In this case, the cDNA may be left overnight at 4°C or for longer time at -20°C.

Note

The miRCURY LNA™ SYBR® Green master mix does not include the ROX passive reference dye.

First-strand cDNA synthesis

- using the miRCURY™ First-strand cDNA synthesis kit and the miR-specific or control-specific RT primer supplied with the miRCURY LNA™ microRNA primer set.

Protocol

Important: Keep reagents and reactions on ice (or at 4°C) at all times.

See tip 4

Step 1 Dilute template RNA	Adjust each of the template RNA samples to a concentration of 1-10 ng in 4.5 µL using nuclease free water.
Step 2 Prepare reagents listed in Table 1	Place the 5× RT Reaction buffer, miR-specific or control-specific RT primer (supplied with the primer set), dNTP mix and Nuclease-free water on ice and thaw for 15-20 min. Immediately before use, remove the enzymes (Reverse transcriptase and RNase inhibitor) from the freezer, mix by flicking the tubes and place on ice. The rest of the reagents are mixed by vortexing. Spin down all reagents.
Step 3 Combine reagents according to Table 1	If performing first-strand cDNA synthesis on multiple RNA samples, it is recommended to prepare a RT master mix of the relevant reagents (indicated in Table 1). The following procedure is recommended: 1. Prepare the RT master mix and place it on ice. It is recommended to include 10% excess of all reagents to allow for pipetting losses. 2. Dispense 5.5 µL of RT master mix into nuclease free tubes. 3. Place 4.5 µL of template RNA in each tube.

See tip 5 & 6

Table 1 - First strand cDNA synthesis reaction

Reagent	Volume (µL)	Final conc./amount
Template total RNA (adjusted)	4.5	1-10 ng
5 × RT Reaction buffer	2	1×
miR-specific or control-specific reverse primer (Vial A, red)	2	
dNTP mix	0.5	500 µM
RNase inhibitor	0.5	10 U
Reverse transcriptase	0.5	20 U
Total volume	**10 µL**	

Step 4
Mix and spin reagents

Mix the reaction by gentle vortexing or pipetting to ensure that all reagents are mixed thoroughly. After mixing, spin down.

Step 5
Incubate for 30 min at 50°C followed by heat-inactivation of the reverse transcriptase for 5-10 min at 85°C

Although not recommended, the protocol can be paused at this stage. The cDNA may be left overnight at 4°C or for longer time at -20°C.

Real-time PCR amplification

- using the miRCURY LNA™ SYBR® Green master mix, the Universal primer, and the LNA™ PCR primer from the miRCURY LNA™ microRNA primer set or the control PCR primers.

Important: Keep reagents and reactions on ice (or at 4°C) at all times.

See tip 4

Step 6 Prepare reagents listed in Table 2	Place all reagents on ice and thaw for 15-20 min. Protect the SYBR® Green master mix vials from light. Immediately before use, mix the SYBR® Green master mix by inverting the tube and the rest of the reagents by vortexing. Spin down all reagents.
Step 7 Dilute cDNA template 1:10 in nuclease free water	It is recommended to dilute the cDNA (produced by the protocol above) to allow pipetting of a larger volume of the reaction into the subsequent real-time PCR reaction as this generally results in more accurate pipetting and reproducible results.
Step 8 Combine reagents according to Table 2	When multiple real-time PCR reactions are performed it is recommended to prepare a master mix of all other reagents than the cDNA template (as indicated in Table 2 a or b). The following procedure is recommended: 1. Prepare the PCR master mix (see Table 2 a and b) and place it on ice. It is recommended to include 10% excess of all reagents in the master mix to allow for pipetting losses. 2. Place 16 µL of PCR master mix in PCR tubes or wells of a PCR plate. 3. Add 4 µL cDNA template to each tube.

See tip 7 & 8

Table 2a - microRNA primer sets - real-time PCR reaction

	Reagent	Volume (µL)	Final conc./amount
	Diluted cDNA template	4	
PCR master mix	SYBR® Green master mix	10	1×
	LNA™ PCR primer (Vial B, blue)	1	
	Universal PCR primer (Vial C, blue)	1	
	Passive reference dye (stock conc. 1 mM) (optional - see Tip 7, p. 27)		depending on instrument
	Uracil-DNA-glycosylase (UDG) (optional - see Tip 8, p. 28)		0.5 U
	Nuclease-free water	Up to 16 µL	
	Total volume	**20 µL**	

Table 2b - Endogenous control primer sets - real-time PCR reaction

	Reagent	Volume (µL)	Final conc./amount
	Diluted cDNA template	4	
PCR master mix	SYBR® Green master mix	10	1×
	Control PCR primers (Vial B, blue)	2	
	Passive reference dye (stock conc. 1 mM) (optional - see Tip 7, p. 27)		depending on instrument
	Uracil-DNA-glycosylase (UDG) (optional - see Tip 8, p. 28)		0.5 U
	Nuclease-free water	Up to 16 µL	
	Total volume	**20 µL**	

Step 9
Mix and spin reagents

Mix the reaction by gentle vortexing or pipetting to ensure that all reagents are mixed thoroughly. After mixing, spin down.

Step 10
Real-time PCR amplification.

- Perform 40 cycles of PCR amplification followed by melt curve analysis according to table 3a if Uracil DNA Glycosylase (UDG) is NOT used.
- If UDG is used, perform PCR amplification according to table 3b (page 24) and see comments below the table.

Table 3a - Real-time PCR cycle conditions (without UDG)

Process step	LC480 Instrument settings	ABI Instrument settings
Polymerase Activation/Denaturation	95°C, 10 min	95°C, 10 min
40 amplification cycles	95°C, 10 s 60°C, 20 s Optical read	95°C, 20 s 60°C, 60 s Optical read
Melting curve analysis*		

* Optional: Melting curve analysis of the PCR product(s) may be performed to verify their specificity and identity. Melting curve analysis is an analysis step built into the software of instruments. Please follow the instructions provided by the supplier.

Note: The T_m of a PCR product depends on buffer composition and salt concentration. T_m values obtained when using miRCURY LNA™ SYBR® Green master mix may differ from those obtained using other reagents.

Note: The LC480 Instrument settings are optimized for fast-cycling real-time PCR instruments. The ABI Instrument settings are suitable for most other types of real-time PCR machines with standard cycling capabilities.

Step 11
Analyze data

Analyze data using the software supplied with the real-time PCR instrument.

Table 3b - Real-time PCR Cycle conditions using UDG*

Process step	LC480 Instrument settings	ABI Instrument settings
UDG degradation	37°C, 10 min	37°C, 10 min
Polymerase Activation/Denaturation	95°C, 10 min	95°C, 10 min
40 amplification cycles Melting curve analysis	95°C, 10 s 60°C, 20 s Optical read	95°C, 20 s 60°C, 60 s Optical read
Final	72°C, forever	72°C, forever

* The following comments are relevant when Uracil DNA Glycosylase (UDG) is used:
  - This protocol is based on the use of a UDG that has maximal enzymatic activity at 37°C and is inactivated at 95°C. Other UDGs may have different temperature optima, and the UDG degradation step should be adjusted accordingly.
  - The final step (72°C, forever) is only required if end point analysis by gel electrophoresis is desired. The UDG enzyme will recover activity when returning to lower temperatures resulting in breakdown of newly generated PCR amplicon. The PCR reactions should be removed within a few hours and transferred to -20°C.

Note: The LC480 Instrument settings are optimized for fast-cycling real-time PCR instruments. The ABI Instrument settings are suitable for most other types of real-time PCR machines with standard cycling capabilities.

Step 11 Analyze data	Analyze data using the software supplied with the real-time PCR instrument.

Data analysis

Recommended references for guidance to data analysis

1. www.exiqon.com/microrna-real-time-pcr

2. IUL Biotechnology Series, **A-Z of Quantitative PCR.** Edited by Stephen A. Bustin. First edition. ISBN 0-9636817-8-8

3. http://www.gene-quantification.com/

Basic guide to normalization with miRCURY LNA™ Endogenous Controls

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.

In general it is recommended to employ as many controls (reference genes) as possible to guarantee proper real-time PCR quantitation. The controls should be empirically validated for each study. A panel of different programs exist for evaluating the best performing endogenous controls (e.g. GeneNorm, NormFinder and BestKeeper) and for applying multiple endogenous controls for normalizing target expression (e.g. qBASE and REST). Since the method(s) of choice for normalizing real-time PCR data are highly individual, it is recommended to visit the following web-site for detailed information about the approaches, methods and softwares available for real-time PCR quantitation: http://www.gene-quantification.info/.

One simplified approach for normalizing target expression with the miRCURY LNA™ endogenous controls is to apply the comparative quantitation "$\Delta\Delta C_T$" method also known as the $2^{-\Delta\Delta C_T}$ method. This relies on comparing the differences in cycle number threshold (C_T) values obtained for the targets(s) and selected control in a sample of interest with the C_T values obtained in a control or calibrator sample (e.g.

tumor versus normal tissue). When using this approach, it is critical that the level of the selected control is not regulated by the experimental conditions. Hence, in order to normalize microRNA expression levels, real-time PCR is performed by using miRCURY LNA™ microRNA primer set(s) of interest and miRCURY LNA™ endogenous control primer set(s) in parallel.

Data is normalized as follows:

1. C_T values for all samples are extracted and the ΔC_T is calculated as the difference in C_T-value between microRNA target and endogenous control:
   $\Delta C_T = C_T$ (target microRNA) - C_T (endogenous control)

2. $\Delta\Delta C_T$ is calculated:
   $\Delta\Delta C_T = \Delta C_T$ (sample of interest) – ΔC_T (control or calibrator sample)

3. Normalization of target gene expression in sample of interest is determined as: $2^{-\Delta\Delta C_T}$

4. The normalized level of expression in the calibrator or control sample is set to 1 and the change of target expression is determined as:
   Fold change in target microRNA expression = 1 – normalized target microRNA expression in sample of interest.

Tips and troubleshooting

Tip 1

RNA extraction and template preparation

Purification and preparation of total RNA that includes small RNAs (<200 nt) from a biological sample is the first critical step for a successful expression profiling analysis. Therefore the method used for the RNA sample preparation is critical to the success of the experiment. The following points need to be considered before starting the experiment:

- If not using one of the miRCURY™ microRNA Isolation kits, it is important that the method used to isolate RNA from a sample should be a quantitative recovery of small RNAs and should not result in a substantial loss of the small RNA fraction. This also applies when using commercially available kits. Make sure the total RNA preparation is guaranteed to contain microRNA.
- If commercially available purified RNA is used, it is important to make sure that the RNA is representative of the microRNAs in the species and/or tissue from which it is isolated.
- If samples prepared with different RNA isolation methods are compared, it is recommended to measure a small RNA reference, which has a consistent and unvarying expression level to allow for comparison of all other microRNA levels in the different sample preparations.
- The isolation of RNA and reaction steps preceding the real-time PCR reaction should be performed in rooms separate from where the real-time PCR experiments take place.
- It is recommended to assess the integrity of the isolated RNA before proceeding with the real-time PCR experiments. This may be performed either on the Agilent Bioanalyzer or by denaturing agarose gel electrophoresis.
- If necessary, treat RNA preparations with DNases to remove contaminating DNA that may interfere with the real-time PCR results.
- Purified total RNA should be dissolved in nuclease-free water at a stock concentration of at least 1 µg/µL. See recommendations for storage conditions in Tip 2.

Tip 2

General guidelines for handling and storage of RNA

The following precautions should be taken to prevent RNase contamination and degradation of the RNA sample and reagents:

- Always wear latex or vinyl gloves.
- Use nuclease-free, low nucleic acid binding, plasticware and filter barrier pipette tips.
- Keep tubes capped when possible. Always spin tubes before opening.
- For storage of RNA it is recommended to precipitate the RNA with ethanol and keep the RNA at –20°C.
- For long-time storage the RNA may be stored at -80°C. Avoid repeated freezing and thawing cycles.

Tip 3

Good PCR laboratory practice

To reduce the risk of contaminating PCRs with "old" PCR amplicons and consequently obtain false results:

- Always wear a clean lab coat. Use separate lab coats, when setting up PCR reactions and handling PCR products.
- Change gloves whenever you suspect they have been contaminated.
- Establish and maintain designated areas for PCR set-up, PCR amplification, and gel electrophoresis of PCR products.
- Never bring amplified PCR products into the PCR set-up area.
- Open and close all sample tubes carefully. Try not to splash or spray PCR samples.
- Keep reactions and components capped whenever possible. Always spin tubes before opening.
- Use filter barrier pipette tips to avoid aerosol-mediated contamination of your pipetting device.
- Clean laboratory benches and equipment regularly.
- Adapt protocols using dUTP and Uracil DNA glycosylase to degrade "old" PCR amplicon in the initial steps of the PCR reaction.

Tip 4

Keep reagents and reactions cool at all times.

In order to ensure optimal performance of the miRCURY LNA™ microRNA PCR system it is important that the reagents and reactions are kept on ice (or at 4° C) as much as possible during the protocol (apart from steps specifically involving raised temperatures). It is recommended that equipment (e.g. centrifuges and PCR blocks) is cooled before use to avoid exposure to increased temperatures for significant periods of time.

Tip 5

First-strand cDNA synthesis

To avoid loss of sample, it is recommended that the first-strand reaction is performed in nuclease-free low nucleic acid-binding (siliconized) microcentrifuge tubes.

Tip 6

Recommended controls

The following controls are recommended to be included in the experimental set-up:

- **Reverse transcription controls**, i.e. first-strand cDNA synthesis reactions performed without reverse transcriptase. If a PCR product is amplified from this control reaction it indicates genomic DNA contamination of the template RNA.
- **Non-template controls in the real-time PCR amplification**, i.e. real-time PCR reactions performed without any cDNA template. This control will reveal PCR product contamination of the reaction.

Tip 7

Passive reference dye (ROX)

Many real-time PCR instruments will only produce reliable results when a passive reference dye such as ROX is added to the PCR reaction. The reference dye is used to normalize signals from individual PCR-wells in order to enable comparison of real-time PCR amplification signals across an entire PCR-plate. It is recommended to examine whether your real-time PCR instrument has this type of requirement.

The amount of ROX to include in the PCR reaction depends on the requirements of the real-time PCR instrument and must be adjusted accordingly. In general, it is recommended to follow the supplier's instructions for preparation and concentrations of ROX solutions. Table 7 lists recommendations for amounts of ROX from a selected number of instruments. Typically, real-time PCR instruments that allow excitation at individual wavelengths for individual dyes (most filter wheel based instruments) require less ROX than instruments that use a single excitation wavelength for all fluorophores (most laser based instruments use excitation at 488 nm).

Note

The miRCURY LNA™ microRNA PCR SYBR® Green master mix (product# 201000) does not contain a passive reference dye.

Table 7

The amount of ROX passive reference dye to be added to each vial of SYBR® Green master mix (2× conc.)

Instrument	Stock concentration	Volume of ROX to be added per vial	Final concentration in PCR reaction
ABI 7000 System ABI 7300 System	100 µM	7.8 µL	300 nM
ABI 7700 System ABI 7900HT System	100 µM	10.4 µL	400 nM
ABI 7500 System Stratagene Mx3000 Stratagene Mx3005P Stratagene Mx4000	10 µM	13 µL	50 nM

Tip 8

Uracil DNA glycosylase (UDG)-based contamination protection

The ability of uracil-DNA glycosylase (UDG) to cleave the N-glycosidic bond between the uracil base and the phosphodiester backbone of DNA has traditionally been used to avoid PCR carryover. In order to remove DNA from previous PCR reactions it is recommended to include UDG in your PCR reaction and replace dTTP with dUTP. The SYBR® Green master mix (vial) is suited for the UDG-based prevention of carryover contamination

Troubleshooting guide

Problem	Suggestion
PCR signal in samples amplified from first-strand synthesis reactions performed without reverse transcriptase	This would indicate contamination of the template RNA with genomic DNA. Perform DNAse treatment of the RNA sample.
PCR signal in no-template PCR reaction	This could indicate contamination of the cDNA template or PCR reagents with amplified PCR product. To avoid this problem good PCR laboratory practice should be followed (see Tip 3, p. 28). Furthermore, the use of Uracil-DNA glycosylase (UDG) is recommended (see Tip 8, p. 30 for details). Exposing the reactions to elevated temperatures (i.e. room temperature) for more than a few minutes during any part of the protocol increases the risk of background signals. It is very important that the reagents and reactions are kept cool (on ice or 4°C) at all times (see Tip 4, p. 26 for details).
Generated signals are weak	• On some real-time PCR cyclers, gain-settings are adjustable. Make sure the gain settings of your real-time PCR cycler have been set to accommodate the signals generated from the specific assay. • RNA samples may contain PCR inhibitors. Further purification or an alternative RNA extraction method may be necessary.
No fluorescent signal is detected during the PCR	Confirm that you have a PCR product by running an aliquot of your PCR reaction on an agarose gel.

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Problem	Suggestion
No fluorescent signal detected during the PCR, but a PCR amplicon can be detected by agarose gel electrophoresis	• Check that the filter in the real time PCR cycler was set to either SYBR® Green or FAM/FITC. • Check that the optical read is at the correct step of the real-time PCR cycles. • Adjust the baseline in the real-time PCR cycler software.

FAQs

What kind of real-time PCR instruments are compatible with the miRCURY LNA™ microRNA PCR System?

The miRCURY LNA™ microRNA PCR System is compatible with all instruments capable of reading green fluorophores such as fluorescein/FITC/FAM and SYBR® Green. The miRCURY LNA™ microRNA PCR System has been tested and found to work on real-time PCR instruments from several leading suppliers of this type of instrument.

What kind of settings should I use on my real-time PCR instrument?

If your real-time PCR instrument supports fluorophores such as fluorescein/FITC/FAM or SYBR® Green your instrument must be set to detect these fluorophores.

Is the miRCURY LNA™ real-time PCR system compatible with other SYBR® Green master mixes?

We do not recommend using other master mixes for real-time PCR analysis with the miRCURY LNA™ primer sets. The primer sets have been optimized and validated using the miRCURY LNA™ microRNA SYBR® Green Master mix and the performance of the primer sets could be compromised by using a different master mix (which may contain different salt and/or enzyme concentrations).

Why is the Universal primer not added to the PCR reaction for the Endogenous controls?

The Endogenous controls have been designed with two gene specific PCR primers and therefore do not require the addition of the Universal reverse primer which is added to the microRNA PCR reactions. The Endogenous control primer mix is provided in the Endogenous control primer set as "PCR primers" (label marked with blue line). Please see page 20 for details on how to set up the reactions.

What is the difference between the 5S reference assay (positive control) included with the SYBR® Green master mix kit and the 5S rRNA endogenous control primer set?

The 5S reference assay included in the miRCURY LNA™ SYBR® Green master mix is based on reverse transcription using the random hexamer primer supplied with the miRCURY LNA™ first strand cDNA synthesis kit. The 5S Endogenous control primer set is based on a gene specific reverse transcription step followed by PCR amplification with specific forward and reverse primers. The 5S reference assay is mainly a positive control assay to check that the components of the system are working properly. The 5S Endogenous control has been developed and validated for use in normalizing microRNA expression experiments.

My RNA is already enriched for miRNAs, how much should I use in the real-time PCR experiments?

The miRCURY LNA™ microRNA PCR system is developed for use on total RNA and we do not recommend enriching for small RNAs. Samples of enriched microRNAs are difficult to quantitate accurately making it very tricky to ensure the same amount of sample is added to each reaction. If necessary, a total RNA equivalent should be used for the enriched sample, e.g. use a proportional amount of enriched sample resulting from 1-10 ng of total RNA. It may be necessary to try a couple of different amounts of enriched sample to ensure that the results fall within the linear range of the assay.

Why should I use UDG (UNG) in my PCR amplification reactions?

Uracil-DNA glycosylase (UDG or UNG) is recommended if there is a signal in the "no template" control PCR reaction which could be due to contamination of the reaction with previously amplified PCR products. The use of UDG also provides an added advantage to a hot start by degrading all PCR products made prior to the first full cycle. UDG removes contaminating templates by cleaving the N-glycosidic bond between the uracil base and the phosphodiester backbone of DNA. Please refer to page 20 of for details on how to set up reactions using UDG and page 22 for instructions regarding cycling conditions when using UDG.

What is the recommended experimental set up for microRNA real-time PCR?

In general, the following experimental set up is recommended for each biological replicate: 2 RT reactions followed by 2 PCR reactions for each RT reaction (giving rise to 4 data points for each microRNA and sample). However, since it is the RT reaction which usually gives rise to most of the variation, it may be advisable to perform 3 RT reactions with 1-2 PCR reactions for each sample, if amount and economy allow. It is further recommended to always include at least three biological replicates (separate RNA extractions) of each sample type in order to allow statistical analysis of the results. If small changes in microRNA expression are expected, it may be necessary to include more replicates to ensure a significant result. In general it is recommended that replicates should be included at any stage during sample procurement, processing, RNA isolation, etc. that could give rise to variation between samples.

For a complete list of FAQs and further guidance, please go to www.exiqon.com/microrna-real-time-pcr

Note

Information on the miRCURY LNA™ microRNA PCR System and technical data is available at www.exiqon.com. For technical assistance, please e-mail support@exiqon.com. Alternatively call +45 45 650 929 (outside North America) or +1 781 376 4150 (in North America).

Appendix: Protocol for 5S rRNA reference assay

Before starting the experiment

The protocol is optimized for an input of 1 - 10 ng total RNA. The amount of total RNA to be used may vary depending on the microRNA expression levels in the cells or tissue to be analyzed. For highly expressed microRNAs it is possible to use as little as 10 pg total RNA as starting material. If a larger amount is used, and the microRNA of interest is highly expressed, we recommend diluting the cDNA a 100-fold or more.

Total RNA should be prepared using a method that preserves small RNA species. The miRCURY™ RNA Isolation kits are recommended for use with the miRCURY LNA™ microRNA PCR System. DNAse treatment may be recommended, please see Tip 1.

See tip 1

RNA work requires specific handling and precautions should be taken to prevent RNase contamination and degradation of the RNA sample and reagents. Find information on how to handle RNA in the tips section (p. 24). The tips sections also list simple guidelines for good laboratory practice for optimal performance of PCR experiments.

See tip 2 & 3

The miRCURY LNA™ 5S rRNA reference protocol is a two-step protocol consisting of:

1. Random-primed first-strand synthesis of small RNAs to cDNA
2. Real-time PCR amplification of the 5S cDNA product.

It is critical for the success of the experiment to follow the protocol setup below. The protocol should be carried out in a continuous workflow from first-strand cDNA synthesis to real-time PCR amplification (around 3 hours). However, if necessary the assay can be paused immediately after the first-strand cDNA synthesis. In this case, the cDNA may be left overnight at 4°C or for longer time at or -20°C.

First-strand cDNA synthesis

- using the miRCURY LNA™ First-strand cDNA synthesis kit with random hexamer primer.

Protocol

Important: Keep reagents and reactions on ice (or at 4°C) at all times.

See tip 4

Step 1
Dilute template RNA

Adjust each of the template RNA samples to a concentration of 1-10 ng in 4.5 μL using nuclease free water.

Step 2
Prepare reagents listed in Table 4

Place the 5× RT Reaction buffer, Random hexamer primer, dNTP mix and Nuclease-free water on ice and thaw for 15-20 min. Immediately before use, remove the enzymes (Reverse transcriptase and RNase inhibitor) from the freezer and mix by flicking the tubes and place on ice. The rest of the reagents are mixed by vortexing. Spin down all reagents.

See tip 4 & 5

Step 3
Combine reagents according to Table 4

If performing first-strand cDNA synthesis on multiple RNA samples, it is recommended to prepare a RT master mix of the relevant reagents (indicated in Table 4). The following procedure is recommended:

1. Prepare the RT master mix and place it on ice. It is recommended to include 10% excess of all reagents to allow for pipetting losses.
2. Dispense 5.5 μL of RT master mix into microcentrifuge tubes.
3. Add 4.5 μL of template RNA in each tube.

Step 4
Mix and spin reagents

Mix the reaction by gentle vortexing or pipetting to ensure that all reagents are mixed thoroughly. After mixing, spin down.

Table 4 - Random First-strand cDNA synthesis reaction

Reagent	Volume (µL)	Final conc./amount
Template total RNA (adjusted)	4.5	10 ng
Nuclease-free water	1	
5× RT Reaction buffer	2	1×
Reverse transcriptase	0.5	10 U
RNase inhibitor	0.5	20 U
Random hexamer primer (Supplied with the miRCURY LNA™ First-strand cDNA synthesis kit)	1	60 µM
dNTP mix	0.5	500 µM
Total volume	**10 µL**	

Step 5
Incubate for 10 min at 25°C followed by 30 min at 50°C. Heat-inactivate the reverse transcriptase for 5 min at 85°C

Although not recommended, the protocol can be paused at this stage. The cDNA may be left overnight at 4°C or for longer time at -20°C.

Real-time PCR amplification

- using the miRCURY LNA™ SYBR® Green master mix and the 5S rRNA, Reference primer mix.

Important: Keep reagents and reactions on ice (or at 4°C) at all times.

See tip 4

Step 6 Prepare reagents listed in Table 5	Place all reagents on ice and thaw for 15-20 min. Protect the SYBR® Green master mix vials from light. Immediately before use, mix the SYBR® Green master mix by inverting the tube and the rest of the reagents by vortexing. Spin down all reagents.
Step 7 Dilute cDNA template 1:10 in nuclease free water	It is recommended to dilute the cDNA (produced by the protocol above) to allow pipetting of a larger volume of the reaction into the subsequent real-time PCR reaction as this generally results in more accurate pipetting and reproducible results.

See tip 6 & 7

Step 8
Combine reagents according to Table 5

When multiple real-time PCR reactions are performed it is recommended to prepare a master mix of all other reagents than the cDNA template (as indicated in Table 5). The following procedure is recommended:

1. Prepare the PCR master mix and place it on ice. It is recommended to include 10% excess of all reagents in the master mix to allow for pipetting losses.
2. Place cDNA template (4 µL) in the tubes.
3. Add 16 µL of PCR master mix to each tube.

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Step 9
Mix and spin reagents

Mix the reaction by gentle vortexing or pipetting to ensure that all reagents are mixed thoroughly. After mixing, spin down.

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Table 5 - Real-time PCR reaction

	Reagent	Volume (µL)	Final conc./ amount
	Diluted cDNA template	4	
PCR master mix	SYBR® Green master mix	10	1×
PCR master mix	Reference primer mix, 5S rRNA (Supplied with the miRCURY LNA™ SYBR® Green master mix)	1	
PCR master mix	Passive reference dye (stock conc. 1 mM) (optional – see Tip 7, p. 27)		depending on instrument
PCR master mix	Uracil-DNA-glycosylase (UDG) (optional - see Tip 8, p. 28)		0.5 U
PCR master mix	Nuclease free water	Up to 16 µL	
	Total volume	**20 µL**	

Step 10
Real-time PCR amplification.

- Perform 40 cycles of PCR amplification followed by melt curve analysis according to table 6a if Uracil DNA Glycosylase (UDG) is NOT used.
- If UDG is used, perform PCR amplification according to table 6b (page 42) and see comments below the table.

Table 6a- Real-time PCR cycle conditions (without UDG)

Process step	LC480 Instrument settings	ABI Instrument settings
Polymerase Activation/Denaturation	95°C, 10 min	95°C, 10 min
40 amplification cycles	95°C, 10 s 60°C, 20 s Optical read	95°C, 20 s 60°C, 60 s Optical read
Melting curve analysis*		

* Optional: Melting curve analysis of the PCR product(s) may be performed to verify their specificity and identity. Melting curve analysis is an analysis step built into the software of instruments. Please follow the instructions provided by the supplier.

Note: The T_m of a PCR product depends on buffer composition and salt concentration. T_m values obtained when using miRCURY LNA™ SYBR® Green master mix may differ from those obtained using other reagents.

Note: The LC480 Instrument settings are optimized for fast-cycling real-time PCR instruments. The ABI Instrument settings are suitable for most other types of real-time PCR machines with standard cycling capabilities.

Table 6b- Real-time PCR Cycle conditions using UDG*

Process step	LC480 Instrument settings	ABI Instrument settings
UDG degradation (Optional step)	37°C, 10 min	37°C, 10 min
Polymerase Activation/Denaturation	95°C, 10 min	95°C, 10 min
40 amplification cycles Melting curve analysis	95°C, 10 s 60°C, 20 s Optical read	95°C, 20 s 60°C, 60 s Optical read
Final	72°C, forever	72°C, forever

* The following comments are relevant when Uracil DNA Glycosylase (UDG) is used:
  - This protocol is based on the use of a UDG that has maximal enzymatic activity at 37°C and is inactivated at 95°C. Other UDGs may have different temperature optima, and the UDG degradation step should be adjusted accordingly.
  - The final step (72°C, forever) is only required if end point analysis by gel electrophoresis is desired. The UDG will restore activity when returning to lower temperatures resulting in breakdown of newly generated PCR amplicon. The PCR reactions should be removed within a few hours and transferred to -20°C.

Step 11 Analyze data	Analyze data using the software supplied with the real-time PCR instrument.

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