GenePharma

Hairpin-it™ miRNAs qPCR Quantitation Kit

For the detection and quantification of microRNAs using real-time PCR detection instruments.

Catalog No. QPM-010/ QPM-011/ QPM-012/ QPM-013

User Manual

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Kit Contents and Storage

Cat. No. QPM-010	**Size: 100rxns**
Cat. No. QPM-011	**Size: 200 rxns**
Cat. No. QPM-012	**Size: 300 rxns**
Cat. No. QPM-013	**Size: 500 rxns**

Hairpin-it™ miRNAs qPCR Quantitation Kit Reagents

Hairpin-it™ miRNAs qPCR Quantitation Kit Reagents box includes the following items. Store the components at –20℃. **Reagents must be stored in the dark.**

Reagent	Amount			Storage
	100rxns	200rxns	500 rxns	
Real-time PCR Master Mix（5×）*	1.0 ml	2×1.0 ml	5×1.0 ml	4℃
miRNA specific primer Set（10μM）	100μl	200μl	1.0 ml	−20℃
miRNA RT primer（10μM）	15μl	50μl	100μl	−20℃
Taq DNA polymerase （5U/μl）	50μl	100μl	200μl	−20℃
Synthetic miRNA Standard	1nmol			−20℃
1X RNA Dilution buffer	2×1.0 ml	4×1.0 ml	10×1.0 ml	4℃

*Include dNTP Mixture， Mg^{2+}, Molecular Beacon probe.

Accessory Products

Accessory Products

Some of the reagents supplied in the Hairpin-it™ miRNAs qPCR Quantitation Kit as well as other products suitable for use with the kit are available separately from Genepharma. Ordering information is provided below. For more information, refer to our Web site (**www.genepharma.com**).

Item	Amount	Catalog no.
U6 snRNA qPCR Normalization Kit	20 rxns	QPM-090
	50 rxns	QPM-091
5S rRNA qPCR Normalization Kit	25μl×50 rxns	QPM-080
	25μl×100 rxns	QPM-081
miRNAs qPCR Quantitation Core Kit	100 rxns	QPM-030
	200 rxns	QPM-031
	500 rxns	QPM-032
Custom miRNAs Quantitation Kit	40μl×50 rxns	QPM-040
	40μl×100 rxns	QPM-041
	40μl×200 rxns	QPM-042

Introduction

Overview

Introduction

The Hairpin-it™ miRNAs qPCR Quantitation Kit is a sensitive and specific method using real-time PCR for the detection and quantification of microRNAs (miRNA) from total RNA samples. MiRNAs are small, single-stranded, ~19–23 nt RNA molecules encoded in the genomes of plants, animals, and viruses. Mature miRNAs enter the RNA-induced silencing complex (RISC) and guide the RISC to induce translational repression or endonucleolytic cleavage of specific target mRNAs. Unlike commonly used methods for detection of miRNAs, the Hairpin-it™ miRNAs qPCR Quantitation Kit is more rapid and sensitive.

This Kit contains miRNA specific RT and PCR primer set, with a Molecular Beacon probe included. The stem-loop like miRNAs RT primer and the miRNAs specific Beacon probe ensure the RT and PCR reaction would not be interfered by the miRNAs precursors. The reaction template could be total RNA or cell lysates.

To normalize for RNA content among different experimental samples, the **U6 snRNA qPCR Normalization Kit** (Cat # QPM-050) and **5S rRNA qPCR Normalization Kit** (Cat # QPM-060) are available separately. For more information about these kits, see: http:// www.genepharma.com.

The system enables highly sensitive detection from as few as 10 copies of a target miRNAs, with a broad dynamic range that supports accurate quantification of high-copy mRNA from up to 1 μg of total RNA:

□ **High specificity** ensurenced by stemloop RT primer and miRNAs molecular Beacon probe; even highly homogized miRNAs can be accurately dicriminated.

□ **Higher dynamic range and sensitivity** of miRNAs quantification than conventional method(such as Northern blot & micro-array) with broad dynamic range of at least seven orders of magnitude, and in this ready to use formulation can quantify as few as 7 copies of a miRNA target in as little as 0.1 pg of total RNA.

□ **Less sample requairment** Total RNA, cell lysate or purified samll RNA can work as the qPCR Quantitation Kit's template, even the genomic DNA contamination would not interfere in miRNAs quantification.

□ **miRNA synthetic standard** can work as a standard curve when you need to know the absolute number of a miRNA in one cell or work as positive control when you need to know the relative expression ratio between miRNA and U6 snRNA or 5S rRNA..

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Schematic Description of Hairpin-it™ miRNAs qPCR Quantitation Assay

Hairpin-it™ miRNAs real-time Quantitation assay of miRNAs includes two steps, stem–loop RT and real-time PCR. Stem–loop RT primers bind to at the 3' portion of miRNA molecules and are reverse transcribed with reverse transcriptase. Then, the RT product is quantified using real-time PCR that includes miRNA-specific forward primer, reverse primer and a dye-labeled Molecular Beacon probes.

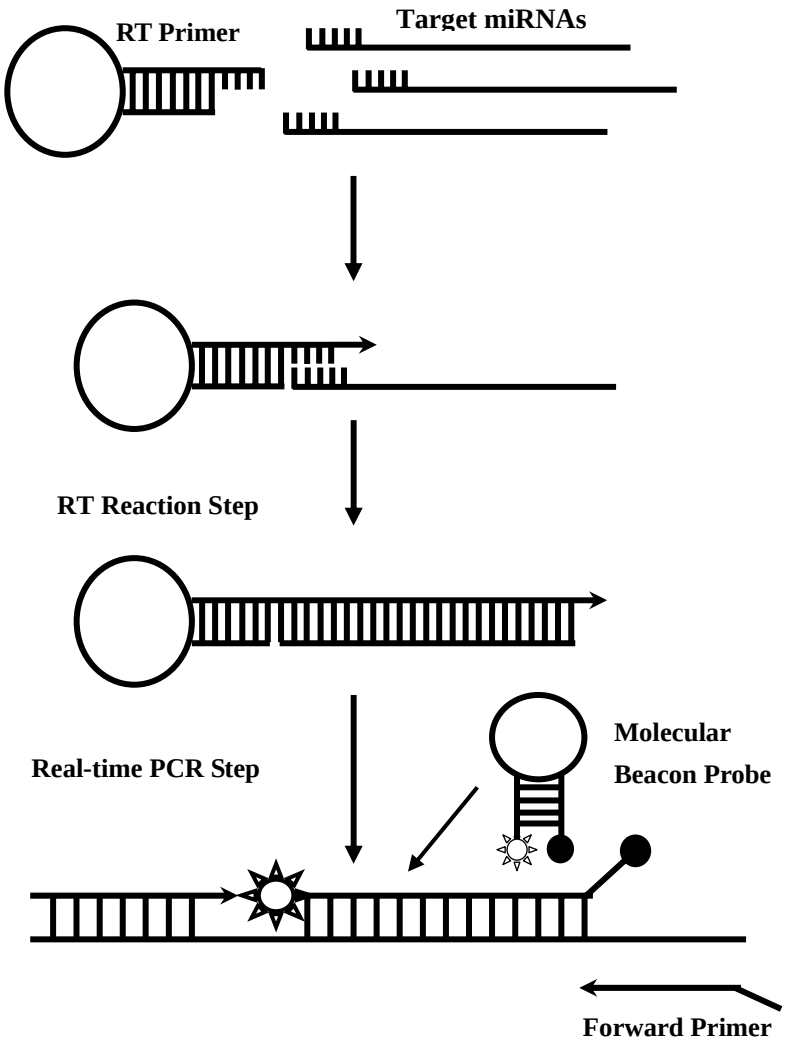

Fig 1. Schematic of Hairpin-it™ miRNAs real-time Quantitation assay

Components of the Kit

Introduction	This section provides more information about the reagents supplied in the Hairpin-it^{TM} miRNAs qPCR Quantitation Kit.
Real-time PCR Master Mix 5×Conc.	The 5×Real-time PCR Master Mix consists of a proprietary buffer system, $MgCl_2$, dNTPs, and a miRNA specific Molecular Beacon probe. The mix includes 0.2 mM of each dNTP and 5mM $MgCl_2$, and has been confirmed to be work well for most targets in restrict lab experiments. However, the optimal Magnesium concentration may range from 3 to 6 mM, and so if necessary, use the separate tube of 25mM magnesium sulfate to increase the magnesium concentration.
	It's very important for you to store the 5×Real-time PCR Master Mix in the dark.
microRNA Specific RT primer 10μM	The miRNAs RT primer is a stem-loop like RT primer, with a miRNA specific region in the 3' end and provide better RT specificity and efficiency than linear ones. Base stacking of the stem enhance the thermal stability of the RNA–DNA hetero-duplex. Furthermore, spatial constraint of the stem–loop would likely improve the assay specificity in comparison to conventional linear RT primers.
miRNA specific primer & probe Set 10μM	The pre-designed miRNAs primer and Molecular Beacon probe set are specific for mature miRNAs and can discriminate among related miRNAs that differ by as little as one nucleotide. For the sequence of primer sets, please log on http:// www.genepharma.com
Synthetic microRNA Standard 1 nmol	The synthetic miRNAs standard can work as a miRNA control when spiked into the total RNA extracted from tissue or cells. If the exact copy number of the miRNA in a certain amount RNA sample was wanted, you can dilute the synthetic miRNAs standard into 5~7 order of magnitude to make the standard curve and then calculate the copy number depend on the Ct value you got in the experiment.
RNA Dilution buffer 1×	The 1X RNA Dilution Buffer is supplied with the kit for use in diluting the miRNA standard for the concentration magnitude of a standard curve. It is preferred to store the miRNA dilution at –80℃ or –20℃。
Taq DNA polymerase 5U/μl	For most PCR reaction, the final concentration of DNA polymerase was ussually 0.05U/μl per reaction volume.

User-Supplied Required Materials

Reverse Transcriptase	For detection and quantification of the miRNAs in your sample, a RT reaction use the miRNA specific RT primer provided in the kit was needed. We recommended using traditional Reverse Transcriptase such as MMLV.
RNase Inhibitor	To keep the activity of Reverse Transcriptase, adding RNase Inhibitor in your RT reaction was recommended. The final concentration usually was 0.25 U/μl.
Rox reference dye	To normalize the fluorescence difference from well to well, adding Rox reference dye in your qPCR system was recommended when using the Real-Time PCR instruments such as ABI PRISM 7000/7300/7500/7900, MX3000p/4000.
Optical PCR plates or tubes	As to eliminate the impact on fluorescence detection caused by PCR tubes or plates, using optical PCR plates and tubes was recommended to corporate the requirements of the Real-Time PCR instruments such as ABI PRISM 7000/7300/7500/7900, MX3000p/4000.

Method

Performing miRNAs RT Reaction

Introduction

The Hairpin-it™ miRNAs real-time Quantitation assay of miRNAs includes two steps, stem–loop RT reaction and real-time PCR detection. Stem–loop RT primers bind to at the 3' end of miRNA molecules and are reverse transcribed with reverse transcriptase. Then, the RT product is quantified using real-time PCR that includes miRNA-specific forward primer, reverse primer and a dye-labeled Molecular Beacon probes.

To exactly quantifying the copy number of microRNA in a certain RNA sample, a standard curve should be established.

To profiling microRNA's expression relative to a said house-keeping gene, U6 snRNA or 5S rRNA qPCR Normalization Kit should be needed, see page 1 "Accessory products ". For more detailed information about these two kits, please log on http:// www.genepharma.com.

Handling the synthetic microRNA standard

The synthetic microRNA standard is supplied as 1 nmol dry powder Oligo stock. Following the guideline below when handling the synthetic microRNA standard stock.

- **Dilution:** Carefully open the tube, add 1ml RNA dilution buffer to dilute the stock to 1μM. Prepare another 1.5ml tube to dilute the 1μM microRNA standard solution to 0.1μM as work concentration to a probably volume.
- **Storage:** It is important to store the 1μM microRNA standard solution and the 0.1μM work concentration solution at –20℃. And it is preferred to store the solution at –70℃ for longer time.
- **RNase-free conditions:** Take precautions to ensure that the stock solution does not become contaminated with RNase.
  a. Use RNase-free sterile pipette tips and supplies for all manipulations.
  b. Wear gloves when handling reagents and solutions.

Handling the microRNA RT primer

The microRNA RT primer is supplied as 10μM solution. Prepare another 1.5ml tube to dilute the 10μM microRNA RT primer solution to 1μM as work concentration to a probably volume using RNase free H_2O. Store the 10μM microRNA RT primer solution and the 1μM work concentration solution at –20℃. For a standard microRNA RT reaction, the final concerntration of RT primer is 50 nM.

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Performing miRNAs RT Reaction, continued

Preparing The RT Reaction Mix

The template for the Hairpin-it™ miRNAs real-time Quantitation assay can be total RNA, cell lysate or purified microRNA. The RNA template's input range can vary from 0.2 ng to 200 ng or more as for as the requirement of the experiment. Other than the stability at 42℃, there is no special requirement of Reverse Transcriptase for the RT reaction. We recommended a standard 25μl RT reaction size.

The following table provides RT reaction Mix volumes for a standard 25μl reaction size.

Component	Final Con.	Vol /1 rxns
5×RT Buffer	1×	5μl
DTT（0.1M）	0.01 M	2.5μl
dNTP（10mM）	0.25 mM	0.75μl
Mg^{2+}（25mM）	3 mM	3μl
MiR-RT primers（1μM）[1]	50 nM	1.25μl
RNasin（40U/μl）	0.4 U/μl	0.25μl
MMLVReverse Transcriptase（200U/μl）	100 U	0.5μl
RNA Sample （0.2 ng to 200 ng total RNA）[2]	0.2 ng to 200 ng	Xμl
RNase Free H_2O		To 25μl

[1]See the **Important** note on handling the RT primer on page 6.

[2]Add 1 pg to 1μg total RNA or mRNA to each reaction, the exact volume is feasible.

[3]Votex the RT reaction reagents and Mix before RT reaction.

Performing miRNAs RT Reaction

Standard RT Reaction Program

30 min at 16_℃，30 min at 42_℃，5 min at 85_℃

Keep all components, reaction mixes and samples on ice. After assembly, transfer the reaction mixes to a thermal cycler preheated to the cDNA synthesis temperature and immediately begin RT reaction.

Performing miRNAs Real-Time PCR Reaction

Handling the microRNA RT product

For the Hairpin-it™ miRNAs real-time Quantitation assay, pipette 4μl microRNA RT reaction product as the template for real-time PCR step subsequently. Store the surplus microRNA microRNA RT reaction product at –20℃.

Preparing Real-Time Reaction Mix

The following table provides Real-time PCR reaction Mix volumes for a 40μl reaction size. Note that preparation of a master mix is crucial in quantitative applications to reduce pipetting errors.

Component	Final Con.	Vol /1 rxns
5×Real-time PCR Buffer [1]	1×	8μl
miR specific Primer set(10μM) [2]	0.2μM	0.8μl
microRNA RT product		4μl
Taq DNA polymerase（5U/μl）	0.5 U/μl	0.4μl
dd H_2O		To 40μl

[1]The 1×Buffer contains 3mM Mg2+, 0.2mM dNTP, 0.2 mM Molecular Beacon probe.

[2]The miR specific Primer set includes PCR forward and reverse primers.

For multiple reactions, prepare a master mix of common components, add the ppropriate volume to each tube or plate well, and then add the unique eaction components.

Performing miRNAs Real-time PCR Reaction

Program the real-time PCR instrument to perform PCR amplification as shown below.

94℃ for 3minute hold, 40 cycles of:

94℃, 15 seconds

55℃, 25 seconds

72℃, 25 seconds

Fluorescence detection step is at 55℃.

ROX Reference Dye

ROX Reference Dye can be included in the reaction to normalize the fluorescent reporter signal, for instruments that are compatible with that option. ROX is often supplied at a 25μM concentration. Use the following table to determine the amount of ROX to use with a particular instrument:

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Performing miRNAs Real-Time PCR Reaction, continued

Instrument	Amount of ROX per 40 µl reaction	Final ROX Conc.
ABI 7000, 7300, 7900HT	0.8 µl	500 nM
ABI7500 Mx3000P, Mx4000	0.08 µl	50 nM

To accurately pipette 0.08 µl per reaction, dilute ROX 1:10 immediately before use and use 0.8 µl of the dilution.

Construction a standard curve

The synthetic miRNAs standard provided by this kit can work as a miRNA control when spiked into the total RNA extracted from tissue or cells. If the exact copy number of the miRNA in a certain amount RNA sample was wanted, you can construct a standard curve follow the recommendation step below:

1. Add 1ml RNA dilution buffer to dilute the synthetic miRNAs standard to 1 µM stock. Prepare another 1.5ml tube to dilute the 1 µM microRNA standard solution to 0.1 µM as work concentration to a probably volume.
2. Dilute the 0.1 µM synthetic miRNAs to 0.01 µM by Spiking it into extracted total RNA sample, e.g. adding 1 µl 0.1 µM synthetic miRNAs stock per 10 µl total RNA sample.
3. Dilute the spiked RNA sample into 6~8 order of magnitude with RNase free H_2O.
4. Add **2 µl RNA sample dilution** to 25 µl RT reaction system.
5. Add **4 µl RT reaction product** to 40 µl real-time PCR system.
6. For the standard curve, the 2 µl 0.01 µM spiked RNA sample added into 25 µl RT reaction system represents **5×10^8 copies per reaction**.

1. For multiple reactions, prepare a master mix of common components, add the appropriate volume to each tube or plate well on ice, and then add the unique reaction components (*e.g.*, template). Preparation of a master mix is *crucial* in qRT-PCR to reduce pipetting errors.
2. Make sure that all components are at the bottom of the tube/plate; centrifuge briefly if needed.

Appendix

Technical Service

World Wide Web

Visit the Invitrogen Web Resource using your World Wide Web browser. At the site, you can:

- □ Download manuals in Adobe Acrobat (PDF) format
- □ Explore our catalog with full color graphics
- □ Get the scoop on our hot new products and special product offers
- □ Obtain citations for Invitrogen products
- □ Request catalog and product literature

The Genepharma URL is **www.genepharma.com**

Quality Control

The product is tested functionally by qRT-PCR using total HeLa RNA as template. Kinetic analysis must demonstrate a linear dose response with decreasing target concentration and detection of GAPDH mRNA in 1 pg of total HeLa RNA.

Contact Us

For more information or technical assistance, please call, write, fax, or email. Additional international offices are listed on our web page (www.genepharma.com).

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