GenoSensor Corporation

GenoExplorer™ miRNA qRT-PCR Kit

for Catalog #'s 2001, 2002, 2003, 2004

Version A
April 2007

User Manual

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Literature Citation

When describing a procedure for publication using these products, we would appreciate that you refer to them as the GenoExplorer™ miRNA qRT-PCR Kit.

Patents and Trademarks

Introduction

Overview The GenoExplorer™ miRNA qRT-PCR System is a highly robust and efficient system for amplifying specific microRNA molecules or other small non-coding RNA from small quantities of total RNA to generate sufficient amounts of material for downstream study. This system is engineered for use with as little as 10 ng of isolated total RNA as starting material. The procedure is simple and requires no purification and allows for detection of either mature or precursors microRNAs in the sample in less than three hours.

MicroRNAs are a class of non-coding single stranded RNA molecules that regulate their targets by translational inhibition and mRNA destabilization. Increasing evidence suggests that microRNAs may also play a major role in cellular transformation and carcinogenesis by acting either as oncogenes or tumor suppressors. They are transcribed as primary miRNAs which are then processed to a shorter, hairpin pre-miRNAs approximately 70-90 nucleotides structures by a nuclear enzyme Drosha, then further processed by a cytoplasmic enzyme Dicer RNAse III-like endonuclease to approximately 20-22 nucleotides single stranded Mature miRNAs. Despite hundreds of miRNAs that have been discovered, the actual mechanism by which they regulate cellular functions, such as regulation of developmental timing, pattern formation, and secretion, are not well understood. Several aspects of miRNAs made them elusive and difficult to detect and study, such as their small size, lack of poly-adenylated tails, their propensity to bind targets with imperfect sequence homology, and also their sequence homology within families, the detection technology must be able to distinguish sequences that differ only by 1–2 nucleotides

Product System

Workflow The GenoExplorer™ miRNA qRT-PCR kit contains the first-strand cDNA synthesis and SYBR® Green I qPCR reagent mix.

Following isolation of total RNA, all the miRNAs in the sample are modified by linking an adaptor at the 5’ end using ligase and polyadenlyated using poly A polymerase. The universal RT Primer is then used to synthesize cDNA from the tailed miRNA population by reverse transcriptase. The first-strand cDNA is ready for analysis in qPCR using SYBR Green detection reagents. A specific miRNA primer and the universal primer are used for PCR reactions (Fig 1).

The designed primer sets are optimized to amplify the specific miRNA for either mature or precursor forms. Specific primers for any miRNAs in all species can be chosen and ordered from the GenoExplorer™ miRNA qRT-PCR Primer Sets.

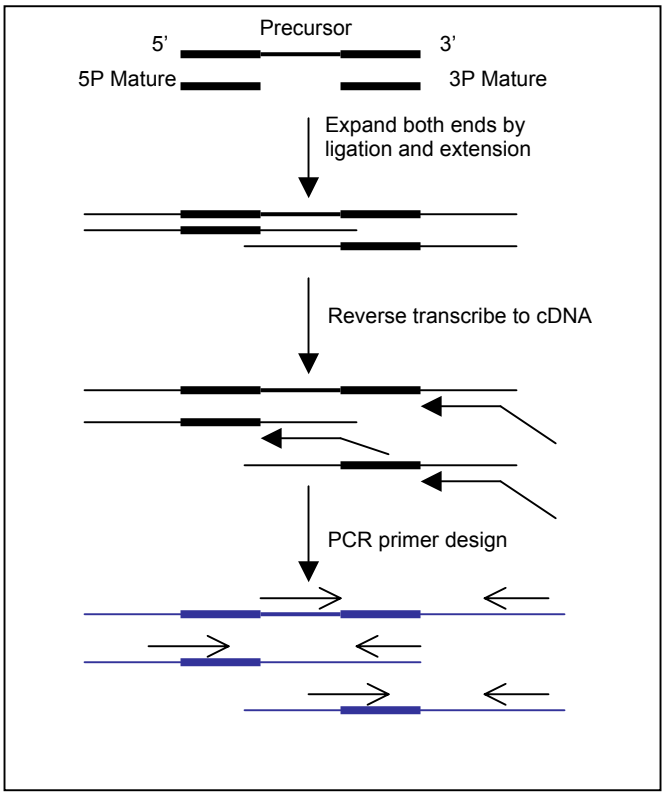

Fig 1. GenoExplorer™ microRNA qRT-PCR principle and primer design.

Advantages of the Kit

Accurate and Specific Quantification

The GenoExplorer™ miRNA qRT-PCR Kit (patented) provides a simple and highly specific method for miRNA quantification. Mature miRNAs situate at either 5' or 3' end or both ends of precursor miRNAs. Both ends of miRNA molecules are elongated in this method by 5' ligation and 3' poly(A) extension. This modification allows primer design for specific miRNA isoforms.

The method generates a miRNA cDNA library. The library will be further analyzed for specific mature or precursor miRNAs using PCR.

Simplified Protocol

The system does not need additional chloroform or column purification. The simple protocol makes the quantitation achievable within one day. All required reagents are included. Specific PCR primers, however, need to be purchased separately based on

specific miRNA targets (refer GenoExplorer™ miRNA qPCR primer sets Cat # 2003) as well as internal reference controls (Cat # 2004).

Flexibility for Real-Time or End-Point Measurement
The assay offers flexibility to do real-time or end-point PCR measurement by a variety of instrument.

Product Specification
The kit provides enough reagents for 10 cDNA synthesis using total or enriched RNA and 100 PCR reactions. cDNA is used for any specific PCR primer pairs. Specific primers are available with separate orders (Cat # 2003 and 2004).

Kit Components and Storage Conditions

- **GenoExplorer™ miRNA qRT-PCR Kit (Cat# 2001)**

Components	Amount	Storage
Poly(A) extension mix	30 µl	-20º C
RT priming mix	80 µl	-20º C
Annealing buffer	60 µl	-20º C
2X RT mix	100 µl	-20º C
2X GenoExplorer SYBR Green qPCR mix	750 µl	-20º C

- **GenoExplorer™ miRNA First-Strand cDNA Core Kit (Cat# 2002)** has everything in Cat# 2001 but not 2X SYBR Green qPCR mix

- **GenoExplorer™ miRNA qPCR Primer Sets (Cat# 2003)**

Components	Amount	Storage
Forward primer (specific miRNA primer)	100 µl	-20º C
Universal reverse primer	100 µl	-20º C

- **GenoExplorer™ miRNA qPCR Reference Primer Sets (Cat# 2004)**

Components	Amount	Storage
Forward primer (specific reference primer)	100 µl	-20º C
Universal reverse primer	100 µl	-20º C

Shipping and Storage

GenoExplorer™ miRNA qRT-PCR kits are shipped on dry ice. Components should be stored at temperatures shown in the above table. At proper storage conditions, components are stable for 1 year from the date received. Expiration dates are also noted on product labels.

Safety Warnings and Precautions

For research use only. Not recommended or intended for the diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals. Consider all chemicals as potentially hazardous. Only persons trained in laboratory techniques and familiar with the principles of good laboratory practice should handle these products. Wear suitable protective clothing such as laboratory overalls, safety glasses, and gloves. Exercise caution to avoid contact with skin or eyes: if contact should occur, wash immediately with water (Material Safety Data Sheet for products is available upon request).

Additional Required Materials

Total RNA containing the small RNA
RNase-free water
Adjustable pipettors
RNase-free tips
RNase-free polypropylene microcentrifuge tubes (0.2, 0.5 or 1.5 ml)
Graduated cylinder
Microcentrifuge
Incubator set at 37º C
Incubator set at 42º C
Incubator or heating block set at 75º C
Heating block at 95º C
Thermocyclers (real-time feature is optional)
Minicentrifuge
Electrophoresis system (optional)

The GenoExplorer™ qRT-PCR kit is open to a variety of equipment. The examples given are only suggestions rather than specific recommendations. Please contact technical support if you have specific questions.

Related Products from GenoSensor

GenoExplorer™ microRNA Array Full Kit (Cat# 1101 – 1199)
GenoExplorer™ microRNA Array Labeling Kit (Cat# 1301)
GenoExplorer™ microRNA Biochips Kit (Cat# 1201 – 1299)
GenoExplorer™ microRNA Probe Set (Cat# 1401 –1499)
GenoExplorer™ Reagents for Hybridization Assay (Cat# 1501 –1504)
GenoExplorer™ microRNA qRT-PCR Kit (Cat# 2001)
GenoExplorer™ microRNA First-Strand cDNA Core Kit (Cat# 2002)
GenoExplorer™ microRNA qPCR Primer Sets (Cat# 2003)
GenoExplorer™ microRNA qPCR Reference Primer Sets (Cat# 2004)

Quick Start Protocol

Part I. Extension Procedure

Add the following reagent in a 0.2 ml DNase/RNase-free tube on ice

- Total RNA (10ng – 1µg) …… X µl
- Poly(A) extension mix ……. 3 µl
- DNase/RNase-free water (to 10 µl total) …. X µl (total 10 µl)

Mix well and incubate at 37 ºC for 30 minutes
Heat at 95 ºC for 5 min and cool on ice

Part II. First-Strand cDNA Synthesis Procedure

Add the following reagent in a 0.2 ml DNase/RNase-free tube on ice

- Extended RNA (from previous step Part I) … 2 µl
- RT priming mix ……. 8 µl (total 10 µl)

Incubate at 46 ºC for 10 min
Chill on ice and add the following

- 2X first-strand mix ……. 10 µl (total 20 µl)

Incubate at 42 ºC for 60 min
Heat at 95 ºC for 5 min
Chill on ice or store at –20 °C

Part III. qPCR Procedure

Directly use previous cDNA or dilute it 1:10 (see detailed protocol)

- cDNA template (from previous step Part II)… 2 µl
- 2X GenoExplorer SYBR qPCR mix …….. 7.5 µl
- Forward primer …….. 1 µl
- Reverse Primer …….. 1 µl
- DNase/RNase-free water …… 3.5 µl (total 15 µl)

Perform PCR using either real-time or end-point measurement

Standard Cycling Program

Denature: 94 ºC for 15 minutes
30 – 45 cycles of:
    Denature: 94 ºC, 30 seconds
    Anneal: 59 ºC, 15 seconds
    Elongate: 72 ºC, 30 seconds
Elongate: 72 ºC, 1 minute
Store: 4 ºC
Note: Annealing temperature may be variable

GenoExplorer™ miRNA qRT-PCR Detailed Protocol

General Description

GenoExplorer™ miRNA qRT-PCR system employs elongation of both ends of miRNAs by a 5'end ligation and 3' poly(A) extension approach, followed by reverse transcription. This offers greater selectivity for PCR primer design and specificity to quantify either mature or precursor miRNA forms. The amplified PCR is specific for individual forms without mixture of both. The modified miRNAs are then reverse transcribed to cDNA. Specific miRNAs are amplified and quantified by PCR.

Handling RNA Samples

When working with RNA, always use proper microbiological aseptic techniques. Use RNase- and DNase-free reagents, water, glassware and plasticware. Use non-powdered gloves during all steps of sample labeling, chip hybridization, washing, detection, and scanning.

RNA Preparation

Total RNA or enriched small RNA samples are recommended. In this protocol, starting from total RNA (10ng – 1μg) was described.

High quality and sufficient amounts of RNA samples is crucial for experiments with microarrays. RNA quality can be evaluated by visualizing the RNA on a gel, as well as by calculating the A_{260}/A_{280} ratio. On a denaturing gel (or on an ordinary agarose gel in denaturing buffer) the RNA should appear as two bright distinct bands that represent the 28S and 18S ribosomal species. The 28S band should be brighter than the 18S band. Tailing of these major bands down the gel, or a background smear behind these bands that gets heavier at lower molecular weights can indicate degradation of the RNA. Degraded RNA will produce high background and low signal intensity microarray results.

PART I. Extension of miRNA

In this step, the miRNA is extended.

Required Materials

The following materials are supplied in the GenoExplorer™ miRNAqRT-PCR kit

- Poly(A) extension mix

The following materials are supplied by the user

- 10ng to 1µg of total RNA
- Microcentrifuge
- Heat block or water bath set at 37 °C
- RNase-free pipette tips
- 0.2 and 1.5-ml DNase/RNase-free microcentrifuge tubes
- DNase/RNase-free water

Extension Procedure

At room temperature, add the poly(A) extension reagents in a 0.2 ml reaction tube

1. **Component Amount**
   - Total RNA (10ng – 1µg) ……. X µl
   - Poly(A) extension mix ……. 3 µl
   - DNase/RNase-free water (to 10 µl total) …… X µl (total 10 µl)

2. Mix gently (do not vortex) and centrifuge the tube briefly to collect the contents.
3. Incubate the tube in a heat block or water bath at 37 °C for 30 minutes.
4. Heat at 95 °C for 5minutes, cool the reaction on ice and proceed immediately to **First Strand cDNA Synthesis.**

Note: Each extended reaction provides enough for five cDNA synthesis reactions.

PART II. First Strand cDNA synthesis

This step is to synthesize the first strand cDNA from the extended miRNAs

Required Materials

The following materials are supplied in the kit

- RT priming mix
- Annealing buffer
- 2X RT mix

The following materials are provided by the user

- Thermal cycler or incubator
- Microcentrifuge
- Ice
- 0.2 and 1.5-ml RNase-free microcentrifuge tubes
- RNase-free pipette tips

First-Strand cDNA Synthesis Procedure

1. Centrifuge the 10 µl of extended RNA from previous step and place on ice. Use 2 µl of the 10 µl for the following step.
2. **Component Amount**
   - Extended RNA (from previous step Part I) ……. 2 µl
   - RT priming mix …… 8 µl
3. Incubate the tube at 46 ºC for 10 minutes.
4. Place the tube on ice for 1 minute.

Note:
- In most cases, Annealing Buffer is not required. If non-specific PCR products are seen, adding the Annealing Buffer and reducing the RT priming mix to keep final volume of 10 µl are recommended.
- This procedure is for a single reaction. However, for multiple reactions, prepare a master mix with a 5% overage for accurate pipetting.

5. Add the following to the tube, to a final volume of 20 µl
   - 2X RT mix …… 10 µl
6. Briefly spin the tube briefly to collect the contents.
7. Transfer the tube to a thermal cycler preheated to 42 ºC and incubate for 60 minutes.
8. Incubate at 95 ºC for 5 minutes to stop the reaction then chill the reaction on ice.
9. Store aliquots at –20 ºC or proceed immediately to **qPCR.**

Note: For starting material of 100ng or less of total RNA, use 2 µl of cDNA from previous step for qPCR reaction. For amounts of 1µg or more of total RNA, make 1:10 dilution of cDNA by adding DNase/RNase-free water to 200 µl (final volumes) and use 2µl of the diluted cDNA for qPCR reaction. For amount of 100ng - 1µg total RNA, dilution should be based on the abundance of your target RNA in the sample.

PART III. qPCR

This step is to quantitatively amplify specific miRNA transcripts from the first strand cDNA. The PCR can be performed by either real time or end point.

Required Materials

The following materials are supplied in the kit

- 2X GenoExplorer SYBR qPCR mix

The following materials are needed to prepare or purchase separately

- Specific PCR primers (purchase GenoExplorer™ miRNA qPCR Primer Sets Cat # 2003).
- Reference control primers (purchase GenoExplorer™ miRNA qPCR Reference Primer Sets Cat # 2004).

The following materials are provided by the user

- Thermal cycler
- Microcentrifuge
- Ice
- 0.2 and 1.5-ml RNase-free microcentrifuge tubes
- RNase-free pipette tips
- DNase/RNase-free water

PCR Procedure

1. Add the following components to each DNase/RNase-free PCR tube or plate well
   Component Amount
   - cDNA template (from step Part II, or diluted) …… 2 µl
   - 2X GenoExplorer SYBR qPCR mix …….. 7.5 µl
   - Forward primer (3 µM) …… 1 µl
   - Reverse Primer (3 µM) …… 1 µl
   - DNase/RNase-free water …… 3.5 µl (total 15 µl)

2. Cap or seal the tube/plate, and gently mix. Make sure that all components are at the bottom of the tube/plate. Centrifuge briefly if needed.
3. Place reactions in a preheated real-time instrument

Suggested Cycling Instrument. The real-time qPCR instrument conditions shown below were executed on an Applied Biosystems ABI 7900 384-well plate system, but also apply to an ABI 7500 or an ABI 7300 real-time PCR system. Other real-time instrument may also be applicable.

Real-time qPCR Program Parameters

Standard Cycling Program
Denature: 94 ºC for 15 minutes
30 – 45 cycles of:
Denature: 94 ºC, 30 seconds
Anneal: 59 ºC, 15 seconds
Elongate: 72 ºC, 30 seconds
Elongate: 72 ºC, 1 minutes
Store: 4 ºC
Note: annealing temperature may be variable

Run the program. After cycling, hold the reaction at 4 ºC until further analysis.

Note on Annealing Temperature
This suggested cycling program recommends an annealing temperature of 59ºC. Increasing the annealing temperature may result in better discrimination of closely related miRNA sequences, but with a slight loss in sensitivity.

Appendix

Troubleshooting Guide

No amplification product	Missing reaction component	Repeat reaction setup.
	Questionable template quality	Analyze starting materials
	Inhibitory substance in reaction	Decrease sample volume. Purify DNA either by alcohol precipitation or dialysis.
	Insufficient number of cycles	Run additional cycles
	Incorrect thermocycler programming	Verify times and temperatures
	Errors in block temperature	Calibrate heating block
	Contaminated tubes or solutions	Autoclave tubes and use filtered pipette tips
	Primer Annealing temperature too High	Lower annealing temperature in 2° C increments
Non-specific Products	Premature Taq-DNA polymerase replication	Add components on-ice (chilled), place PCR-reaction mixtures to preheated (94 °C) thermocycler
	Primer Annealing temperature too low	Raise annealing temperature in 2° increments
	Insufficient mixing of reaction buffer	Reaction buffer must be thoroughly mixed prior to use
	Excess PCR primers	Reduce Forward and Reverse primer concentration to 0.1-0.4 µM
	Excess RT-Primer (RT-Priming Mix)	Reduce RT-Priming mix to 8-4µl and bring the volume to 8µl with Water
	Exogenous DNA contamination	• Use non-aerosol tips • Set dedicated area for reaction setup • Wear gloves.

Technical Service

For more information or technical assistance, please call, write, fax, or email.

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