USER GUIDE

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NCode™ miRNA First-Strand cDNA Synthesis and qRT-PCR Kits

For polyadenylation and reverse transcription of miRNAs for use in two-step quantitative RT-PCR

Catalog numbers MIRC-10, MIRC-50, MIRQ-100, and MIRQER-100

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Kit Contents and Storage

Shipping and Storage

Kit components are shipped on dry ice and should be stored at –20ºC, *except* SYBR® GreenER™ qPCR SuperMix Universal (provided with MIRQER-100), which may be stored at either 4ºC or –20ºC.

Kit Configurations

MIRC-10 and MIRC-50: The NCode™ miRNA First-Strand cDNA Synthesis Kits include components for polyadenylation and cDNA synthesis, plus the Universal qPCR Primer. Other qPCR reagents must be ordered separately.

MIRQ-100: The NCode™ SYBR® Green miRNA qRT-PCR Kit includes the components provided with MIRC-10, plus Platinum® SYBR® Green qPCR SuperMix-UDG.

MIRQER-100: The NCode™ SYBR® GreenER miRNA qRT-PCR Kit includes the components provided with MIRC-10, plus SYBR® GreenER™ qPCR SuperMix Universal.

	Number of Reactions		
Cat. no.	**Polyadenylation**	**cDNA Synthesis**	**qPCR**
MIRC-10	10	20	—
MIRC-50	50	100	—
MIRQ-100	10	20	100
MIRQER-100	10	20	100

NCode™ miRNA First-Strand cDNA Synthesis Kits

The following reagents and amounts are provided:

Component	MIRC-10	MIRC-50
5X miRNA Reaction Buffer	50 µL	250 µL
25 mM $MnCl_2$	50 µL	250 µL
10 mM ATP	10 µL	250 µL
Poly A Polymerase	8 µL	40 µL
Annealing Buffer	20 µL	100 µL
SuperScript® III RT/RNaseOut™ Enzyme Mix	40 µL	200 µL
2X First-Strand Reaction Buffer (includes $MgCl_2$ and dNTPs)	200 µL	1 mL
Universal RT Primer (25 µM)	60 µL	300 µL
Universal qPCR Primer (10 µM)	250 µL	1.25 mL
DEPC-treated water	2 mL	2 × 2 mL

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Platinum® SYBR® Green qPCR SuperMix-UDG

MIRQ-100 includes the components provided with MIRC-10, plus the following:

Component	Amount
Platinum® SYBR® Green qPCR SuperMix-UDG	2 × 1.25 mL
50-mM Magnesium Chloride ($MgCl_2$)	1 mL
20X UltraPure™ BSA (Bovine Serum Albumin)	300 µL
ROX™ Reference Dye	100 µL

SYBR® GreenER™ qPCR SuperMix Universal

MIRQER-100 includes the components provided with MIRC-10, plus the following:

Component	Amount
SYBR® GreenER™ qPCR SuperMix Universal	2 × 1.25 mL
ROX™ Reference Dye	100 µL

Important

Minimize exposure of SYBR® Green and SYBR® GreenER™ reagents and ROX™ Reference Dye to direct light, to avoid loss of fluorescent signal intensity.

Note

Each polyadenylation reaction provides enough tailed RNA for six cDNA synthesis reactions, and each cDNA synthesis reaction provides enough cDNA for multiple qPCR reactions.

Product Use

For Research Use Only. Not intended for any human or animal diagnostic or therapeutic uses.

Accessory Products

Additional Products

The NCode™ system is an integrated miRNA expression profiling system that includes miRNA isolation, amplification, purification, quantification, labeling, and array hybridization components. Additional products are available separately from Life Technologies. Ordering information is provided below.

For more information, visit our website at **www.lifetechnologies.com** or contact Technical Support (page 23).

Product	Size	Cat. no.
TRIzol® Reagent	100 mL 200 mL	15596-026 15596-018
RNase AWAY® Reagent	250 mL	10328-011
Custom Primers	visit **www.lifetechnologies.com/oligos**	
Platinum® SYBR® Green qPCR SuperMix-UDG	100 reactions 500 reactions	11733-038 11733-046
SYBR® GreenER™ qPCR SuperMix for ABI PRISM®	100 reactions 500 reactions	11760-100 11760-500
SYBR® GreenER™ qPCR SuperMix for iCycler® Instrument	100 reactions 500 reactions	11761-100 11761-500
SYBR® GreenER™ qPCR SuperMix Universal	100 reactions 500 reactions	11762-100 11762-500
RNaseOUT™ Recombinant Ribonuclease Inhibitor	5,000 units	10777-019
Fluorescein NIST-Traceable Standard (50 µM)	5 × 1 mL	F36915
Quant-iT™ Ribogreen® RNA Assay Kit	200–2,000 cuvette assays	R-11490
RediPlate™ 96 Ribogreen® RNA Quantitation Kit	96-well plate (8 × 12 strip wells)	R-32700
PureLink® miRNA Isolation Kit	25 preps	K1570-01
NCode™ miRNA Amplification System	20 reactions	MIRAS-20
NCode™ miRNA Labeling System	20 labeling and hybridization reactions	MIRLS-20
NCode™ Multi-Species miRNA Microarray V2	5 slides	MIRA2-05
NCode™ Multi-Species miRNA Microarray Control V2	10 µL	MIRAC2-01
NCode™ Multi-Species miRNA Microarray Probe Set V2	3 × 384-well plates / 500 pmol per well	MIRMPS2-01

Introduction

System Overview

The NCode™ miRNA First-Strand cDNA Synthesis Kits and qRT-PCR Kits provide qualified reagents for the polyadenylation of microRNAs (miRNAs) from total RNA and synthesis of first-strand cDNA from the tailed miRNAs for use in real-time quantitative PCR (qPCR).

These kits have been optimized for the detection and quantification of miRNA from 10 ng to 2.5 µg of total RNA using a SYBR® Green or SYBR® GreenER™ detection platform. Isolation of small RNAs is typically not required, though it may enhance detection of some rare miRNAs.

SuperScript® III Reverse Transcriptase (RT) in the cDNA synthesis reaction ensures high specificity and high yields of cDNA from small amounts of starting material.

Platinum® SYBR® Green qPCR SuperMix-UDG (included with catalog no. MIRQ-100) ensures optimal qPCR performance using SYBR® Green I dye, with excellent sensitivity and a linear dose response over a wide range of target concentrations.

SYBR® GreenER™ qPCR SuperMix Universal (included with catalog no. MIRQER-100) contains a novel fluorescent double-stranded DNA (dsDNA) binding dye for both higher sensitivity and lower PCR inhibition than SYBR® Green I dye. It can be used on real-time PCR instruments calibrated for SYBR® Green I dye without any change of filters or settings.

Workflow Overview

Following isolation of total RNA, all the miRNAs in the sample are polyadenlyated using poly A polymerase and ATP. Following polyadenylation, SuperScript® III RT and a specially-designed Universal RT Primer are used to synthesize cDNA from the tailed miRNA population.

The first-strand cDNA is ready for analysis in qPCR using SYBR® Green or SYBR® GreenER™ detection reagents, the Universal qPCR Primer provided in the kit, and a forward primer designed by the user that targets the specific miRNA sequence of interest (see page 3 for design guidelines).

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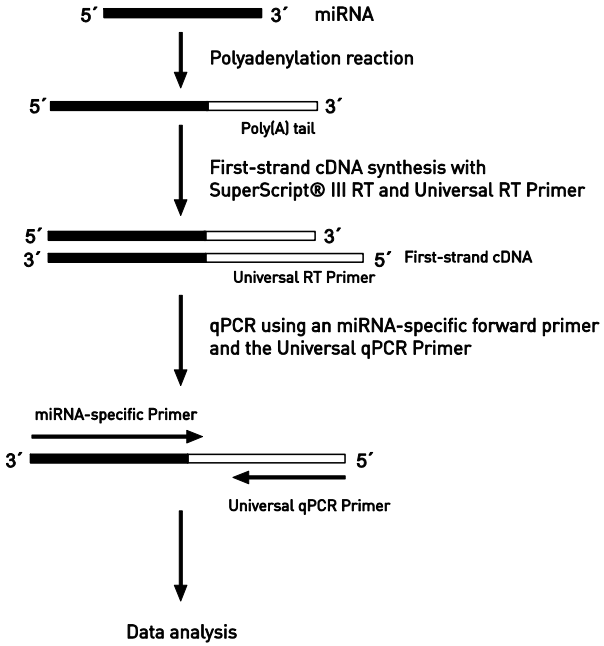
Workflow Diagram



Advantages of the Kit

The NCode™ kits have the following advantages:

- Starting material can range from 10 ng to 2.5 µg of total RNA; enrichment of miRNAs is typically not required
- No proprietary primers or primer-probe assays required for qPCR; you can design your own primers for any miRNA sequence from any species
- Can discriminate between miRNAs that differ by a single nucleotide, for profiling closely related templates
- **Catalog nos. MIRC-10 and MIRC-50:** The Universal qPCR Primer included in the kit gives you the flexibility to order your qPCR detection reagents separately
- **Catalog no. MIRQ-100:** Platinum® SYBR® Green qPCR SuperMix-UDG included in the kit ensures high sensitivity and performance in qPCR using SYBR® Green I fluorescent dye
- **Catalog no. MIRQER-100:** SYBR® GreenER™ qPCR SuperMix Universal included in the kit ensures optimal sensitivity and performance in qPCR using a novel fluorescent dsDNA binding dye

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Forward Primer Design for qPCR

The forward primer in qPCR is specific for the miRNA sequence of interest, and must be ordered separately by the user. As a starting point, we recommend ordering a DNA oligo that is **identical to the entire mature miRNA sequence**. (Note that this is the complement of the reverse-transcribed miRNA sequence from the cDNA synthesis reaction.)

For example, note the following primer design for the miRNA hsa-miR-124a:

`miRNA sequence`	`uuaaggcacgcggugaaugcca`
`Primer sequence`	`ttaaggcacgcggtgaatgcca`

In most cases, using an oligo that is identical to the entire mature miRNA is optimal. In some cases, truncating the primer sequence may be necessary (see page 14).

Note that the Universal qPCR Primer supplied with each kit is used as the reverse primer in qPCR.

Visit **www.lifetechnologies.com/oligos** to order the miRNA-specific forward primer from Life Technologies.

SuperScript® III RT

SuperScript® III Reverse Transcriptase is an engineered version of M-MLV RT with reduced RNase H activity and increased thermal stability (Gerard *et al.*, 1986; Kotewicz *et al.*, 1985). The enzyme can be used to synthesize first-strand cDNA at temperatures up to 55ºC, providing increased specificity, higher yields of cDNA, and more full-length product than other reverse transcriptases.

Platinum® SYBR® Green qPCR SuperMix-UDG

Platinum® SYBR® Green qPCR SuperMix-UDG is a reaction mix containing all components, except primers, for the amplification and detection of DNA in qPCR (Ishiguro *et al.*, 1995; Wittwer *et al.*, 1997). It combines the hot-start technology of Platinum® *Taq* DNA polymerase with integrated UDG carryover prevention and SYBR® Green I fluorescent dye.

The SuperMix is supplied at a 2X concentration and contains Platinum® *Taq* DNA polymerase, SYBR® Green I dye, $MgCl_2$, dNTPs (with dUTP instead of dTTP), uracil DNA glycosylase (UDG), and stabilizers. See the insert provided with Platinum® SYBR® Green qPCR SuperMix-UDG for more details.

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SYBR® GreenER™ qPCR SuperMix Universal

SYBR® GreenER™ qPCR SuperMix Universal is a ready-to-use cocktail containing all components, except primers and template, for the amplification and detection of DNA in qPCR. It combines a chemically modified "hot-start" version of *Taq* DNA polymerase with integrated uracil DNA glycosylase (UDG) carryover prevention technology and a novel fluorescent dye to deliver excellent sensitivity in the quantification of target sequences, with a linear dose response over a wide range of target concentrations.

SYBR® GreenER™ qPCR SuperMix Universal is supplied at a 2X concentration and contains hot-start *Taq* DNA polymerase, SYBR® GreenER™ fluorescent dye, $MgCl_2$, dNTPs (with dUTP instead of dTTP), UDG, and stabilizers.

MicroRNAs

MicroRNAs (miRNAs) are a recently discovered class of small, ~19–23-nucleotide non-coding RNA molecules. They are cleaved from hairpin precursors and are believed play an important role in translation regulation of target mRNAs by binding to partially complementary sites in the 3´ untranslated regions (UTRs) of the message (Lim, 2003). Several groups have hypothesized that there may be up to 20,000 non-coding RNAs that contribute to eukaryotic complexity (Bentwich *et al.*, 2005; Imanishi *et al.*, 2004; Okazaki *et al.*, 2002).

Though hundreds of miRNAs have been discovered, little is known about their cellular function. They have been implicated in regulation of developmental timing and pattern formation (Lagos-Quintana *et al.*, 2001), restriction of differentiation potential (Nakahara & Carthew, 2004), regulation of insulin secretion (Stark *et al.*, 2003), and genomic rearrangements (John *et al.*, 2004).

Several unique physical attributes of miRNAs—including their small size, lack of poly-adenylated tails, and tendency to bind their mRNA targets with imperfect sequence homology—have made them elusive and challenging to study. In addition, strong conservation between miRNA family members means that any detection technology must be able to distinguish between ~22-base sequences that differ by only 1–2 nucleotides. Recent advances in microarray and qPCR detection have enabled the use of these technologies for miRNA screening.

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Other Products in the NCode™ System

The NCode™ SYBR® Green miRNA qRT-PCR Kit and NCode™ miRNA First-Strand cDNA Synthesis Kit were designed and developed in conjunction with the following Life Technologies products (for ordering information, see page vi):

- **The NCode™ miRNA Labeling System** is a robust and efficient system for labeling and hybridizing miRNA to NCode™ microarrays for expression profiling analysis. Using this kit, you ligate a short, highly specific tag sequence to each miRNA, and then hybridize highly fluorescent Alexa Fluor® dye molecules to the tagged miRNA. The high specificity of the binding sequence and high fluorescence of the dye molecules ensure maximum signal and strong signal correlations.
- **The NCode™ miRNA Amplification System** is a robust system for amplifying senseRNA molecules from minute quantities of miRNA. The system provides consistent and accurate ≥1000-fold amplification while preserving the relative abundance of the miRNA sequences in the original sample, allowing you to compare relative quantities across experiments. The resulting amplified miRNA is in the sense orientation, for direct compatibility with NCode™ microarray probe sequences.
- **The NCode™ Multi-Species miRNA Microarray V2** consists of 5 Corning® Epoxide-Coated Glass Slides, each printed with optimized probe sequences targeting all of the known mature miRNAs in miRBase, Release 9.0 (**http://microrna.sanger.ac.uk**), for human, mouse, rat, *D. melanogaster*, *C. elegans*, and Zebrafish. The probes were designed using an algorithm that generates miRNA sequences with enhanced hybridization properties (Goff *et al.*, 2005). Each slide comes blocked and ready to use.
- **The NCode™ Multi-Species miRNA Microarray Probe Set V2** includes the probe sequences provided on the microarray listed above, dried down in 384-well plates at 500 pmoles per well and ready for printing on standard DNA microarray surfaces.
- **The NCode™ Multi-Species miRNA Microarray Control V2** is a synthetic 22-nucleotide miRNA sequence that has been designed and screened as a positive control for use with NCode™ system.

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Materials Supplied by the User

The following materials are required for use with these kits:

- 10 ng to 2.5 µg of total RNA
- Forward PCR primer designed for the miRNA target of interest (see page 3 for design guidelines)
- 1 mM Tris, pH 8.0
- Microcentrifuge
- Heat block, water bath, and/or thermal cycler
- RNase-free pipette tips
- 1.5-mL RNase-free microcentrifuge tubes
- Disposable gloves
- Ice
- Optional: RNaseOUT™ Recombinant Ribonuclease Inhibitor, for the negative RT control
- qPCR instrument
- Appropriate PCR plates/tubes for instrument

Additional materials required for catalog nos. MIRC-10 or MIRC-50.

- qPCR reagents that include SYBR® Green or SYBR® GreenER™ binding dyes (see page vi)

Methods

Isolating Total RNA

Introduction

High quality total RNA is essential for qRT-PCR analysis. In this step, you obtain total RNA or isolate it from a sample.

Note about Isolating Small RNA Molecules

Isolating small RNA molecules from total RNA prior to use of this kit is not required, and may in fact limit the detection of some miRNAs in qRT-PCR. However, for extremely low-abundance miRNAs, multiple total RNA samples (~10 µg each) may be pooled and the miRNA may be enriched from the pooled sample for detection in qRT-PCR. The PureLink® miRNA Isolation Kit is available for this purpose (see page vi).

General Handling of RNA

When working with RNA:

- Use disposable, individually wrapped, sterile plasticware.
- Use aerosol resistant pipette tips for all procedures.
- Use only sterile, new pipette tips and microcentrifuge tubes.
- Wear latex gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin.
- Use proper microbiological aseptic technique when working with RNA.
- Dedicate a separate set of pipettes, buffers, and enzymes for RNA work.
- Use RNase-free microcentrifuge tubes. If it is necessary to decontaminate untreated tubes, soak the tubes overnight in a 0.01% (v/v) aqueous solution of diethylpyrocarbonate (DEPC), rinse the tubes with sterile distilled water, and autoclave the tubes.

You can use RNase AWAY® Reagent, a non-toxic solution available from Life Technologies, to remove RNase contamination from surfaces. For further information on controlling RNase contamination, see (Ausubel *et al.*, 1994; Sambrook *et al.*, 1989).

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Isolating Total RNA

To isolate total RNA, we recommend TRIzol® Reagent (Cat. nos. 15596-026 and 15596-018). Ordering information is provided on page vi.

The PureLink® Micro-to-Midi™ Total RNA Purification System (Cat. no. 12183-018) or PureLink® 96 Total RNA Purification Kit (Cat. no. 12173-011) may also be used.

Amount of Total RNA Required

Use 10 ng to 2.5 µg of total RNA, depending on the abundance of your miRNA targets. The optimal sample range is 100 ng to 1 µg of total RNA.

Poly(A) Tailing of miRNA

Introduction

In this step, you add a poly(A) tail to the miRNA in your total RNA sample.

Required Materials

The following materials are supplied in the NCode™ miRNA First-Strand cDNA Synthesis Kit:

- 5X miRNA Reaction Buffer
- 25 mM $MnCl_2$
- 10 mM ATP
- Poly A Polymerase (PAP)
- DEPC-treated water

The following materials are supplied by the user:

- 10 ng to 2.5 µg of total RNA
- 1 mM Tris, pH 8.0
- Microcentrifuge
- Heat block or water bath set at 37°C
- RNase-free pipette tips
- 1.5-mL RNase-free microcentrifuge tubes

Important

The following reaction uses the **manganese chloride ($MnCl_2$)** supplied in the First-Strand cDNA Synthesis Kit, *not* the magnesium chloride ($MgCl_2$) supplied with Platinum® SYBR® Green qPCR SuperMix-UDG (included with cat. no. MIRQ-100). Be careful to select the vial of $MnCl_2$ for use in the following reaction.

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Note

Each reaction requires 0.5 µL of Poly A Polymerase. To avoid pipetting 0.5 µL of enzyme, do one of the following:

- For multiple reactions, prepare a master mix of all components except RNA, including the equivalent of 0.5 µL of enzyme per reaction
- For a single reaction, dilute the Poly A Polymerase 1:1 with DEPC-treated water. (Dilute 1 µL of Poly A Polymerase with 1 µL of DEPC-treated water, and use 1 µL of the dilution per reaction.)

Poly(A) Tailing Procedure

Use the following procedure to add poly(A) tails to the total RNA:

1. Based on the quantity of total RNA, dilute a volume of 10 mM ATP in 1 mM Tris (pH 8.0) according to the following formula:

   ATP dilution factor = 5000/____ ng of total RNA

   Example: If you are starting with 100 ng of total RNA, the ATP dilution factor is 5000/100 ng = 50. Dilute the ATP 1:50 by adding 1 µL of 10 mM ATP to 49 µL of 1 mM Tris, pH 8.0.

2. Add the following at room temperature to the tube of total RNA. For multiple reactions, prepare a master mix of common components to enable accurate pipetting.

Component	Volume
RNA	x µL
5X miRNA Reaction Buffer	5 µL
25 mM $MnCl_2$	2.5 µL
Diluted ATP (from Step 1)	1 µL
Poly A Polymerase (see **Note** above)	0.5 µL
DEPC-treated water	to 25 µL

3. Mix gently and centrifuge the tube briefly to collect the contents.
4. Incubate the tube in a heat block or water bath at 37°C for 15 minutes.

After incubation, proceed immediately to **First-Strand cDNA Synthesis**, next page.

First-Strand cDNA Synthesis

Introduction

In this step, you reverse transcribe the polyadenylated miRNA to generate first-strand cDNA.

Note

The following reaction uses 4 µL of the 25-µL poly(A) tailing reaction from Step 4, previous page. Each poly(A) tailing reaction provides enough polyadenylated miRNA for up to six cDNA synthesis reactions.

Required Materials

The following materials are supplied in the NCode™ miRNA First-Strand cDNA Synthesis Kit:

- Annealing Buffer
- Universal RT Primer (25 µM)
- 2X First-Strand Reaction Buffer
- SuperScript® III RT/RNaseOUT™ Enzyme Mix

The following materials are provided by the user:

- Thermal cycler or water bath preheated to 65ºC
- Microcentrifuge
- Ice
- 1.5-mL RNase-free microcentrifuge tubes
- RNase-free pipette tips
- Optional: RNaseOUT™ Recombinant Ribonuclease Inhibitor, for the negative RT control

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First-Strand cDNA Synthesis

Use the following procedure to reverse transcribe the polyadenylated miRNA from Step 4, page 10:

1. Add the following to an RNase-free microcentrifuge tube:

Component	Amount
Polyadenylated RNA from Step 4, page 10	4 µL
Annealing Buffer	1 µL
Universal RT Primer (25 µM)	3 µL
Total volume	8 µL

2. Incubate the tube at 65ºC for 5 minutes.
3. Place the tube on ice for 1 minute.
4. Add the following to the tube, for a final volume of 20 µL:

Component	Amount
2X First-Strand Reaction Mix	10 µL
SuperScript® III RT/RNaseOUT™ Enzyme Mix*	2 µL

*For negative RT controls, use 1 µL of sterile, distilled water and 1 µL of RNaseOUT™ Recombinant Ribonuclease Inhibitor instead of the Enzyme Mix.

5. Spin the tube briefly to collect the contents.
6. Transfer the tube to a thermal cycler preheated to 50ºC and incubate for 50 minutes.
7. Incubate at 85ºC for 5 minutes to stop the reaction.

Chill the reaction on ice. Store aliquots at –20ºC or proceed directly to qPCR.

qPCR — Guidelines and Recommendations

Introduction

This section provides guidelines and recommendations for qPCR using either Platinum® SYBR® Green qPCR SuperMix-UDG or SYBR® GreenER™ qPCR SuperMix Universal.

Required Materials

The following materials are provided in all kits:

- Universal qPCR Primer

The following materials are provided with MIRQ-100:

- Platinum® SYBR® Green qPCR SuperMix-UDG
- ROX™ Reference Dye

The following materials are provided with MIRQER-100:

- SYBR® GreenER™ qPCR SuperMix Universal
- ROX™ Reference Dye

The following materials are provided by the user:

- Forward PCR primer designed for miRNA of interest (see next page for detailed design guidelines)
- qPCR instrument
- Appropriate PCR plates/tubes for instrument
- RNase-free pipette tips
- cDNA from Step 7, page 12

Note

Minimize exposure of ROX™ Reference Dye and the SYBR® Green and SYBR® GreenER™ SuperMixes to direct light. Exposure to direct light for an extended period of time may result in loss of fluorescent signal intensity.

Ordering qPCR Reagents Separately

The NCode™ miRNA First-Strand cDNA Synthesis Kits were designed and developed for use with SYBR® Green and SYBR® GreenER™ SuperMixes. If you are using catalog nos. MIRC-10 or MIRC-50, you can order these SuperMixes separately. See page vi for ordering information.

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qPCR Primers

Reverse primer: The Universal qPCR Primer supplied with each kit is used as the reverse primer in qPCR. It is supplied at 10 µM and used at a final concentration of 200 nM.

Forward primer: The forward primer in qPCR is specific for the miRNA sequence of interest, and must be ordered separately by the user. As a starting point, we recommend ordering a DNA oligo that is **identical to the entire mature miRNA sequence**. (Note that this is the complement of the reverse-transcribed miRNA sequence from the cDNA synthesis reaction.)

For example, note the following primer design for the miRNA hsa-miR-124a:

miRNA sequence	uuaaggcacgcggugaaugcca
Primer sequence	ttaaggcacgcggtgaatgcca

In most cases, using an oligo that is identical to the entire mature miRNA is optimal. In some cases, truncating the primer sequence may be necessary (see below).

Visit **www.lifetechnologies.com/oligos** to order the miRNA-specific forward primer from Life Technologies. A final primer concentration of 200 nM is effective for most reactions.

Truncating the Forward Primer

We have seen optimal results with primers that have a melting temperature (Tm) of 55–68ºC. For some GC-rich miRNA sequences, it may be necessary to design a forward primer that is **truncated by 3–4 bases on the 3´ end** to reduce the Tm. If you detect a higher-than-average amount of primer dimers in your qPCR, or if the miRNA sequence is GC-rich, try designing a truncated forward primer.

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Instrument Settings

Platinum® SYBR® Green qPCR SuperMix-UDG and SYBR® GreenER™ qPCR SuperMix Universal can be used with a variety of real-time instruments, including but not limited to:

- Applied Biosystems® 7000, 7700, and 7900HT
- Applied Biosystems® 7300 and 7500 Real-Time PCR Systems
- Applied Biosystems® GeneAmp® 5700
- Bio-Rad iCycler® Instrument
- Agilent Mx3000P®, Mx3005P®, and Mx4000
- Qiagen Research Rotor-Gene® System
- Bio-Rad DNA Engine Opticon®, Opticon™ 2, and Chromo4™ Real-Time Detector
- Cepheid SmartCycler® Instrument

Optimal cycling conditions will vary with different instruments. For additional information, visit **www.lifetechnologies.com/qpcr**.

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™ Reference Dye is supplied with both Platinum® SYBR® Green qPCR SuperMix-UDG and SYBR® GreenER™ qPCR SuperMix Universal at a 25 µM concentration. It is composed of a glycine conjugate of 5-carboxy-X-rhodamine, succinimidyl ester in 20 mM Tris-HCl (pH 8.4), 0.1 mM EDTA, and 0.01% Tween® 20.

Use the following table to determine the amount of ROX™ Reference Dye to use with a particular instrument:

Instrument	Amount of ROX™ per 50-µL reaction	Final ROX™ Conc.
Applied Biosystems® 7000, 7300 7700, and 7900HT	1.0 µL	500 nM
Applied Biosystems® 7500; Agilent Mx3000®, Mx3005P®, and Mx4000	0.1 µL*	50 nM

*To accurately pipet 0.1 µL per reaction, we recommend diluting ROX™ Reference Dye 1:10 immediately before use and use 1 µL of the dilution.

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Melting Curve Analysis

Melting curve analysis should always be performed after qPCR to identify the presence of primer dimers and analyze the specificity of the reaction. Melting curve analysis can identify primer dimers by their lower annealing temperature compared to that of the amplicon. The presence of primer dimers decreases PCR efficiency and obscures analysis and determination of cycle thresholds. For more information, visit **www.lifetechnologies.com/qpcr**.

Fluorescein

The Bio-Rad iCycler® requires the use of fluorescein as a reference dye to normalize the fluorescent reporter signal with SYBR® Green and SYBR® GreenER™ SuperMixes. Fluorescein NIST-Traceable Standard is available from Life Technologies as a 50-µM solution (see page vi). (If you are ordering a SYBR® GreenER™ SuperMix as a separate component, you can order SYBR® GreenER™ qPCR SuperMix for iCycler® instrument, which includes fluorescein in the mix; see page vi.)

We recommend using a final concentration of 50 nM as a general starting point in qPCR. Optimal results may require a titration between 10 and 100 nM.

qPCR Using SYBR® Green SuperMix

Introduction

This section provides a general protocol for qPCR on Applied Biosystems real-time instruments using Platinum® SYBR® Green qPCR SuperMix-UDG.

Note

- Since PCR is a powerful technique capable of amplifying trace amounts of DNA, all appropriate precautions should be taken to avoid cross-contamination.
- For multiple reactions, prepare a master mix of common components (with a ~10% overage for accurate pipetting), add the appropriate volume to each tube or plate well, and then add the unique reaction components (e.g., template). Preparation of a master mix is strongly recommended in qPCR to reduce pipetting errors.
- Note the lower amount of ROX™ Reference Dye required for the Applied Biosystems® 7500.

Template Dilution

Dilute the cDNA 1:10 as described on the next page and use 5 µL of the dilution in a 50-µL qPCR (i.e., 1% v/v cDNA).

Note on Annealing Temperature

The following cycling program recommends an annealing temperature of 60ºC. Raising the annealing temperature to 63–65ºC may result in better discrimination of closely related miRNA sequences, but with a slight loss in sensitivity.

Cycling Program

The cycling program below is designed for Applied Biosystems real-time instruments. This program may also be used as a starting point for other real-time instruments.

Standard Cycling Program for Applied Biosystems® Instruments	**Fast Cycling Program (for the Applied Biosystems® 7500 in Fast Mode)**
50ºC for 2 minutes (UDG incubation) 95ºC for 2 minutes 40 cycles of: 95ºC, 15 seconds 60ºC*, 30 seconds (60 seconds for the 7900HT)	Select *Fast Mode* on Thermal Profile tab 50ºC for 2 minutes (UDG incubation) 95ºC for 2 minutes 40 cycles of: 95ºC, 3 seconds 60ºC*, 30 seconds
Melting curve analysis: See instrument documentation	
*See **Note on Annealing Temperature** above.	

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qPCR Protocol

Follow the steps below to perform qPCR using Platinum® SYBR® Green qPCR SuperMix-UDG. Volumes for a single 50-µL reaction are listed. Volumes can be scaled as needed (e.g., scaled down to a 20-µL reaction volume for 384-well plates).

1. Dilute the cDNA (from Step 7, page 12) 1:10 in DEPC-treated water. Use 5 µL of diluted cDNA per 50-µL reaction (i.e., 1% v/v cDNA).
2. Add the following components to each DNase/RNase-free PCR tube or plate well.

Component	Amount	
Platinum® SYBR® Green qPCR SuperMix-UDG	25 µL	(1X final conc.)
Forward primer, 10 µM	1 µL	(200 nM final conc)
Universal qPCR Primer, 10 µM	1 µL	(200 nM final conc)
ROX™ Reference Dye (optional)	1 µL/0.1 µL	(see page 15)
Template (diluted 1:10, Step 1)	5 µL	(1% v/v cDNA)
DEPC-treated water	to 50 µL	

3. 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.
4. Place reactions in a preheated real-time instrument programmed as described on the previous page. Run the program.

After cycling, hold the reaction at 4ºC until further analysis. Analyze the cycle threshold (Ct) values, slope of the standard curve, Y-intercept, and correlation coefficient (R^2) for your qPCR experiments using the software provided with your instrument.

qPCR Using SYBR® GreenER™ SuperMix

Introduction

This section provides a general protocol for qPCR on Applied Biosystems® real-time instruments using SYBR® GreenER™ qPCR SuperMix Universal.

Note

- Since PCR is a powerful technique capable of amplifying trace amounts of DNA, all appropriate precautions should be taken to avoid cross-contamination.
- For multiple reactions, prepare a master mix of common components (with a ~10% overage for accurate pipetting), add the appropriate volume to each tube or plate well, and then add the unique reaction components (e.g., template). Preparation of a master mix is strongly recommended in qPCR to reduce pipetting errors.
- Note the lower amount of ROX™ Reference Dye required for the Applied Biosystems® 7500.

Template Volume

Dilute the cDNA 1:10 as described on the next page and use 5 µL of the dilution in a 50-µL qPCR (i.e., 1% v/v cDNA).

Note on Annealing Temperature

The following cycling program recommends an annealing temperature of 57ºC. Raising the annealing temperature to 59ºC may result in better discrimination of closely related miRNA sequences, but with a slight loss in sensitivity.

DNA Polymerase Activation

The hot-start DNA polymerase used in SYBR® GreenER™ qPCR SuperMix is activated during the 10-minute incubation at 95°C before PCR cycling.

Continued on next page

qPCR Protocol

Follow the steps below to perform qPCR using SYBR® GreenER™ qPCR SuperMix Universal. Volumes for a single 50-µL reaction are listed. Volumes can be scaled as needed (e.g., scaled down to a 20-µL reaction volume for 384-well plates).

1. Program the real-time instrument as shown below. The cycling program is designed for Applied Biosystems® instruments. It may also be used as a starting point for other real-time instruments.

   50ºC for 2 minutes (UDG incubation)
   95ºC for 10 minutes (UDG inactivation and DNA polymerase activation)
   40 cycles of:
   95ºC, 15 seconds
   57ºC*, 60 seconds

   Melting curve analysis: See instrument documentation

   *See **Note on Annealing Temperature,** previous page.

2. Dilute the cDNA (from Step 7, page 12) 1:10 in DEPC-treated water. Use 5 µL of diluted cDNA per 50-µL reaction (i.e., 1% v/v cDNA).

3. Add the following components to each DNase/RNase-free PCR tube or plate well.

Component	Amount	
SYBR® GreenER™ qPCR SuperMix	25 µL	(1X final conc.)
Forward primer, 10 µM	1 µL	(200 nM final conc.)
Universal qPCR Primer, 10 µM	1 µL	(200 nM final conc.)
ROX™ Reference Dye (optional)	1 µL/0.1 µL	(see page 15)
Template (diluted 1:10, Step 2)	5 µL	(1% v/v cDNA)
DEPC-treated water	to 50 µL	

4. 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.

5. Place reactions in a preheated real-time instrument programmed as described on the previous page. Run the program.

After cycling, hold the reaction at 4ºC until further analysis. Analyze the cycle threshold (Ct) values, slope of the standard curve, Y-intercept, and correlation coefficient (R^2) for your qPCR experiments using the software provided with your instrument.

Appendix

Troubleshooting

Problem	Possible Cause	Suggested Solution
Signals are present in no-template controls, and/or multiple peaks are present in the melting curve graph	Template or reagents are contaminated by nucleic acids (DNA, cDNA)	• Use melting curve analysis and/or run the PCR products on a 4% agarose gel after the reaction to identify contaminants. • Take standard precautions to avoid contamination when preparing your PCR reactions. Ideally, amplification reactions should be assembled in a DNA-free environment. We recommend using aerosol-resistant barrier tips.
	Primer dimers or other nonspecific products are present	• Primer contamination or degraded primers can lead to artifacts. Check the purity of your primers by gel electrophoresis. • Be sure to dilute the cDNA 1:10 in DEPC-treated water before qPCR, as specified in the protocol. Use 5 µL of diluted cDNA per 50-µL reaction (i.e., 1% v/v cDNA). • Increasing the annealing temperature in the qPCR may increase the specificity in the case of miRNA sequences that differ by only a few bases (note that the sensitivity of the reaction may decrease).
No amplification curve appears on the qPCR graph	There is no PCR product	Run the reaction on a gel to determine whether PCR worked. Then proceed to the troubleshooting steps below.
No PCR product is evident, either in the qPCR graph or on a gel	The protocol was not followed correctly	Verify that all steps have been followed and the correct reagents, dilutions, volumes, and cycling parameters have been used.
	Template contains inhibitors, nucleases, or proteases, or has otherwise been degraded.	Purify or re-purify your template.
	Primer design is suboptimal	Verify your primer selection. We recommend using validated pre-designed primers or design primers using dedicated software programs or primer databases.

Continued on next page

Problem	Possible Cause	Suggested Solution
PCR product is evident in the gel, but not on the qPCR graph	qPCR instrument settings are incorrect	Confirm that you are using the correct instrument settings (dye selection, reference dye, filters, acquisition points, etc.).
	Problems with your specific qPCR instrument	See your instrument manual for tips and troubleshooting.
PCR efficiency is above 110%	Template contains inhibitors, nucleases, or proteases, or has otherwise been degraded.	Purify or re-purify your template. Inhibitors in the template may result in changes in PCR efficiency between dilutions
	Nonspecific products may be amplified.	• Use melting curve analysis if possible, and/or run the PCR products on a 4% agarose gel after the reaction to identify contaminants. • Increasing the annealing temperature in the qPCR may increase the specificity in the case of miRNA sequences that differ by only a few bases (note that the sensitivity of the reaction may decrease).
PCR efficiency is below 90%	The PCR conditions are suboptimal	Verify that the reagents you are using have not been freeze-thawed multiple times and have not remained at room temperature for too long. Verify that the amount of primers you are using is correct.

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Notes