Takara Bio USA, Inc.

Mir-X™ miRNA First-Strand Synthesis and TB Green® qRT-PCR User Manual

Cat. Nos. 638313, 638314, 638315 & 638316
(050919)

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I. Introduction

MicroRNAs (miRNAs) are members of a rapidly growing class of small non-coding RNAs, 19–23 nt in length, known to contribute to the regulation of gene expression in plants and animals. First discovered in *C. elegans*, miRNAs have been subsequently shown to be encoded in the genomes of higher vertebrates, including humans. miRNAs act on target mRNAs in a sequence-specific manner to either promote their cleavage and degradation or reduce their translational efficiency.

The **Mir-X miRNA qRT-PCR TB Green Kit** contains the components needed to quantify miRNAs (or other RNAs) isolated from any source. The **Mir-X miRNA First-Strand Synthesis Kit** (included in the Mir-X miRNA qRT-PCR TB Green Kit and also sold separately) is used for converting miRNAs, and other RNA molecules, into cDNA to enable specific RNAs to be quantified by real-time PCR (Figure 1). In a simple, single-tube reaction, RNA molecules are polyadenylated and reverse transcribed using poly(A) polymerase and our SMART® MMLV Reverse Transcriptase; both of which are included in the **mRQ Enzyme Mix**. The **TB Green Advantage® qPCR Premix and mRQ 3' Primer** are then used in real-time qPCR, along with your miRNA-specific 5' primer(s), to quantify specific miRNA sequences in the cDNA. Any other RNA species present in the original RNA sample, such as the mRNA targets of the miRNAs, can also be amplified and quantified using specific 5' primers.

The Mir-X miRNA kits are exceptionally easy to use and require minimal hands on time, making them perfect for high-throughput applications. Fewer manipulations means less chance of errors and highly reproducible results. The entire miRNA quantification procedure (cDNA synthesis and qPCR) can be completed within a half a day.

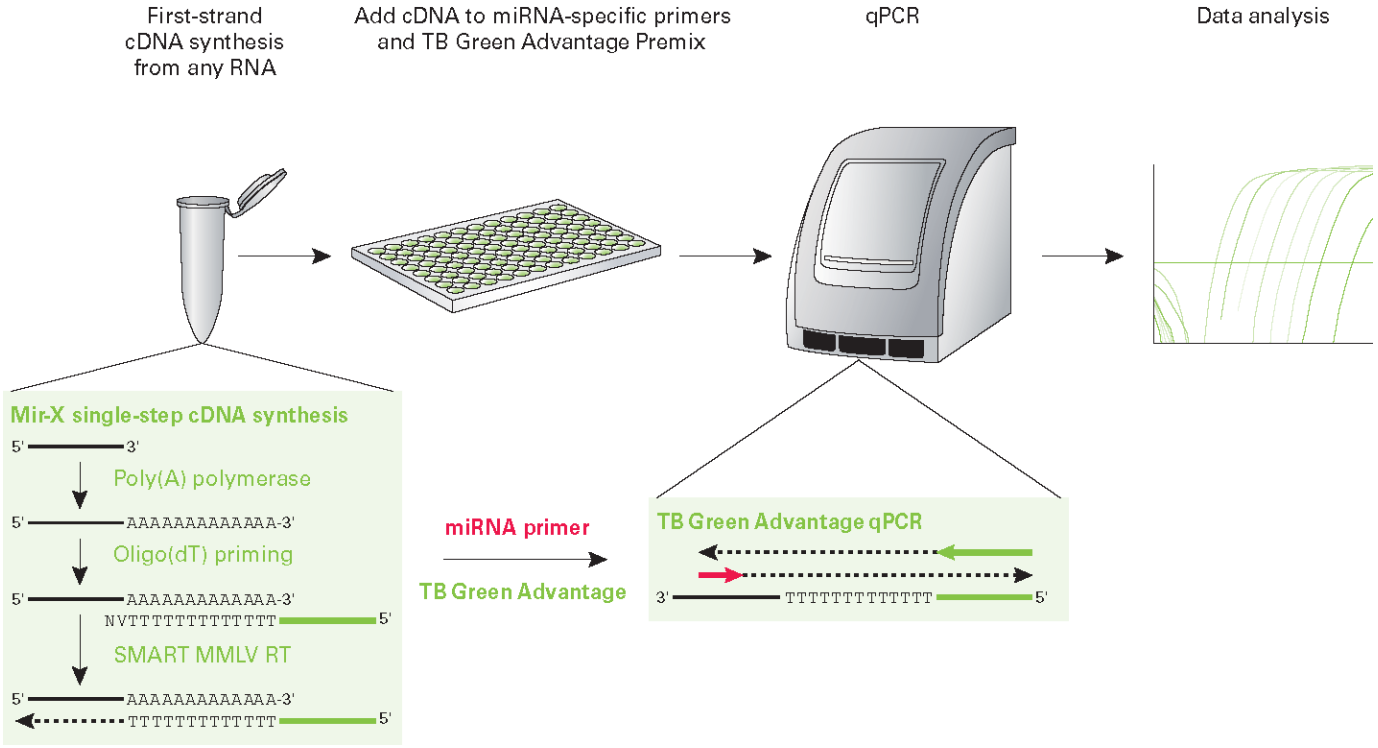

Figure 1. Mir-X miRNA qRT-PCR TB Green kits use a single-step, single-tube reaction to produce first-strand cDNA, which is then specifically and quantitatively amplified using a miRNA-specific primer and TB Green Advantage qPCR chemistry. In the Mir-X cDNA synthesis reaction, RNAs are poly(A)-tailed using poly(A) polymerase, and then copied using a modified oligo(dT) primer and SMART MMLV Reverse Transcriptase.

II. List of Components

Store all components at –20°C.

Mir-X miRNA First-Strand Synthesis Kit (20 rxn; Cat. No. 638313)

- 25 µl mRQ Enzyme Mix
- 100 µl mRQ Buffer (2X)
- 250 µl mRQ 3' Primer (10 µM)
- 50 µl U6 Forward Primer (10 µM)
- 50 µl U6 Reverse Primer (10 µM)

Mir-X miRNA First-Strand Synthesis Kit (60 rxn; Cat. No. 638315)

- **3 each Mir-X miRNA First-Strand Synthesis Kit (20 rxn; Cat. No. 638313)**

Mir-X miRNA qRT-PCR TB Green Kit (200 rxn; Cat. No. 638314)

- **1 each Mir-X miRNA First-Strand Synthesis Kit (20 rxn; Cat. No. 638313)**
  - 25 µl mRQ Enzyme Mix
  - 100 µl mRQ Buffer (2X)
  - 250 µl mRQ 3' Primer (10 µM)
  - 50 µl U6 Forward Primer (10 µM)
  - 50 µl U6 Reverse Primer (10 µM)
- **1 each TB Green Advantage qPCR Premix (200 rxn; Cat. No. 639676)**
  - 625 µl TB Green Advantage qPCR Premix (2X; 4 each)
  - 100 µl ROX Reference Dye LSR (50X)
  - 100 µl ROX Reference Dye LMP (50X)

Mir-X miRNA qRT-PCR TB Green Kit (600 rxn; Cat. No. 638316)

- **3 each Mir-X miRNA First-Strand Synthesis Kit (20 rxn; Cat. No. 638313)**
- **3 each TB Green Advantage qPCR Premix (200 rxn; Cat. No. 639676)**

III. Additional Materials Required

- Work areas and pipettors free of contaminating DNA, RNA, and nucleases
- 0.5 M EDTA
- Cloned DNase I (RNase-free) (Takara Bio, Cat. No. 2270A) [Optional]
- Microfuge tubes, 0.2 ml, RNase-free
- RNase-free water
- Thermal cycler, for incubating cDNA synthesis reactions
- Real-time qPCR instrument (e.g. Stratagene Mx3000P, or equivalent)

IV. Polyadenylation and cDNA Synthesis

Please read each protocol completely before starting. Successful results depend on understanding and performing all the steps correctly.

A. Good PCR Practices

Because the cDNA produced by these protocols is intended to be used in qPCR analyses, due care must be taken to ensure that the samples and reagents are not contaminated with extraneous DNA from the laboratory environment. Due to the tremendous amplification power and sensitivity of qPCR, even trace amounts of contaminating DNA and RNA will be amplified and will affect Ct and final copy number values. Before you begin, prepare work areas free of potentially contaminating DNA, RNA, and nucleases. Ideally, assemble the cDNA synthesis reactions and dilute samples and controls in one work area with a dedicated set of pipettors. Assemble the qPCR reactions in a separate area or in a noncirculating containment hood using a different set of dedicated pipettors. Wear gloves at all times and use PCR pipette tips with hydrophobic filters, and dedicated solutions. We also recommend setting up negative template control (NTC) reactions lacking any template. Finally, perform all post-PCR analyses in a separate area, preferably in a separate room, with different pipettors.

B. RNA Preparations Suitable for miRNA Analysis

Because miRNAs are very small and not polyadenylated, not all RNA preparation methods will yield miRNAs. Acceptable sources of miRNAs include: premade total RNAs available from Takara Bio (e.g. any of our mouse tissue total RNAs); small RNA purified using the NucleoSpin miRNA kit (Cat. Nos. 740971.10, 740971.50 & 740971.250); or total RNA purified using TRIzol or other phenol-based methods. All of these miRNA preparations can be used for miRNA cDNA synthesis and qPCR. However, spin kits or columns used for total or poly(A)+ RNA isolation are not acceptable methods of preparing miRNA, as the small size of the RNAs allow them to pass through any column.

C. Protocol: DNase I Treatment of RNA (Optional)

If your RNA sample was prepared from transfected cells or contains trace amounts of plasmid or significant amounts of genomic DNA, the RNA should be treated with DNase I to reduce or eliminate background signal in subsequent qPCR applications. This is especially important if the miRNA of interest was expressed from a transfected plasmid or a viral vector. Contaminating DNA fragments that contain your miRNA sequence will be amplified in control reactions lacking reverse transcriptase (No RT controls), which can lead to inaccurate qPCR results. For analyses of endogenous miRNAs, DNase I treatment may not be needed.

1. Cloned DNase I (RNase-free) is available from Takara Bio (Cat. No. 2270A).
2. In a 0.2-ml RNase-free tube, combine the following:

Table 1. DNase I Reaction

Reagent	Volume (µl)
RNA sample (3.5–10 µg)	≤44
DNase I Buffer (10X)	5
DNase I (5 U/µl)	0.5
RNase-free ddH_2O	44.5 [RNA µl]
Total volume	**50**

3. In a thermal cycler, incubate 30 min at 37°C, then inactivate the DNase I by adding 1 µl 0.5M EDTA and heating at 80°C for 2 min.
4. The DNase I-treated RNA can be used directly in the Polyadenylation and Reverse Transcription Protocol (Section D).

D. Protocol: Polyadenylation and Reverse Transcription

Prepare a cDNA synthesis reaction for each RNA sample you wish to analyze by qPCR. In addition, if you plan to determine the absolute level of an miRNA using a standard curve, you will need to generate cDNA from dilutions prepared from a known concentration of synthetic miRNA.

1. In an RNase-free 0.2 ml tube, combine the following reagents:

Table 2. Poly(A)/cDNA Synthesis Reaction

Reagent	Volume (µl)
mRQ Buffer (2x)	5
RNA sample (0.25–8 µg)	3.75
mRQ Enzyme	1.25
Total volume	**10**

2. Preferably in a thermal cycler, incubate the tube for 1 hour at 37°C, then terminate at 85°C for 5 min to inactivate the enzymes.
3. Add 90 µl ddH_2O to bring the total volume to 100 µl.
4. Your cDNA is now ready for the miRNA quantification protocols in Section V.

V. Quantification of miRNA by qPCR

Before performing qPCR, determine the quantification method to be used for the samples you wish to analyze. We suggest using either a comparative Ct method (e.g., the delta-delta Ct method or ddCt), or an absolute quantification method that entails constructing a standard curve. Each of these methods requires performing additional qPCR amplifications (U6 snRNA controls for the ddCt method or cDNA prepared from synthetic miRNA for the standard curve method) and are described briefly in Section VI.

For additional information regarding the setup of qPCR reactions using the TB Green Advantage qPCR Premix, including instrument-specific instructions, consult the TB Green Advantage qPCR Premix User Manual.

A. Choosing Your miRNA-Specific 5'-primer for qPCR

We recommend that the entire sequence of your mature miRNA (21–23 nt) be used as your miRNA-specific, 5' primer. The 3' primer for qPCR is the mRQ 3' Primer supplied with the kit.

NOTE: The miRBase Sequence Database is a searchable database of miRNA sequences, maintained by the University of Manchester at: http://www.mirbase.org

B. Protocol: Quantifying miRNA by qPCR

For miRNA quantification, we recommend using the delta-delta Ct method to determine the level of each miRNA relative to the level of U6 snRNA. For this method, you will need to perform a qPCR amplification of U6 for each cDNA sample you wish to analyze. Alternatively, absolute quantification can be performed by using the Ct value(s) obtained for the miRNA in the unknown sample (or sample dilution) to obtain copy number values from a Ct vs. copy number standard curve that is generated from cDNA synthesized from tenfold serial dilutions of a known concentration of synthetic miRNA.

1. Perform all sample, U6, and standard curve reactions in duplicate. Be sure to include appropriate no template controls (NTC) for each primer set. For each reaction, combine the following reagents:

Table 3. Sample qPCR Reaction

Reagent	Volume (µl)
ddH_2O	9
TB Green Advantage Premix (2X)	12.5
ROX Dye (50X)	0.5
miRNA-specific primer (10 µM)	0.5
mRQ 3' Primer (10 µM)	0.5
cDNA	2.0
Total volume	**25**

Table 4. U6 qPCR Reaction

Reagent	Volume (µl)
ddH_2O	9
TB Green Advantage Premix (2X)	12.5
ROX Dye (50X)	0.5
U6 Forward Primer (10 µM)	0.5
U6 Reverse Primer (10 µM)	0.5
cDNA	2.0
Total volume	**25**

2. Cycle the reactions according to the instructions provided by the manufacturer of your real-time qPCR instrument. We routinely use the following conditions with the Stratagene Mx3000P instrument:
   - Denaturation
     - 95°C 10 sec
   - qPCR x 40 Cycles
     - 95°C 5 sec
     - 60°C 20 sec
   - Dissociation Curve
     - 95°C 60 sec
     - 55°C 30 sec
     - 95°C 30 sec

VI. Data Analysis and Calculating miRNA Levels

The two quantification approaches cited here are described only very briefly. For detailed explanations of these and other qPCR strategies, consult a comprehensive qPCR reference. For additional information regarding the set-up of qPCR reactions using the TB Green Advantage qPCR Premix, including instrument-specific instructions, consult the TB Green Advantage qPCR Premix User Manual.

A. Delta-Delta Ct Method

The delta-delta Ct method (ddCt) is an approximation method that measures the relative levels of a miRNA between two samples by comparing them to a second RNA which serves as a normalization standard (e.g., U6). Briefly, the unknown miRNA and the U6 RNA are amplified in each sample to determine the Ct for each. This allows relative levels to be determined using the ddCt calculation. An example is shown below:

Table 5. Sample Data for Delta-Delta Ct Calculation of miRNA Levels

Sample RNA	Ct(miR-122a)	Ct(U6)	dCt
Brain	30	25	5
Liver	20	24	–4

Calculating relative copy numbers using the delta-delta Ct method:

$[\text{miR-122a}]_{\text{Liver}}/[\text{miR-122a}]_{\text{Brain}}$: $2^{-\text{dCt(Liver)}} / 2^{-\text{dCt(Brain)}} = 2^{-(-4)} / 2^{-(5)} = 29 = 512$*

*This result indicates that the level of miR-122a is 512-fold higher in the liver than in the brain.

Theoretically, between successive PCR cycles (i.e., each delta Ct) there is a twofold difference in PCR product. In this case, a normalized Ct difference of 9 between the samples gives 2^9, which is 512-fold. *Important*: a number of assumptions need to be considered before this method can be used correctly. The relative levels of the U6 should not differ between samples. More importantly, the amplification efficiency of the primers should be near 100% (i.e., the amount of product should actually increase twofold with each PCR cycle). If primer efficiency is significantly less than 100%, then this method will not be accurate and either the primers should be redesigned or the absolute quantification method should be used (Section B).

B. Absolute Quantification Method (Standard Curve)

In this method, a calibrated preparation of synthetic miRNA is used to make serial dilutions and generate the standard curve. The plot is then used to determine miRNA copy number from the Ct values obtained from the experiment samples.

1. Determine the average Ct values for the duplicate qPCR reactions of the cDNA samples generated from the diluted synthetic miRNA samples and plot these Ct values vs. input miRNA copy number on a log scale.
2. Determine average Ct values for each duplicate experiment sample, or sample dilution, and extrapolate the corresponding RNA copy number from these Ct values using the standard curve generated in Step 1. Use all Ct values that are below that of the NTC.

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