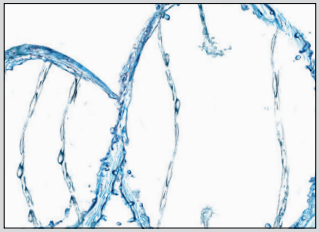

EPIK™ miRNA Select Hi/Lo-ROX Kit

Product Manual

BIOLINE

A Meridian Life Science® Company

EPIK™ miRNA Select Kit

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GENERAL INFORMATION

1. KIT CONTENTS

EPIK™ miRNA Lo/Hi-ROX Kit	KIT	
RT primer-Assay#1	16 µl	Box A
RT primer-Assay#2	16 µl	
RT primer-Assay#3	16 µl	
RT primer-Assay#4	16 µl	
PCR primer mix-Assay#1	200 µl	
PCR primer mix-Assay#2	200 µl	
PCR primer mix-Assay#3	200 µl	
PCR primer mix-Assay#4	200 µl	
EPIK™ 5x RT Buffer	160 µl	Box B
EPIK™ RT Enzyme	40 µl	
2x SensiSMART™ SYBR Master Mix	4 x 1 ml	
DEPC Water	5 x 1.8 ml	

2. DESCRIPTION

Mature microRNAs (miRNAs) are endogenously biosynthesized across many species of eukaryotes. These single-stranded RNAs (~ 22 nucleotides long) are known to play important regulatory roles in animals and plants by targeting mRNA transcripts for cleavage or translational repression. To date, thousands of unique, miRNAs have been identified (www.mirbase.org). Their expression levels vary greatly among species, tissues and in disorders.

Detecting miRNAs remains a significant challenge, mainly due to the short lengths of the nucleotide sequences. Various methods for miRNA measurement are currently available and quantitative real-time PCR remains the method of choice for both convenience and reliability. Stem-loop structure-based assays have been successfully used for the quantification of replicating viruses and mature miRNAs, however these methods rely on sequence-dependent probes or chemically modified primers for optimal specificity and are time-consuming, labour-intensive and suffer from sample-to-sample variability. There are many distinct advantages of EPIK™ miRNA Select Assays over these miRNA-detection methods, including:

- Highly specific; targeting only mature miRNA and not precursors and can discriminate between highly similar miRNAs.
- Ultra-sensitive; can detect mature miRNA from as little as 10 pg of total RNA

- Wide dynamic range of quantitation; all assays can detect mature miRNAs with greater than six logs of dynamic range (1 million fold changes).
- Fast reaction time; the simple two-step protocol takes less than 2 hours from RNA to result.

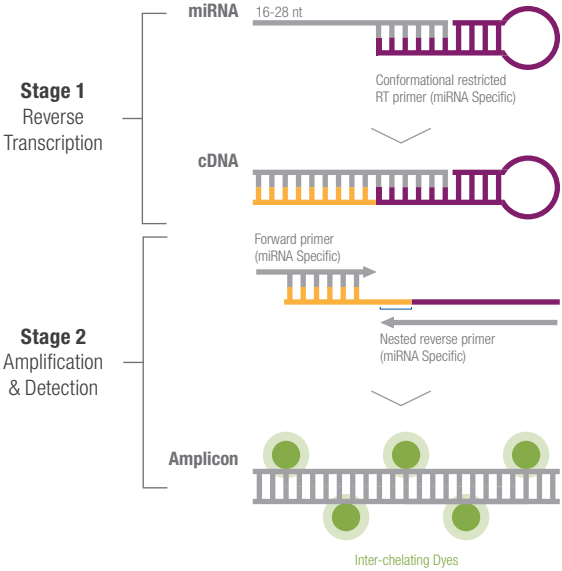

Fig. 1 EPIK™ miRNA Select Assay powered by MiRXES™ technology
The EPIK™ miRNA Select Assay comprises of a set of three specific primers. The conformationally-restricted RT primer allows efficient hybridization only to the mature form of the target miRNA (Stage 1). The miRNA-specific forward and nested reverse real-time PCR primers confer further specificity and enable robust amplification of the target cDNA (Stage 2).

The workflow of EPIK™ miRNA Select Assays consists of 2 independent stages, where users have the choice of pausing between each stage (Fig 1).

All EPIK™ miRNA Select Assays have been validated using both synthetic miRNA templates and total human RNA. Typically the assays detect as few as 100 copies of template per RT reaction with excellent assay efficiency and linearity (Fig. 2). These stem-loop structure-based assays are designed using MiRXES™ proprietary thermodynamics-based algorithms, enabling these assays to demonstrate superior sensitivities and specificities.

For the amplification and detection stage with SensiSMART™ SYBR mixes, a commonly available DNA-binding dye (SYBR Green) is used, rather than a probe-based system. This leads to remarkable sensitivities as well as extremely low background, enabling the accurate detection of very low miRNA levels. In addition, these assays, allow the clear discrimination between miRNA sequences with high similarity.

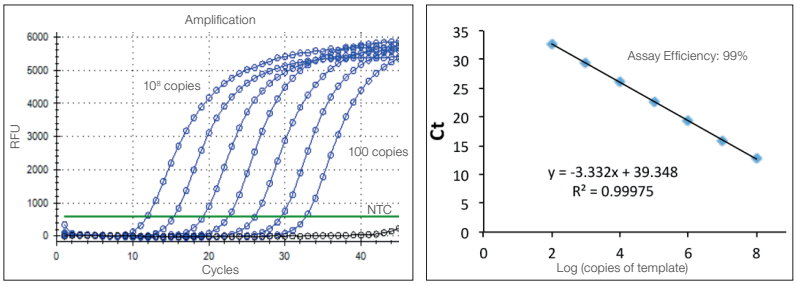

Fig. 2 Performance of EPIK™ miRNA Select Assay for human miRNA.
A synthetic miRNA was reverse-transcribed and amplified by SensiSMART™. The results illustrate the sensitivity and efficiency (99.97%) of the assay, allowing the detection of miRNAs at varying expression levels (10^8 to 100 copies), including low expressers.

Please read this manual carefully to familiarize yourself with the EPIK™ miRNA Select protocol before starting (also available on www.bioline.com/mirna).

3. STORAGE

When stored under the recommended conditions and handled correctly, full activity of reagents is retained until the expiry date indicated on the outer box label. Avoid subjecting any reagent to repeated freezing and thawing. Reagents should be stored at -20 °C.

4. SAFETY INFORMATION

When working with chemicals, always wear suitable personal protective equipment (PPE), including lab coat, gloves and safety glasses.
For detailed information, please consult the material data safety sheets (MSDSs) available on our website at www.bioline.com/mirna.

5. PRODUCT SPECIFICATIONS

EPIK™ miRNA Select Assays are powered by MiRXES™ technology. The conformationally restricted RT primers are designed so that there is specific hybridization to the mature miRNA target. Following a reverse transcription stage, a robust amplification of the newly synthesized cDNA is accomplished using miRNA-specific forward and reverse real-time PCR primers to confer further specificity and sensitivity.

The EPIK™ miRNA Select Assays protocol is optimized for use of up to 100 ng human total RNA per cDNA synthesis reaction (20 µl). The exact amount of human total RNA needed depends on the tissue state and can vary, depending on the type of cell, tissue or biofluid of interest and on the expression levels of the target miRNAs. As low as 10 pg of total RNA is sufficient for accurate quantification of highly expressed targets whereas up to 100 ng may be required for low expression miRNAs.

Each EPIK™ miRNA Select Assay provides RT primer and qPCR primer mix specific to one miRNA target as well as the necessary RT and qPCR reagents.

It is essential that the right reagent type is chosen for the machine you intend to use (see Section 7) (see www.bioline.com/mirna).

6. EQUIPMENT AND REAGENTS TO BE SUPPLIED BY THE USER

The following additional items are required:

- Nuclease-free disposable plasticware
- Microcentrifuge for 1.5 ml tubes
- Plate centrifuge suitable for PCR plates
- Cooling block or ice bucket suitable for PCR plates
- Heating block or thermocycler capable of isothermal heating at 42 °C and 70 °C
- Real-Time PCR machine
- Vortex

7. REAL-TIME PCR MACHINE AND ROX LEVEL

Please ensure that you have the correct SensiSMART™ ROX level for the machine you intend to run the assays on as different qPCR machines from different manufacturers have specific requirements for the method of normalization employed (see www.bioline.com/mirna).

EPIK™ miRNA Select Assays have been optimized for use in SYBR® Green-based real-time PCR on the real-time PCR instruments listed below, each of these instruments having the capacity to analyze the real-time PCR data with the passive reference signal either on or off.

EPIK miRNA Select Hi-ROX	Applied Biosystems: 7000, 7300, 7700, 7900, 7900HT, 7900HT FAST, StepOne™, StepOne™ Plus. -The kit is also compatible with several instruments that do not require the use of ROX, such as the Qiagen (Corbett) Rotor-Gene™ 6000, the Bio-Rad CFX96 or the Roche LightCycler® 480.
EPIK miRNA Select Lo-ROX	Applied Biosystems: 7500, 7500 FAST, Viia7™, QuantStudio® 3&5; QuantStudio™ 6; QuantStudio™ 7 ; QuantStudio™ 12K Flex Real-Time PCR systems -Stratagene (Agilent): Mx4000™, Mx3000P™, Mx3005P™ The kit is also compatible with several instruments that do not require the use of ROX, such as the Qiagen (Corbett) Rotor-Gene™ 6000, the Bio-Rad CFX96 or the Roche LightCycler® 480

8. IMPORTANT NOTES

8.1 Handling RNA

Handle RNA carefully to avoid contamination by RNases, often found on labware, fingerprints and dust. For optimal RNA stability, keep RNA frozen at -20 °C for short-term or -80 °C for long-term storage.

It is important to work quickly when purifying RNA (see hints and tips on working with RNA at www.bioline.com/uk/rna-hints-and-tips).

8.2 Starting material

We recommend using purified miRNA or total RNA rather than attempting direct detection of miRNA in partly purified sample types.
It is recommended that the ISOLATE II miRNA Kits are used for the preparation of the samples, as this allows rapid, unbiased, phenol-free isolation of miRNA.

The EPIK™ miRNA Select protocol is optimized for use of up to 100 ng total RNA per cDNA synthesis reaction (20 µl). Although the ratio between total RNA and specific miRNA is not fixed, measurement of total RNA provides a convenient way of estimating miRNA loading and an approximate methods for normalizing between experiments. If the ISOLATE II miRNA (BIO-52083) is used for example, the large RNA fraction concentration (as ng/µl), can be calculated from the final pure eluate and used as an estimate of total miRNA from the ISOLATE II miRNA column used to extract the miRNA fraction. This will only be correct if the elution volume of the large RNA fraction and miRNA fraction are identical.

9. PROTOCOL

The EPIK™ miRNA Select protocol is a two-step protocol consisting of:

Step 1.	Reverse transcription with miRNA-specific RT-oligonucleotide and EPIK™ cDNA synthesis kit - See section 9.1
Step 2.	Real-Time PCR using SensiSMART™ SYBR Master Mix and amplification primers - See section 9.2

It is critical for the success of the experiment to follow the protocol carefully, from first-strand cDNA synthesis to real-time PCR amplification (approximately 2 hours). However, the procedure can be paused after the first-strand cDNA synthesis and the undiluted cDNA may be stored at -20 °C for up to three days.

Workflow

When working with the EPIK™ miRNA Select Assays, it can be difficult for a single user with a single qPCR machine to run all the plates in one day. We suggest that in order for all the samples to be treated the same, storage should occur just after cDNA synthesis (see step 9.1.6). All the cDNA reactions must be treated identically, so if it is not possible to run all the qPCR within one day, all the cDNA reactions must be frozen at -20 °C once completed. This will ensure that all cDNA reactions are subjected to the same number of freeze-thaw cycles prior qPCR.

The user should allow sufficient time so that all the real-time data can be collected in as short a time as possible, as we recommend that the cDNA from step 9.1.6 is stored at -20 °C for no more than three days.

9.1 First-strand cDNA synthesis (Step 1)

It is important to keep the components and the reactions on ice during the procedure.

9.1.1 Prepare template RNA

Gently thaw template RNA on ice. We recommend the use of 100 ng or less of total RNA per 20 µl RT reaction (see section 8.1). If different RNA samples are used it is recommended to adjust each samples to similar concentration using nuclease free water.

9.1.2 Prepare reagents

1. Gently thaw the EPIK 5x RT Buffer and RT Primer tube(s) on ice. Mix by vortexing (1 second) and spin down.
   Note: In case of precipitate in the EPIK 5x RT Buffer, incubate at 37 °C and vortex.

9.1.3 Assemble reagents

Assemble the reaction as indicated in Table 1a or 1b: The most consistent results can be obtained by preparing a mastermix with template RNA, EPIK 5x RT buffer, water and EPIK RT enzyme in the proportions shown. The EPIK RT Enzyme should be added to the master mix last, right before dispensing of the master mix into the PCR tubes.

Table1a: Singleplex setup

Reagent	Volume
Template RNA (up to 100 ng)	X µl (up to 6 µl)
EPIK 5x RT Buffer	4 µl
RT primer	0.4 µl
EPIK RT Enzyme	1 µl
Nuclease-free water	up to 20 µl

Table 1b: Multiplex setup-as a general rule, in case of multiplexing, 0.4 µl of each RT primer should be used in the 20 µl reaction. The example below is for a quadruplex reaction.

Reagent	Volume
Template RNA (up to 100 ng)	X µl (up to 6 µl)
EPIK 5x RT Buffer	4 µl
RT primer-Assay#1	0.4 µl
RT primer-Assay#2	0.4 µl
RT primer-Assay#3	0.4 µl
RT primer-Assay#4	0.4 µl
EPIK RT Enzyme	1 µl
Nuclease-free water (DEPC)	up to 20 µl

9.1.4 Mix and spin

Thoroughly mix the reagents by gently pipetting up and down. Spin down after mixing.

9.1.5 Incubate and heat inactivate

Incubate reaction at 42 °C for 30 min, followed by heat-inactivation of the reverse transcriptase at 90 °C for 5 min. Keep the undiluted cDNA reactions on ice until the assembly of real-time PCR reaction, or go to step 9.1.6 for storage.

9.1.6 Store cDNA

If desired, undiluted cDNA reactions can be stored at -20 °C for up to three days. It is recommended to store cDNA in "low-nucleic acid binding" (pre-siliconized) tubes.

9.2 REAL-TIME PCR AMPLIFICATION AND DETECTION (Step 2)

In this step, the cDNA is amplified by real-time PCR using SensiSMART SYBR Master Mix.

Important: Keep all reagents on ice (or at 4 °C) at all times during set up.

9.2.1 Prepare reagents

1. Thaw 2x SensiSMART™ SYBR Master Mix and cDNA reactions from Step 1 on ice.
2. Mix by quickly vortexing and spin down.
3. Dilute the cDNA reaction 10 fold with DEPC-water (provided)

9.2.2 Assemble the real-time PCR reagents

1. Prepare each qPCR reaction in the proportion indicated in Table 2. Mix by vortexing and spin down.

Table 2: qPCR setup

Reagent	One reaction
2x SensiSMART™ SYBR Master Mix	10 µl
Diluted cDNA reaction (from 9.2.1.3)	5 µl
PCR primers	2 µl
DEPC water	3 µl
Total volume	**20 µl**

* It is critical to place the PCR on a cooling block or on ice throughout the procedure.

We recommend proceeding with a mastermix and to scale up the volumes accordingly to the number of reactions needed per assay.

9.2.3 Mix and spin

Centrifuge briefly (30 s at 200 x g in a suitable plate centrifuge).

9.2.4 Real-Time PCR amplification

Perform real-time PCR amplification according to the following cycling parameters.

Cycles	Temperature	Time	Notes
1	95 °C	10 min	Polymerase activation
	40 °C	5 min	
40	95 °C	10 s	Denaturation
	60 °C	30 s	Annealing/extension (acquire at end of step)

We recommend adding a melt-curve analysis step to your reaction conditions. This is normally added as a set module during qPCR machine programming and recommendations vary between manufacturers. Please refer to the manufacturer's machine-specific manual for more advice.

To obtain accurate, specific results for the miRNA and control assays, you must ensure that the real-time PCR amplification is performed exactly as set out above. Deviation from the protocol will yield poor results.

9.2.5 Data collection

Collect raw Ct values (also known as Cp or Cq, depending on the PCR instrument) using the software supplied with the real-time PCR instrument. Please note that it is not recommended to use auto Ct settings, but set the threshold manually to one tenth of the average maximal fluorescence value. We recommend that you export the data as an Excel file for further analysis.

9.4.2 Using multiple qPCR machines

If the user has multiple identical qPCR machines, all the reactions (RT and qPCR) should be performed in parallel. Take steps to ensure that all the RT reactions and qPCR reactions are treated identically. You should make sure that the same number of freeze/thaw steps is applied across replicates.

A TECHNICAL SUPPORT AND TROUBLESHOOTING

For technical assistance or more information on these products, please email us at tech@bioline.com

B ASSOCIATED PRODUCTS

Product	Size	Cat. #
ISOLATE II miRNA Kit	25 prep	BIO-52083
ISOLATE II RNA/DNA/Protein Kit	50 prep	BIO-52085
ISOLATE II Biofluid Kit	50 prep	BIO-52086
ISOLATE II FFPE RNA/DNA Kit	50 prep	BIO-52087

C PRODUCT WARRANTY AND DISCLAIMER

Bioline warrants that its products will conform to the standards stated in its product specification sheets in effect at the time of shipment. Bioline will replace any product that does not conform to the specifications free of charge. This warranty limits Bioline's liability to only the replacement of the product.

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1. Trademarks: SensiSMART™, EPIK™ (Bioline Reagents Ltd), SYBR® (Molecular Probes), StepOne™, QuantStudio®, ViiA7™ (ABI), Mx4000, Mx3000P and Mx3005P (Stratagene), iCycler™, MyiQ5™, Opticon™, Chromo4™, Miniopticon™, (Bio-Rad), LightCycler®, ROX™ (Roche), RealPlex™ (Eppendorf), MX4000 (Stratagene).

2. Purchase of this product includes limited right to use the supplied amount of SYBR® Green I stain patented by Molecular Probes, Inc.

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4. EPIK™ products are manufactured by Bioline Reagents Ltd.

5. Notice to Purchaser: No rights are conveyed with respect to US patent 5,928,907

6. The technology employed in this product is covered by Patent No: 185776, SG. Patents pending in other nations.

Ordering Information

Product	Size	Cat. #
EPIK miRNA Select Lo-ROX Kit	4 x 100 reactions	BIO-66045
EPIK miRNA Select Hi-ROX Kit	4 x 100 reactions	BIO-66046

PM0216V1.0

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