EPIK™ Cancer miRNA Panel Assay

Bioline

Abstract

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

Step 1. Reverse transcription with miRNA-specific RT-oligonucleotides and EPIK™ cDNA synthesis kit

Step 2. Real-Time PCR using SensiSMART™ Master Mix and amplification primers

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.

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Guidelines

KIT CONTENTS

EPIK™ Cancer miRNA ASSAY COMPONENTS KIT	
Cancer Assay Plates*	8 x 96-well plates**
RT Primer Pool - lyophilized	4 tubes (A, B, C and D)
RNA Spike - lyophilized	1 tube
EPIK 5x RT Buffer	1 x 32 µL
EPIK RT Enzyme	1 x 8 µL
2x SensiSMART™ SYBR Master Mix	4 x 1 mL
DEPC Water	3 x 1.8 mL

* For well definitions please see web site www.bioline.com/mirna
** Three 96-well configurations are available, depending on the real-time PCR machine used.

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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 Panel 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.

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

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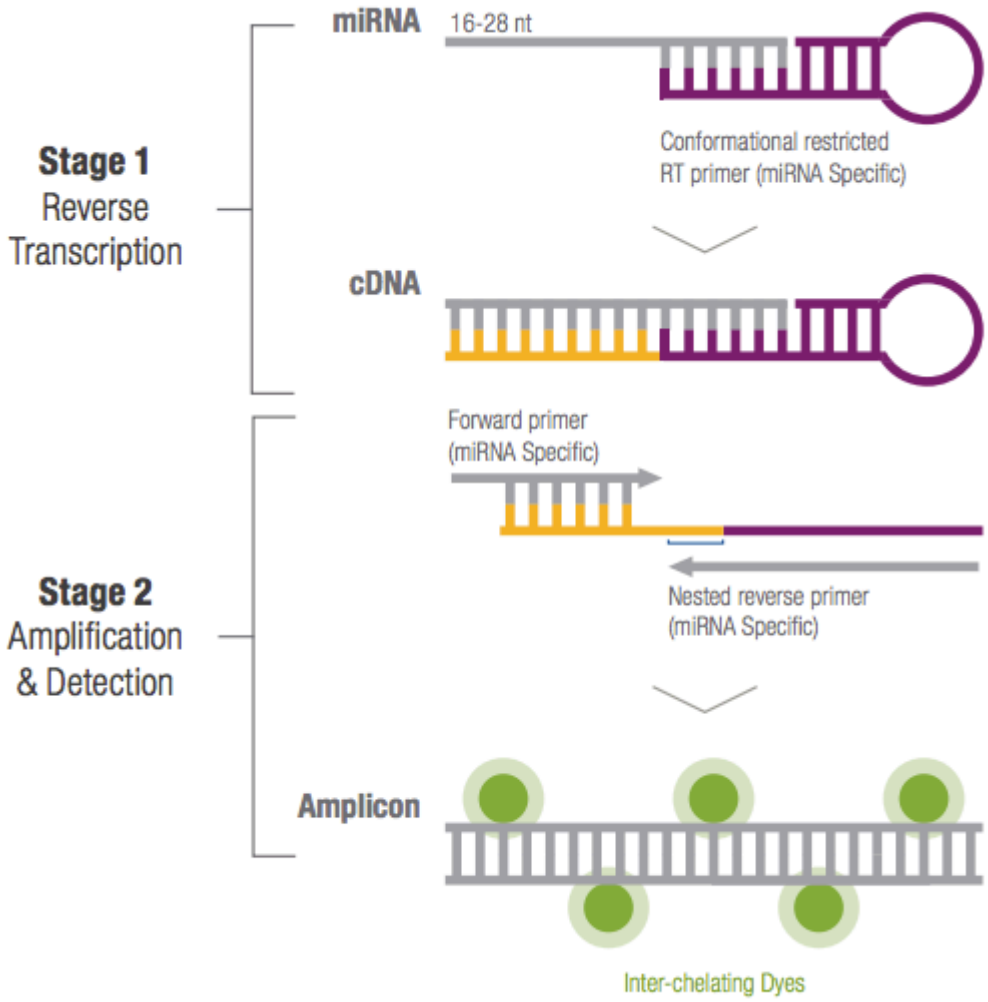

Fig. 1 EPIK™ miRNA Panel Assay powered by MiRXES™ technology. The EPIK™ miRNA Panel Assay comprises of a set of three specific primers. The conformationallyrestricted RT primer allows efficient hybridization only to the mature form of the target miRNA (Stage 1). The miRNA-specific forward and nested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

All EPIK™ miRNA Panel 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™ qPCR 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.

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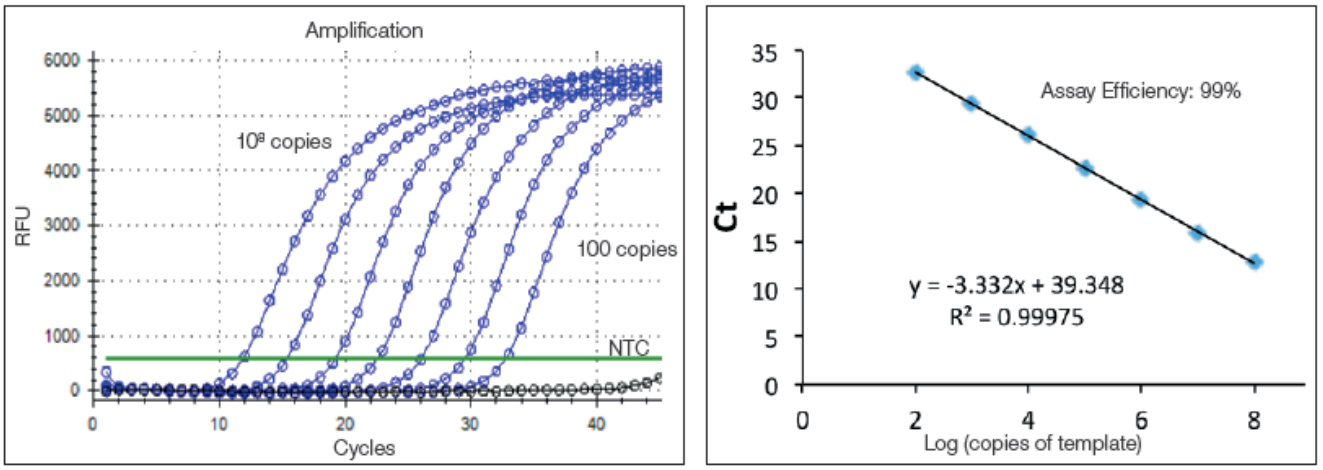

Fig. 2 Performance of EPIK™ miRNA Panel Assay for human miRNA. A synthetic miRNA was reverse-transcribed and amplified by SensiSMART™. The results illustrate the sensitivity and efficiency (99.97%) of the assay, allowing the detection of miRNAs at varying expression levels (10^8 to 100 copies), including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

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

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 plate or reagent to repeated freezing and thawing. Reagents should be stored according to their label, with plates and the reagent box being stored at -20 °C and the RNA Spike stored at -80 °C after reconstitution.

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.

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PRODUCT SPECIFICATIONS

EPIK™ miRNA Panel 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 Panel Assay 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.

The qPCR primers for EPIK™ miRNA Panel Assays are supplied lyophilized in 96-well white plates, which are compatible with the machines listed below (see www.bioline.com/mirna).

Plates with white wells provide improved signal-to-noise ratio in qPCR experiments and give the highest chance of detection of low-copy number miRNA. The white plates supplied can be used in most machines without re-calibration and as a direct substitute for clear plates.

It is essential that the right plate type and the right reagent type are chosen for the machine you intend to use.

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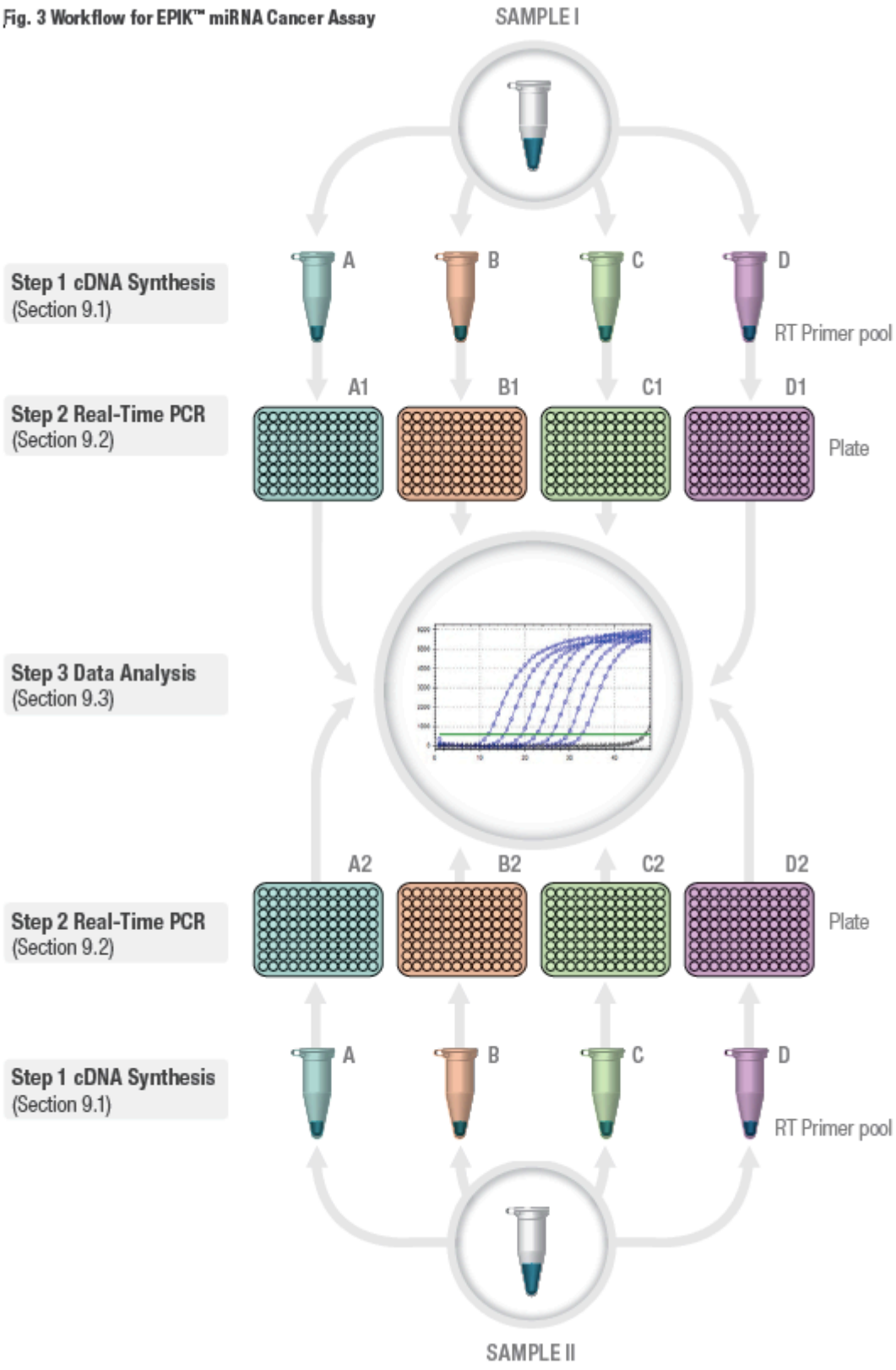
Fig. 3 Workflow for EPIK™ miRNA Cancer Assay



EQUIPMENT AND REAGENTS TO BE SUPPLIED BY THE USER

- The following additional items are required:
- Nuclease-free disposable plasticware
- Plate seals suitable for qPCR

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- Microcentrifuge for 1.5 mL tubes
- Plate centrifuge suitable for 96-well plates
- Cooling block or ice bucket suitable for 96-well plates
- Heating block or thermocycler capable of isothermal heating at 42 °C and 70 °C
- Vortex

REAL-TIME PCR MACHINE AND ROX LEVEL

Please ensure that you have the correct plate type and SensiSMART™ ROX level for the machine you intend to run the assays on. Different qPCR machines from different manufacturers have specific requirements for the method of normalization employed, as well as for the plate size and shape. Using the wrong plate may damage your instrument (see www.bioline.com/mirna).

ROX

EPIK™ miRNA Panel 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.

- The EPIK™ Hi-ROX miRNA Panel Assays can be used on the ABI StepOne Plus.
- The EPIK™ Lo-ROX miRNA Panel Assays can be used on: ABI 7500, 7500 Fast QuantStudio® 3&5 Real-Time PCR System; QuantStudio™ 6 Real-Time PCR system; QuantStudio™ 7 Real-Time PCR system; QuantStudio™ 12K Flex Real-Time PCR system and ViiA7™. The EPIK™ Lo-ROX miRNA Panel Assays can also be used on the BioRad® CFX96 and Roche LightCycler® 480 that do not require the use of ROX.

96-well plates

The type of qPCR plate depends on which qPCR machine is to be used to run the experiment. The plates supplied will only fit machines fitted with a 96-well block. For advice on other plate types (384-well or 48-well) please contact Bioline Technical Support. We do not provide these assays for Rotor-Gene qPCR machines at the present time.

Due to the low volume of the qPCR reaction, we recommend the use of one of the following machines in combination with the following plate types:

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Plate type 1 (0.1 mL, “low profile”)†	ABI StepOne Plus; ABI ViiA7 FAST; ABI 7500 FAST; QuantStudio® 3 Real-Time PCR System FAST; QuantStudio® 3&5 Real-Time PCR System FAST; QuantStudio™ 6 Real-Time PCR system FAST; QuantStudio™ 7 Real-Time PCR system FAST; QuantStudio™ 12K Flex Real-Time PCR system FAST
Plate type 2 (0.2 mL)††	ABI ViiA7; ABI 7500; QuantStudio® 3 Real-Time PCR System; QuantStudio® 3&5 Real-Time PCR System; QuantStudio™ 6 Real-Time PCR system; QuantStudio™ 7 Real-Time PCR system; QuantStudio™ 12K Flex Real-Time PCR system
Plate type 3 (0.1 mL)	BioRad CFX96; Roche LC480 (96-well block only)

† This plate may also fit the following machines, though this operation is performed entirely at the users’ own risk, and results may be variable. Bioline does not accept any responsibility for damage caused by using the incorrect plate type: ABI 7900HT FAST. Please contact Bioline Technical Support for more advice.
† † This plate may also fit the following machines, though this operation is performed entirely at the users’ own risk, and results may be variable. Bioline does not accept any responsibility for damage caused by using the incorrect plate type: ABI 7900HT; ABI 7300; ABI 7700; ABI 7000; BioRad iCycler IQ; BioRad IQ4; BioRad IQ5; BioRad Opticon; BioRad Chromo; Eppendorf Mastercycler ep realplex; Stratagene Mx3000; Stratagene MX3005; Stratagene MX4000; Techne Quantica. Please contact Bioline Technical Support for more advice.

Each plate is supplied with dried down PCR primers specific for the following

reactions:

- 88 miRNA-specific detection primer pairs (arrayed in columns 1 to 11, rows A to H)
- 2 RNA Spike control primers pairs (in duplicate) for the detection of the RNA

  Spike (column 12, rows A to D)
- 2 primer pairs (in duplicate) detecting artificial control DNA for plate-to-plate calibration (column 12, rows E to H)

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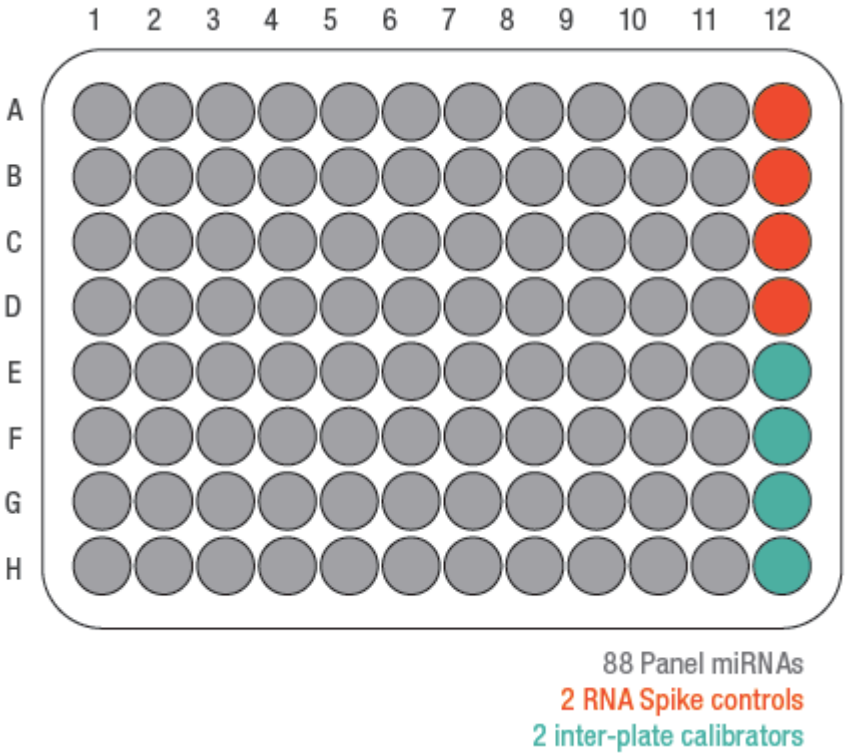

Fig. 4 EPIK™ miRNA Panel Plate layout. Wells A1 to H11 (red) each contain specific miRNA qPCR primers. Wells A12 and B12 contain replicate RNA Spike Control 1 (orange). Wells C12 and D12 contain replicate RNA Spike 2 (orange). Wells E12 and F12 contain replicate Inter-plate Calibrator 1 (green). Wells G12 and H12 contain replicate Inter-plate Calibrator 2 (green).

The EPIK™ Cancer miRNA Assay comprises eight plates. The miRNAs most likely to be found in cancer samples (as shown in the published literature) are arrayed over plates 1 to 4, to give a total of 352 separate miRNA-specific reactions. The 4 plates are supplied in duplicate so that the user can either:

- Perform a preliminary analysis of the experimental variation in one sample
- Or perform an initial screen against two conditions as a singlicate experiment

  (e.g. comparison of cancerous versus non-cancerous)

IMPORTANT NOTES

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.

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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).

Handling and storing starting material

We recommend using purified miRNA or total RNA with this panel 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 Panel Assay 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.

Controls and calibrators

The RT-qPCR miRNA detection procedure outlined in this manual includes a synthetic RNA control (RNA Spike) which provides an accurate and convenient internal control for the experiment (for the suitability of such controls, see Redshaw *et al* (2013) Biotechniques **54**: 155-164 and Ho *et al* (2013) PLoS One **8**: e72463.

The RNA Spike once reconstituted from its lyophilized state can be used in two modes:

- as an RNA spike to be used during the sample isolation, i.e. added to the lysis buffer of the RNA isolation kit: after reconstitution in 30 µL of nuclease-free water, 5 µL should be used per sample isolation and this can be used to detect differences in RNA purification.
- an RT control for pre-purified miRNA, i.e. added during the reverse transcription stage: once reconstituted in 30 µL of nuclease-free water, addition of the RNA Spike in the reverse transcription stage (1 µL per RT reaction) will allow normalization between RT reactions, in the same way as reference genes are used in qPCR measurements of mRNA.

Users should not attempt to use RNA Spike in both modes in the same experiment.

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If the user wishes to use their own calibration or plate-to-plate calibrator, then column 12 can be used to aliquot user-supplied primers. Control primers sets supplied in the 96-well plate target artificial sequences with no known homology to natural or artificial sequences deposited in miRBase (release 21, June 2014). As optimisation is required to ensure that user controls function properly, we recommend using the controls supplied.

Analysis

For convenience an Excel spreadsheet can be downloaded from the product page at www.bioline.com/mirna, which can be used to analyse plate to plate variation as well as for analysis and presentation of the data.

WORKFLOW

When working with the EPIK™ Cancer miRNA Panel Assay, 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 panels to be treated the same, storage should occur just after cDNA synthesis. All the cDNA reactions must be treated identically, so if it is not possible to run all the plates 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.

When a qPCR machine is available, remove a cDNA reaction from storage and prepare the qPCR as detailed in the protocol. Thaw the qPCR master mix and cDNA as late as possible before running the qPCR. Once mixed the cDNA:qPCR master mix should be loaded into the PCR plate and run on the instrument immediately. Storage of the ready to run plate at this point, even on ice, may result in non-specific primer-dimer formation.

When the first run is nearly finished, remove the next cDNA reaction tube and repeat the process with the next plate.

The remaining tubes and plates can be deployed in a similar way. 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 is stored at -20 °C for no more than three days. It should be possible for a single user with a single qPCR machine to run all the samples within 72 hours.

In order to minimize the effect of -20 °C storage, we we recommend processing the plates of the same panel as close as possible in time, running successively panels A, B, C and D.

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Using a single qPCR Machine

1. The same qPCR machine type should be used to perform all of the qPCR cycling and analysis. While some plate types will fit in qPCR machines from different manufacturers, you are at risk of damaging your qPCR machine by using the wrong plate type. In addition, data from different makes of qPCR machines are processed using different methods. Using multiple manufacturers' qPCR machines will make your analysis more complicated.
2. Perform the cDNA synthesis.
3. Store all the cDNA reactions at -20 °C once complete.
4. When the qPCR machine is available, remove the first cDNA reaction tube and thaw gently on ice. This storage step ensures that all the samples have been treated in a similar way, in that all the samples are subjected to one cycle of freeze/thaw.
5. Remove the first cDNA reaction tube from ice and follow the protocol steps. Make sure that the plate is sealed with a qPCR-compatible plate seal.
6. Place the plate in the qPCR machine and run the qPCR program.
7. Once prepared, plates should NOT be frozen at -20 °C. Plates should be processed immediately after preparation.
8. Start preparation of the next plate so that it is ready to be placed in the qPCR machine immediately after all the data from the first plate has been collected. We recommend starting 15-20 min before the end of the run.
9. Repeat steps 1 to 8 with the remaining plates. There are 8 plates in total for the Cancer panel, 4 plates for both the Biofluid and Stem Cell panels.

The user should allow sufficient time so that all the real-time data can be collected in as short a time as possible. We recommend that the cDNA is stored at -20 °C for no more than three days. It should be possible for a single user with a single qPCR machine to run all the samples within 72 hours.

Using multipe 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.

Materials

EPIK™ Cancer miRNA Hi-ROX Panel BIO-66032 by Bioline

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Protocol

First-strand cDNA synthesis

Step 1.

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

First-strand cDNA synthesis

Step 2.

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 'Handling and storing starting material' in the Guidelines).

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It is important to ensure that the same amount of RNA is used across the different panels. Adjust each of the template RNA samples to similar concentration using nuclease free water.

First-strand cDNA synthesis

Step 3.

Prepare reagents: Reconstitute the RNA spike (the synthetic miRNA control) by adding 30 µL of nuclease-free water to the tube and vortexing.

First-strand cDNA synthesis

Step 4.

Prepare reagents: Incubate at room temperature for five minutes.

DURATION

00:05:00

First-strand cDNA synthesis

Step 5.

Prepare reagents: Spin down in a microcentrifuge, then re-vortex for at least thirty seconds.

DURATION

00:00:30

First-strand cDNA synthesis

Step 6.

Prepare reagents: Reconstitute the Cancer RT Primer Pools (A, B, C and D) by adding 20 µL of nuclease-free water to the tube and and vortexing.

First-strand cDNA synthesis

Step 7.

Prepare reagents: Incubate at room temperature for five minutes.

DURATION

00:05:00

First-strand cDNA synthesis

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Step 8.
Prepare reagents: Spin down in a microcentrifuge, then re-vortex for at least thirty seconds. Store at -20 °C.

DURATION
00:00:30

First-strand cDNA synthesis
Step 9.
Prepare reagents: Gently thaw the EPIK 5x RT Buffer and Cancer RT primer pool tubes on ice.

First-strand cDNA synthesis
Step 10.
Prepare reagents: Mix by vortexing (1 second) and spin down.

NOTES
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Note: In case of precipitate in the EPIK 5x RT Buffer, incubate at 37 °C and vortex.

First-strand cDNA synthesis
Step 11.

Assemble reagents: For the analysis of 352 cancer miRNAs (EPIK™ Cancer miRNA Panel Assay), 4 RT reactions are required per sample, using RT primer pool A, B, C and D.

First-strand cDNA synthesis
Step 12.

Assemble reagents: Assemble the reaction as indicated in Table 1 (next step): 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 sample tubes.

First-strand cDNA synthesis
Step 13.

Assemble reagents: Dispense 2 µL of each primer pool into 4 separate tubes, then add 18 µL of the master mix.
Prepare 4 reverse transcription reactions as follows:

Table 1:

Reagent	Volume (Tube A)	Volume (Tube B)	Volume (Tube C)	Volume (Tube D)
Template RNA (up to 100 ng)	X µL (up to 6 µL)	X µL (up to 6 µL)	X µL (up to 6 µL)	X µL (up to 6 µL)
EPIK 5x RT Buffer	4 µL	4 µL	4 µL	4 µL

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DEPC water	6 µL - X µL	6 µL - X µL	6 µL - X µL	6 µL - X µL
EPIK RT Enzyme	1 µL	1 µL	1 µL	1 µL
Cancer RT Primer Pool A	2 µL	-	-	-
Cancer RT Primer Pool B	-	2 µL	-	-
Cancer RT Primer Pool C	-	-	2 µL	-
Cancer RT Primer Pool D	-	-	-	2 µL
Total volume	**20 µL**	**20 µL**	**20 µL**	**20 µL**

First-strand cDNA synthesis

Step 14.

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

First-strand cDNA synthesis

Step 15.

Incubate and heat inactivate: Incubate reaction at 42 °C for 30 min

DURATION
00:30:00

First-strand cDNA synthesis

Step 16.

Incubate and heat inactivate: Heat-inactivate the reverse transcriptase at 90 °C for 5 min.

DURATION
00:05:00

First-strand cDNA synthesis

Step 17.

Incubate and heat inactivate: Keep the undiluted cDNA reactions on ice until the assembly of real-time PCR reaction or store as directed in the next step.

First-strand cDNA synthesis

Step 18.

Store cDNA: If desired, undiluted cDNA reactions can be stored at -20 °C for up to three days.

Real-Time PCR Amplification and Detection

Step 19.

In this step, the cDNA is amplified by real-time PCR in the EPIK Cancer miRNA Panel Assay.

Do not remove the plate from the sealed bag provided until you are ready to perform the qPCR step

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for that plate. If multiple qPCR machines are not available, see guidelines for suggestions for workflow.

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

Real-Time PCR Amplification and Detection

Step 20.

Prepare reagents: Thaw 2x SensiSMART™ PCR Master Mix and cDNA reactions (A, B, C or D) as required on ice (See "Workflow" in the Guidelines).

Real-Time PCR Amplification and Detection

Step 21.

Prepare reagents: Mix by quickly vortexing and spin down. cDNA reactions A, B, C and D are to be used with PCR plate A, B, C and D respectively (see Figure 3 in the Guidelines).

Real-Time PCR Amplification and Detection

Step 22.

Assemble the real-time PCR reagents: Mix each cDNA library with 2x SensiSMART™ PCR Master Mix and nuclease-free water in the proportion indicated in Table 2. Mix by vortexing and spin down.

Table 2:

Reagent	Volume (Plate A)	Volume (Plate B)	Volume (Plate C)	Volume (Plate D)
2x SensiSMART PCR Master Mix*	1000 µL	1000 µL	1000 µL	1000 µL
DEPC water	980 µL	980 µL	980 µL	980 µL
cDNA reaction A	20 µL	-	-	-
cDNA reaction B	-	20 µL	-	-
cDNA reaction C	-	-	20 µL	-
cDNA reaction D	-	-	-	20 µL
Total volume	2000 µL	2000 µL	2000 µL	2000 µL

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

Real-Time PCR Amplification and Detection

Step 23.

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Assemble the real-time PCR reagents: Remove the plate from the blue foil bag and place on a cold block or in an ice bucket.

Real-Time PCR Amplification and Detection

Step 24.

Assemble the real-time PCR reagents: Carefully peel back the carrier seal from the top of the plate and discard.

Real-Time PCR Amplification and Detection

Step 25.

Assemble the real-time PCR reagents: Dispense 20 µL cDNA:PCR master mix per well (see Table 2) into the corresponding PCR plate.

Real-Time PCR Amplification and Detection

Step 26.

Assemble the real-time PCR reagents: Seal the plate using a qPCR-compatible seal.

Real-Time PCR Amplification and Detection

Step 27.

Mix and spin: Centrifuge the plate briefly (30 s at 200 x g in a suitable plate centrifuge).

DURATION
00:00:30

Real-Time PCR Amplification and Detection

Step 28.

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

Table 3:

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)

NOTES

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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.

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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.

Data collection

Step 29.

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.

Analysis of results

Step 30.

Verifying the controls: The Ct values in wells E12 and F12 or wells G12 and H12 of each plate (see Guidelines Figure 4 or plate layout) should be approximately the same. Variation between wells replicates of the same sample on the same plate can also give an estimation of experimental error.

It is possible to use the panels as a "plus-minus" screen in order to determine which miRNAs are present or absent from the sample.

The RNA Spike is detected by two distinct assays. When the RNA Spike is used as a control, please ensure that the RNA Spike Ct values from the following wells are similar:

• Wells A12 and B12

• Wells C12 and D12

Variation between these duplicates gives a measure of the experimental error due to pipetting.

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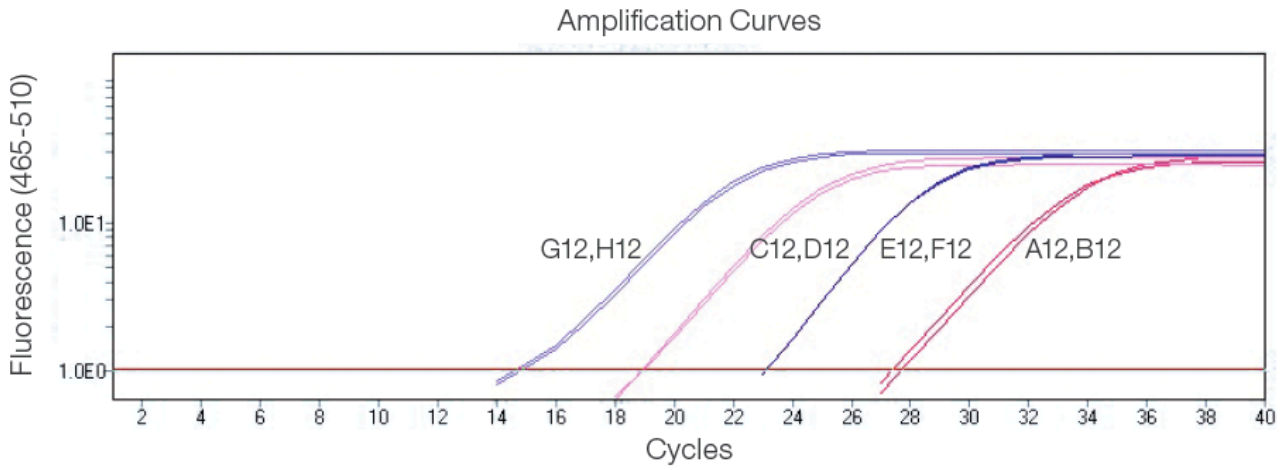

Fig. 5 EPIK miRNA Panel Plate calibrators. Amplification plot showing the inter-plate calibrators (Calibrator 1 - wells E12 and F12 and Calibrator 2 - wells G12 and H12) and spike controls (Spike Control 1 - wells A12 and B12 and Spike Control 2 - wells C12 and D12) from the EPIK Panel Assay. The results show very little variation between the duplicates, indicating low pipetting errors.

Analysis of results

Step 31.

miRNA profile analysis (relative presence or absence):

The most powerful application of the miRNA panels is when the results are used to make an estimate of abundance of each miRNA relative to a control. The panels supplied here allow the simultaneous relative determination of hundreds of miRNA species, either in duplicate with a single sample, or as single-panel assays of two samples. Raw data can then be exported into the Excel spreadsheet provided on our EPIK™ miRNA web page for further analysis (see www.bioline.com/mirna).

The user can deploy the software supplied with their qPCR machine. Some manufacturers supply software which is powerful enough to determine well-towell, plate-to-plate and sample-to-sample variation.

During the analysis, we recommend that at Ct of less than 36 is taken to be a positive reaction and Ct of more than 36 is taken to be negative. Some caution should be applied when examining miRNA with a Ct of 34 and above.

Analysis of results

Step 32.

Absolute quantification of miRNA

These miRNA assays are only suitable for determining relative abundance. The format of the panels does not allow an analysis of the absolute number of miRNA molecules in a sample. These measurements are complex to perform and to establish the limitations of the technology; users should consult the literature on the subject. In order to determine efficiency and other essential

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parameters critical for absolute determination, users should request individual miRNA assays in larger quantities. Please contact Bioline for more details.

Warnings

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.

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