Product Protocol

1 Introduction

1.1. Purpose

This document describes the use of the ID3EAL Multiplex RT RNA Spike-in Kit, for use with all individual ID3EAL miRNA qPCR assays. The synthetic RNA Spike-in controls included in this kit are to be introduced during RNA isolation and/or cDNA synthesis. Spike-in specific qPCR will generate corresponding C_T values which serve as internal calibrators. These are normalized during qPCR data analysis pre-processing, allowing the technical variations affecting each sample to be accounted for[1]. Hence, this kit allows miRNA expression to be measured with greater precision.

1.2. Background

RT-qPCR is the gold standard method for quantifying miRNA expression. It is highly sensitive, specific and typically shows a dynamic range >6 logs. The small size of miRNAs prevent amplification by conventional RT-qPCR. To address this technical challenge, MiRXES developed the ID3EAL miRNA qPCR system, a unique 3-primer technology which has demonstrable market leading performance for sensitive and robust miRNA detection and quantification[2].

Assay Principle

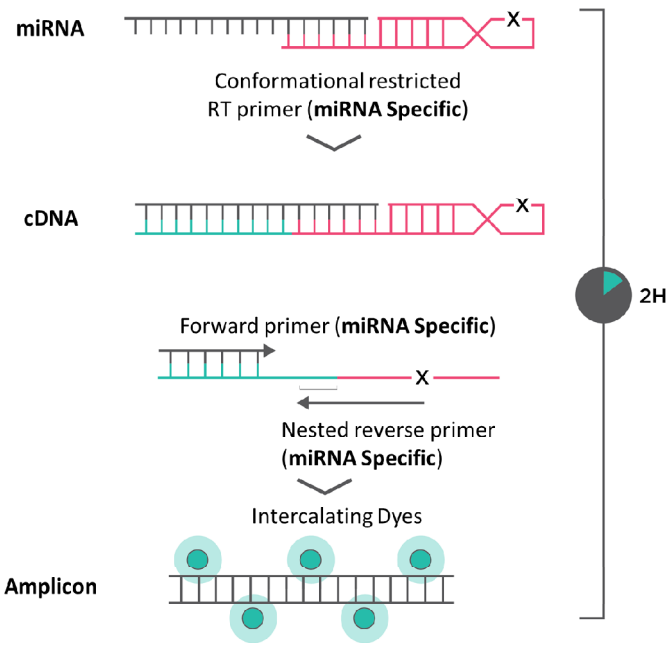


Figure 1. miRNA Assay Principle

Key Benefits

Increased Sensitivity
Optimised RT-qPCR primers and reagents to drive efficient target amplification from limiting amounts (≥ 1 pg) of input RNA.

Improved Specificity
No universal primers. Every assay utilizes 3 miRNA specific primers to discriminate single nucleotide differences.

Fast Detection
RNA to CT in less than 2 hours for faster turnaround time and improved throughput.

Reliable Data
Assays optimised by MiRXES' proprietary technology and wet-lab validated with RNA spike-in templates and biological samples.

Convenience
Compatible with all major qPCR instruments.

The ID3EAL miRNA qPCR system can detect miRNA present in samples at 10^8 – 10^1 copies[3]. At this level of sensitivity, uncontrolled technical variations can lead to noisy data. Consequently, control of technical variables such as inconsistent RNA isolation efficiency,

RNases and presence of RT or qPCR inhibitors, becomes a critical consideration for robust identification of differentially expressed miRNAs.

1.3. Intended Use

The ID3EAL Multiplex RT RNA Spike-in Kit is intended for molecular biology applications. These products are not intended for diagnosis, prevention or treatment of a disease.

All due care and attention should be exercised in the handling of this product. We recommend all users to adhere to national guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

1.4. Principle of the Procedure

Included in the ID3EAL Multiplex RT RNA Spike-in Kit are two tubes of synthetic RNA Spike-in controls:

- ID3EAL Spike-in control for Isolation (INT0415) contain a mix of RNA templates for Iso Spike-in 1 (1104101-ZZZ0000098A) and Iso Spike-in 2 (104101-ZZZ0000099A) qPCR assays.
- ID3EAL Spike-in control for Reverse Transcription (INT0416) contain RNA template for RT Spike-in (1104101-ZZZ0000097A) qPCR assay.

These RNA Spike-in controls are supplied in dried form and need to be reconstituted with the appropriate volume of nuclease free water prior to use (**see 6.1. Reconstitution of RNA Spike-in controls**). After reconstitution, the RNA Spike-in controls can be introduced into the sample preparation workflow during RNA isolation and/or cDNA synthesis (**see 6.2. Use of Spike-in controls**).

The ID3EAL Multiplex RT Spike-in Primers (INT0417) will generate cDNA of Spike-in controls for Isolation (INT0415) and/or Reverse Transcription (INT0416) in the same 20 µL reaction tube. Up to 5 additional miRNA-specific RT assays may be multiplexed in the same tube (**see 6.3. Reverse Transcription Reaction Setup**).

Following reverse transcription, the cDNA is diluted and used in individual ID3EAL qPCR reactions (**see 6.4. qPCR Reaction Setup**).

Read the Product Protocol carefully before using this product.

2 Product Description

- The ID3EAL Multiplex RT RNA Spike-in Kit contains:

Table 1. Kit Components

Part Number	Reagent	No. of tubes	Volume [µl/tube]
INT0415	ID3EAL Spike-in control for Isolation (60)	1	Dried
INT0416	ID3EAL Spike-in control for Reverse Transcription (60)	1	Dried
INT0417	ID3EAL Multiplex RT Spike-in Primers (60)	1	120
1104101-ZZZ0000097A	ID3EAL Individual miRNA qPCR Assay (100), RT Spike-in	1	220
1104101-ZZZ0000098A	ID3EAL Individual miRNA qPCR Assay (100), Iso Spike-in 1	1	220
1104101-ZZZ0000099A	ID3EAL Individual miRNA qPCR Assay (100), Iso Spike-in 2	1	220

- Each kit is sufficient for 60 cDNA synthesis reactions, providing enough cDNA for 4000 x 10 µL individual qPCR assays or 2400 x 20 µL individual qPCR assays.

3 Storage and Transportation

- The ID3EAL Multiplex RT RNA Spike-in Kit is shipped in a cold chain environment. The components of the kit should arrive cold.
- Upon arrival, the ID3EAL Spike-in control for Isolation (INT0415) and ID3EAL Spike-in control for Reverse Transcription (INT0416) should be stored in -90°C to -70°C All other components are to be stored at -25°C to -15°C.
- All components must always be thawed and kept on ice during preparation and use.

4 Material and Devices Required but Not Provided

NOTE : *It is the user's responsibility to validate system performance for any procedures used in their Laboratory.*

Table 2. Material and Devices Required but Not Provided

Type	Materials/Devices
Reagents	ID3EAL cDNA Synthesis System (1103103)
	ID3EAL Individual miRNA RT Primer 1-plex (1103113 / 1106104 / 1106114)
	ID3EAL miRNA qPCR Master Mix (1104202 / 1104205 / 1104212 / 1104215)
	ID3EAL Individual miRNA qPCR Assay (1104101 / 1106173 / 1106163)
	Surface decontaminant such as 10 % Bleach, 70 % Ethanol, RNase Away or equivalent.

RT-PCR Instrument	ID3EAL Multiplex RT RNA Spike-in Kit is compatible for use with real time PCR instrument calibrated for SYBR Green dye chemistry (absorption maxima: ~ 494 nm, emission maxima: ~521 nm)

5 Limitations, Warnings and Precautions

The ID3EAL Multiplex RT RNA Spike-in Kit described here has not been systematically verified for testing on platforms or reagents, other than those mentioned in these instructions.

5.1. Suggested Specimen Type

- Cells, fresh/frozen tissue, FFPE sections, biofluids, exosomes, extracted RNA.

5.2. Biosafety Precautions on Specimen Handling

- Put on protective disposable powder-free gloves, a laboratory coat and eye protection when handling the specimens.
- Perform all manipulations of potentially infectious specimens within a Class II (or higher) biological safety cabinet (BSC).
- Discard sample and assay waste according to your local safety regulations.

5.3. Handling the ID3EAL Multiplex RT RNA Spike-in Kit

- Before initial use, check the product and its components for:
  - Cold state upon arrival
  - Integrity
  - Completeness with respect to number, type and filling
  - Correct labelling
- Use of this product is limited to personnel specially instructed and trained in the techniques of real-time PCR.
- Avoid microbial and nuclease (DNase/RNase) contamination of the specimens and the components of the Kit.
- Always use DNase/RNase-free disposable pipette tips with aerosol barriers.
- Always wear protective disposable powder-free gloves when handling kit components.
- The workflow in the laboratory should proceed in a unidirectional manner. Use separated and segregated working areas for: (i) sample preparation, (ii) reaction setup and (iii) amplification/detection activities.
- Always wear disposable powder-free gloves in each area and change them before entering a different area.
- Dedicate supplies and equipment to the separate working areas and do not move them from one area to another.
- Store the reconstituted RNA Spike-in controls in aliquots separately from all other components of the Kit.
- Do not open the reaction tubes/plates post amplification, to avoid contamination with amplicons.
- Do not use expired components.

6 Protocol

6.1. Reconstitution of RNA Spike-in controls

- ID3EAL RNA Spike-in controls are shipped dry to improve their stability and performance.
- Centrifuge the tube(s) at 1000 x g for 30 sec to ensure RNA pellet is at the bottom of the tube.
- Add nuclease free water to the tube as follows:

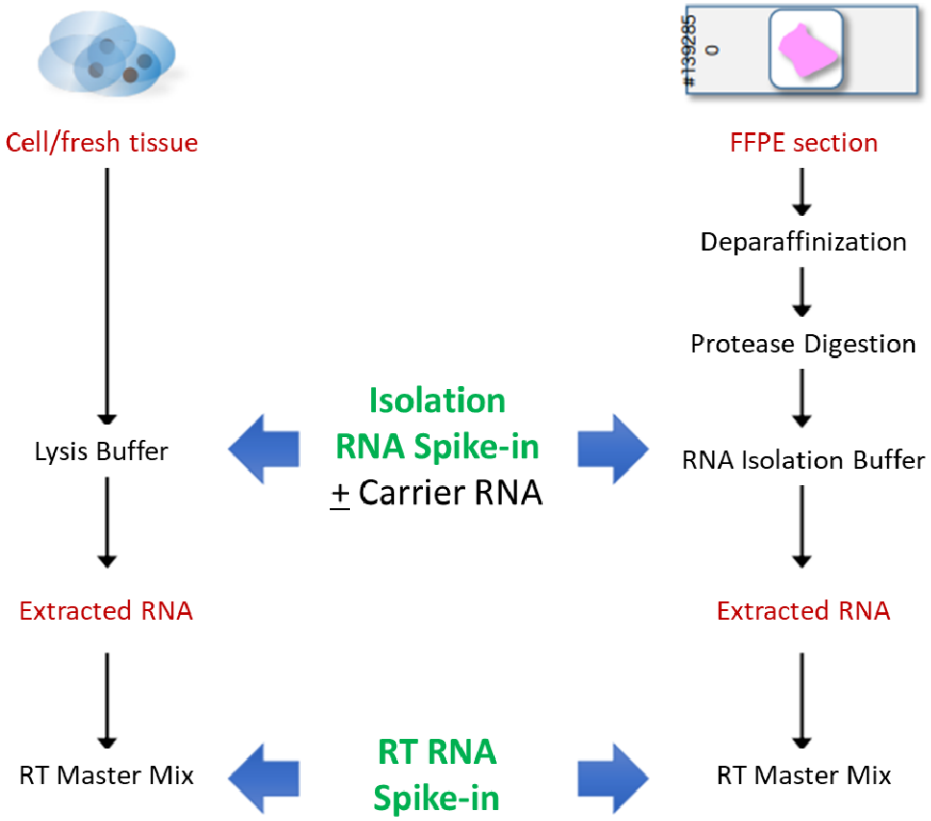
Table 3. Vol. of nuclease free water required.

Part Number	Product	Vol. of nuclease free water
INT0415	ID3EAL Spike-in control for Isolation (60)	330 µL
INT0416	ID3EAL Spike-in control for Reverse Transcription (60)	66 µL

- Vortex well and spin down the solution.
- Store at -80 ˚C in aliquots or use immediately.

6.2. Use of Spike-in controls

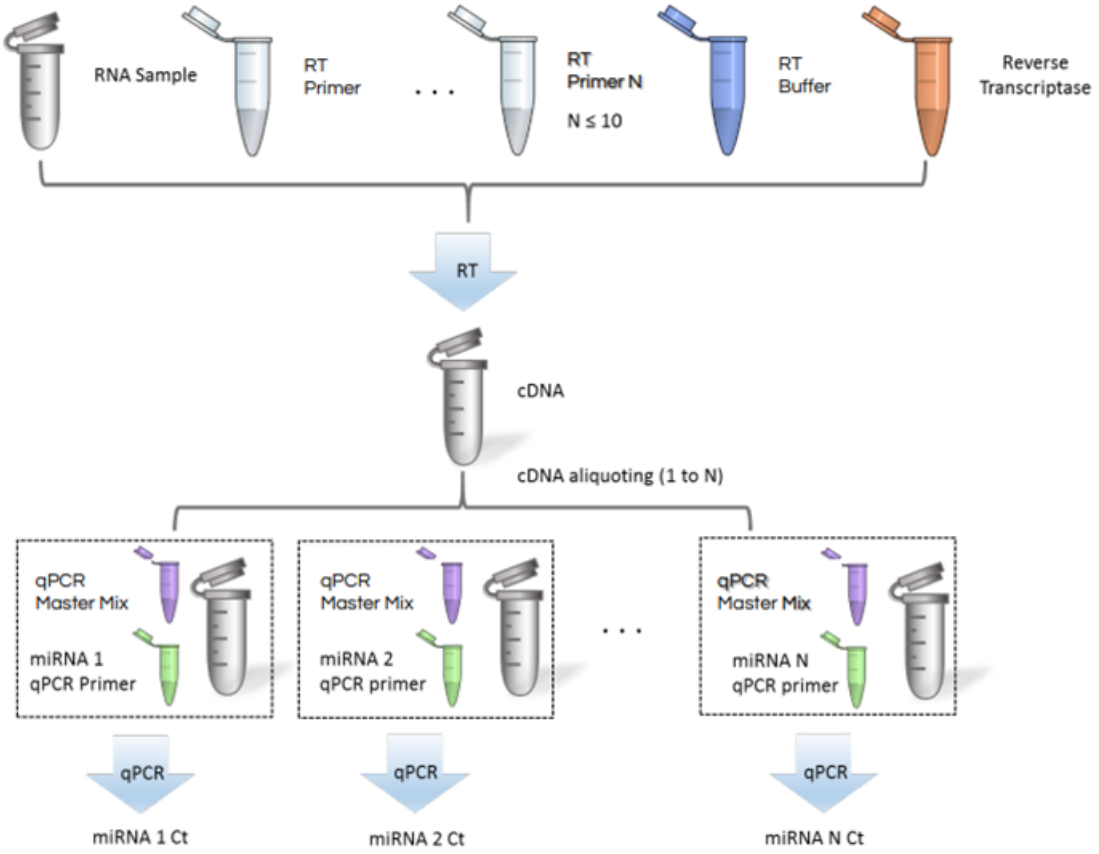
- RNA Spike-in controls can be introduced into the sample preparation workflow during RNA isolation and/or cDNA synthesis.



- To use during RNA isolation, Add 5 µL of ID3EAL Spike-in control for Isolation (INT0415) to each sample lysis buffer or RNA isolation buffer prior to sample mixing. Do not add directly to samples as the Spike-in control can be rapidly degraded by nucleases in the samples. Varying C_T values of Iso Spike-in 1 or Iso Spike-in 2 between samples indicates variations in RNA isolation efficiency or purity.
- To use during reverse transcription, Add 1 µL of ID3EAL Spike-in control for Reverse Transcription (INT0416) to each sample RT master mix. Varying C_T values of RT Spike-in between samples indicates variations in reverse transcription efficiency.

6.3. Reverse Transcription Reaction Setup

- The ID3EAL Multiplex RT Spike-in Primers (INT0417) provided in this kit simplifies the workflow for reverse transcription of Spike-in controls and up to 5 additional non-homologous (< 6 of 8 nt match at 5'/3' ends) miRNA targets.



- Gently thaw template RNA on ice. 100 ng – 1 µg may be used for each RT reaction input.
- Normalize the RNA input for each sample to the same amount. For biofluid extracts, normalize input to 5 µL of sample.
- Thaw ID3EAL miRNA RT Buffer (4x) (1103103), ID3EAL Multiplex RT Spike-in Primers (INT0417) and N x ID3EAL Individual miRNA RT Primer 1-plex (N ≤ 5) (1103113 / 1106104 / 1106114). Mix by vortexing and spin down by centrifugation. If necessary, incubate ID3EAL miRNA RT Buffer at 37˚C and vortex to dissolve any precipitate.

- Assemble the RT reaction according to Table 4. ID3EAL Reverse Transcriptase (20x) (1103103) should be kept at -20˚C and added as the last component of the RT reaction.

Table 4. Reverse transcription reaction setup (per reaction)

Reagent	Volume (µL)
ID3EAL Spike-in control for Reverse Transcription (INT0416)	1
ID3EAL Multiplex RT Spike-in Primers (INT0417)	2
N x ID3EAL Individual miRNA RT Primer 1-plex (N ≤ 5)	(N ≤ 5)
Sample RNA	X
Nuclease free water	11 - N - X
ID3EAL miRNA RT Buffer (4x)	5
ID3EAL Reverse Transcriptase (20x)	1
Total	20

- Mix assembled reagents, vortex for 30 sec and spin for 10 sec.
- Incubate reaction at 42˚C for 30 min followed by heat-inactivation at 95˚C for 5 min.

6.4 qPCR Reaction Setup

- Turn on the thermal cycler and pre-heat the lid to 105°C.
- Transfer all reagents and cDNA to ice.
- Gently thaw cDNA, ID3EAL miRNA qPCR Master Mix (1104202 / 1104205 / 1104212 / 1104215) and ID3EAL Individual miRNA qPCR Assay (1104101 / 1106173 / 1106163) on ice. Mix by vortexing and spin down by centrifugation.
- Dilute cDNA from Step 6.3 by 1:10 in nuclease free water. Pipette diluted cDNA template into each PCR well as indicated in Table 5.

Table 5. qPCR reaction setup (per reaction)

Reagent	Volume (µL)	
	384-well reaction	96-well reaction
ID3EAL Individual miRNA qPCR Assay (10x)	1	2
Diluted cDNA	3	5
Nuclease-free water	1	3
ID3EAL miRNA qPCR Master Mix (2x)	5	10
Total	10	20

- Seal the reaction plate with optical adhesive film.
- Centrifuge the reaction plate in a centrifuge with a microtiter plate rotor for 30 seconds at approximately 1000 x g.
- Perform Real-time PCR amplification with the following cycling parameters.

Table 6. Temperature Profile and Cycling

No. of cycles	Temperature (°C)	Time (Hr:Min:Sec)	Step
1x	95	00:10:00	Polymerase activation
	40	00:05:00	
40x	95	00:00:10	Denaturation
	60	00:00:30	Annealing/extension (Data collection)

- Check that the thermal cycling program is correct before leaving the thermal cycling to run.
- For basic information regarding the setup and programming of the different real-time PCR instruments, please refer to the user manual of the respective PCR instruments.

NOTE: *It is the user's responsibility to validate system performance for any procedures used in their Laboratory.*

7 Data Analysis

A normalizing scaling factor for each sample can be calculated from the RNA spike-in C_T values of every sample. This sample-specific scaling factor can be applied to all qPCR C_T values for the same sample to correct for sample-specific technical variations.

$$Avg.SP_{RT} = \frac{\sum_j^n C_T.SP_{RT}}{n} \quad (1)$$

$$SP.Factor_{RT,j} = Avg.SP_{iso} - SP_{RT,j} \quad (2)$$

(1) $Avg.SP_{RT}$ is the mean C_T value of all RT Spike-in ($C_T.SP_{RT}$) assays in a group of n samples that includes sample j.
(2) $SP.Factor_{RT,j}$ is the RT Spike-in scaling factor for sample j which can be applied to all other qPCR C_T values for sample j.

Scaling factors for Iso Spike-in 1 and Iso Spike-in 2 are calculated in the same manner. Typically, $SP.Factor_{iso1}$ or $SP.Factor_{iso2}$ is used by default. $SP.Factor_{RT}$ is used when C_T values of isolation spike-in controls are not available or usable. The appropriate spike-in scaling factor is determined through an overall analysis of results.

8 Assay Limitations

- Strict compliance with the Product Protocol is required for optimal results.
- Use of this product is limited to personnel specially instructed and trained in the techniques of real-time PCR including testing procedures and interpretation of results prior to performing the assay.
- Good laboratory practice is essential for proper performance of this assay. Extreme care should be taken to preserve the purity of the components of the Kit and reaction setups. All reagents should be closely monitored for impurity and contamination. Any suspicious reagents should be discarded.
- Appropriate specimen collection, transport, storage and processing procedures are required for the optimal performance of this test.
- This assay must not be used on the specimen directly. Appropriate nucleic acid extraction methods have to be conducted prior to using this assay.
- The presence of PCR inhibitors (e.g., heparin) may cause false negative or invalid results.

9 Quality Control

- To ensure consistent product quality, each lot of the ID3EAL Multiplex RT RNA Spike-in Kit had been tested against predetermined specifications.
- Users are strongly discouraged from combining components from assay kits of different lot numbers.

10 Technical Assistance

For technical advice, please contact Technical Support:
techsupport@mirxes.com

11 Disclaimers

MiRXES guarantees the performance of all products in the manner described in our product literature for 24 months from date of manufacture. The purchaser must determine the suitability of the product for its particular use.

MiRXES Pte Ltd is not liable for any damage or loss that may result from your use of the test.

12 Explanation of Symbols and Abbreviations

Manufacturer

13 Reference

1 Gilsbach, R., Kouta, M., Bönisch, H., & Brüss, M. (2006). Comparison of in vitro and in vivo reference genes for internal standardization of real-time PCR data. BioTechniques, 40(2), 173–177. https://doi.org/10.2144/000112052

2 Hong, L. Z., Zhou, L., Zou, R., Khoo, C. M., Chew, A. L. S., Chin, C.-L., & Shih, S.-J. (2021). Systematic evaluation of multiple qPCR platforms, NanoString and miRNA-Seq for microRNA biomarker discovery in human biofluids. Scientific Reports, 11(1), 4435. https://doi.org/10.1038/s41598-021-83365-z

3 Wan, G., 'En Lim, Q., & Too, H.-P. (2010). High-performance quantification of mature microRNAs by real-time RT-PCR using deoxyuridine-incorporated oligonucleotides and hemi-nested primers. RNA, 16(7), Article 7. https://doi.org/10.1261/rna.2001610

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