MiRXES
TO KNOW IS TO ACT

ID3EAL™ Cancer miRNA Knowledge Panel Manual

The fast track to impactful miRNA discoveries

The ID3EAL™ Cancer miRNA Knowledge Panel allows for the analysis of miRNA expression in every hallmark of cancer.

Targeting 352 miRNAs that are highly associated with onco- and tumor suppressor genes and pathways involved in diverse types of cancers, the ID3EAL™ Cancer miRNA Knowledge Panel miRNA can detect miRNA dysregulation across 46 cancer related pathways.

Powered by ID3EAL™ Technology

ID3EAL™ technology uses a proprietary and advanced thermodynamic model to target every miRNA with three (3) sequence-specific primers, ensuring robust detection with high specificity and sensitivity.

Unique conformationally restricted stem-loop primers generate a panel-specific cDNA library while miRNA-specific forward and reverse qPCR primers only amplify the intended targets.

Safety Notes

At MiRXES, we regard the safety of our customers and users of utmost importance.
Appropriate personal protective equipment should be always worn when handling chemicals.
For more information on the product, please consult the relevant safety data sheets, which can be obtained from the distributor, or alternatively, contact the Technical Service Department.
In case of any accidents, contact the relevant authorities in your area or region.

Product Use Limitations

This product is for research use only. No right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. Not for diagnostic use. Handling of this product should be done and observed with care and attention. All users of this product are highly recommended to adhere to the various safety and handling guidelines that pertains to this product.

Product Warranty and Satisfaction Guarantee

MiRXES warrants that its products will conform to the standards stated in its product specification sheets in effect at the time of shipment. MiRXES will replace any product that does not conform to the specifications, free of charge. This warranty limits MiRXES' liability to only the replacement of the product. The technology employed in this product is covered by Patent No: 185776, SG; ZL 201180038333.8, CN; 5851496, JP. Patents pending in other nations. The MiRXES terms and conditions can be obtained on request and provided at the back of our invoices. Any questions related to the product specifications and performances can be answered by contacting the MiRXES Technical Support, your distributor or by visiting www.mirxes.com.

Table of Contents

SAFETY NOTES 2
PRODUCT USE LIMITATIONS 2
PRODUCT WARRANTY AND SATISFACTION GUARANTEE 2
1. KIT CONTENT 4
2. PRODUCT DESCRIPTION 6
3. TECHNOLOGY AND PRINCIPLE 7
4. WORKFLOW OVERVIEW 8
5. RNA ISOLATION AND SPIKE-INS 9
6. RECONSTITUTION PROTOCOL 9
7. ID3EAL™ PANEL RNA SPIKE-IN PROTOCOL 10
8. REVERSE TRANSCRIPTION PROTOCOL 11
9. QPCR PROTOCOL 12
10. PLATE LAYOUT 13
11. DATA ANALYSIS 15
A. WORKFLOW 15
I. BASIC WORKFLOW 15
II. INTERMEDIATE WORKFLOW 15
III. ADVANCED WORKFLOW 16
12. SAFETY NOTES 18
13. PRODUCT USE LIMITATIONS 18
14. PRODUCT WARRANTY AND SATISFACTION GUARANTEE 18

1. Kit Content

ID3EAL™ Cancer miRNA Knowledge Panel (96-well format)

Component	SKU 1105263 12 assays	SKU 1105264 24 assays	Storage Temperature
ID3EAL™ miRNA RT Primers 96-plex	4 x 24 µl	8 x 24 µl	-20˚C
ID3EAL™ Panel Spike-in (lyophilized)	4 tubes	8 tubes	-20˚C dry -80˚C reconstituted
96-well plate*	48 x 96-well plates	96 x 96-well plates	-20˚C
Additional Reagents Kits Required (See below)			
ID3EAL™ cDNA Synthesis System (60)	1 x SKU 1103103	2 x SKU 1103103	-20˚C
ID3EAL™ miRNA qPCR Master Mix (2x)	4x SKU 1104205 or SKU 1104215	8 x SKU 1104205 or SKU 1104215	-20˚C

*Compatible plates are available for ABI 0.1ml, ABI 0.2ml and Roche/Bio-Rad qPCR machines

ID3EAL™ cDNA Synthesis System

Component	SKU 1103103 60 rxns	Storage Temperature
ID3EAL™ Reverse Transcriptase (20x)	3 x 20 µl	-20˚C
ID3EAL™ miRNA RT Buffer (4x)	3 x 100 µl	-20˚C

ID3EAL™ miRNA qPCR Master Mix

Component	SKU 1104205 1200 rxns	SKU 1104215* 1200 rxns	Storage Temperature
ID3EAL™ miRNA qPCR Master Mix (2X)	12 x 1000 µl	12 x 1000 µl	-20˚C

*For use in machines requiring Hi-ROX

ID3EAL™ Cancer miRNA Knowledge Panel (384-well format)

Component	SKU 1105363 12 assays	SKU 1105364 24 assays	Storage Temperature
ID3EAL™ miRNA RT Primers 96-plex	4 x 24 µl	8 x 24 µl	-20˚C
ID3EAL™ Panel Spike-in (lyophilized)	4 tubes	8 tubes	-20˚C dry -80˚C reconstituted
384-well plate*	12 x 384-well plates	24 x 384-well plates	-20˚C
Additional Reagents Kits Required (See below)			
ID3EAL™ cDNA Synthesis System (60)	1 x SKU 1103103	2 x SKU 1103103	-20˚C
ID3EAL™ miRNA qPCR Master Mix (2x)	2 x SKU 1104205 or SKU 1104215	4 x SKU 1104205 or SKU 1104215	-20˚C

*Compatible plates are available for ABI/Bio-Rad qPCR machines

ID3EAL™ cDNA Synthesis System

Component	SKU 1103103 60 rxns	Storage Temperature
ID3EAL™ Reverse Transcriptase (20x)	3 x 20 µl	-20˚C
ID3EAL™ miRNA RT Buffer (4x)	3 x 100 µl	-20˚C

ID3EAL™ miRNA qPCR Master Mix

Component	SKU 1104205 1200 rxns	SKU 1104215* 1200 rxns	Storage Temperature
ID3EAL™ miRNA qPCR Master Mix (2X)	12 x 1000 µl	12 x 1000 µl	-20˚C

*For use in machines requiring Hi-ROX

2. Product Description

MiRNAs play important regulatory functions within the cell and act as novel means of inter-cellular communication when secreted into the local milieu or into circulation. Dysregulation of miRNAs have been observed in every hallmark of cancer. Accurate detection of aberrant miRNA expressions in cancer pathogenesis, treatment response and recurrence could provide insights into the underlying mechanism and identify new drug targets.

The ID3EAL™ Cancer miRNA Knowledge Panel cover miRNAs that regulate key onco- and tumor suppressor genes and pathways. This content was curated from an in-house database of more than 20,000 samples, cross-referenced to published literature and validated by the Clarivate Web of Science database.

The ID3EAL™ Cancer miRNA Knowledge Panel enable researchers to quickly and reliably screen large number of cancer related miRNAs to identify signatures of interest in their cells, animal models or clinical specimens. The unique design features of the ID3EAL™ miRNA assays (Figure 1) offer several advantages that improve detection sensitivity, specificity and reproducibility over chemically modified primers, probe-based assays and small RNA sequencing (L. Z. Hong, et. al., Sci Rep 2021, 11, 4435).

Every assay has been extensively wet lab validated using synthetic miRNA templates and human RNA samples. These assays are supplied with optimized RT and qPCR reagents in a ready-to-use kit to minimize set-up time and maximize performance. Unique RNA Spike-in controls and inter-plate calibrators are built in to monitor and normalize technical variations from RNA isolation to qPCR.

3. Technology and Principle

Re-defining miRNA quantification with Sensitivity, Specificity, and Speed.

Assay Principle

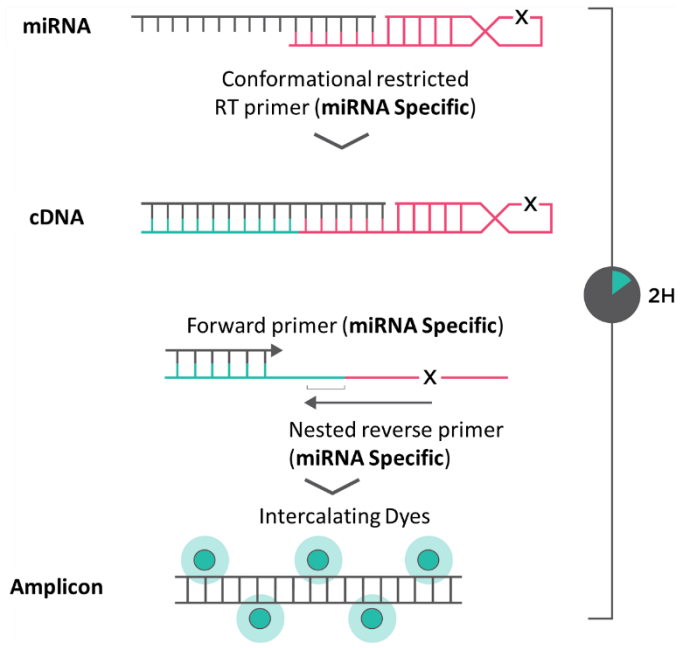


Figure 1. miRNA Assay Principle

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 C_T 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.

Unique Features

Unique RT Primer: Conformational restricted miRNA-specific RT primer efficiently hybridizes to mature but not precursor form of target miRNA.

Specific Real-Time PCR Primers: miRNA specific forward and reverse real-time PCR primers confer further specificity and enable robust amplification of amplicon.

Tailored RT-qPCR Reagents: Optimized RT and qPCR master mixes enhance signal to noise ratio.

4. Workflow Overview

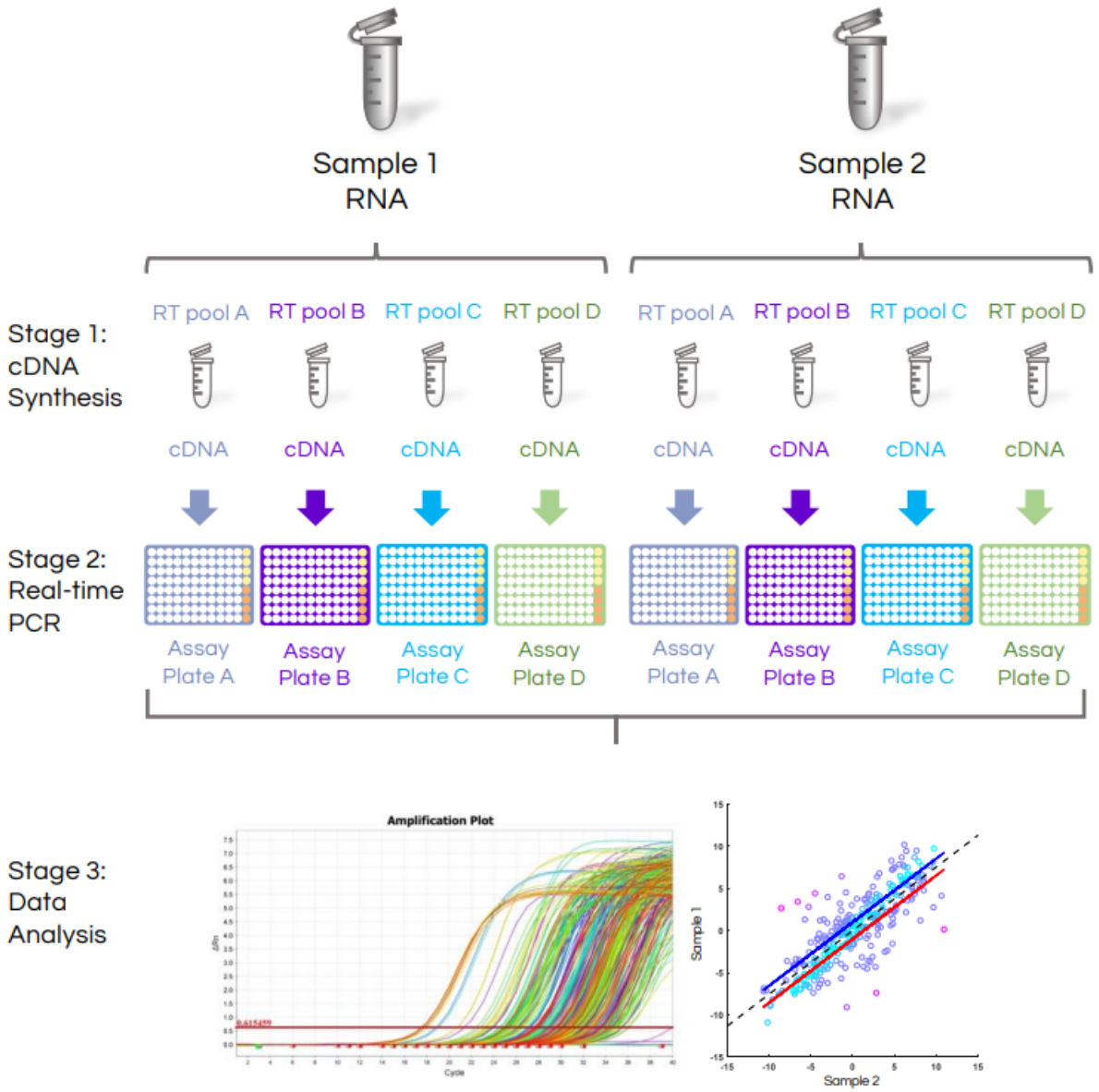


Figure 2. ID3EAL™ Cancer miRNA Knowledge Panel workflow

5. RNA Isolation and Spike-ins

RNA Isolation

The ID3EAL™ Cancer miRNA Knowledge Panel is agnostic to different biological sample types and RNA isolation methods. miRNAs have been detected in RNAs isolated from freshly harvested as well as stored cells and tissues, including FFPE tissues. When selecting methods of RNA isolation, users should ensure the method retains the small RNA fraction. A Bioanalyzer or a denaturing RNA gel can be used to verify the presence of small RNAs.

Though miRNAs are highly stable in native protein bound forms within biological samples, purified miRNAs like all RNAs, are susceptible to degradation by endogenous and exogenous ribonucleases (RNases) as well as chemical degradation. MiRXES recommends handling miRNA samples in dedicated RNA handling equipment in dedicated, isolated areas (e.g., PCR hoods). Filtered pipette tips and nuclease free consumables should be used.

The ID3EAL™ Panel RNA Spike-in contains uniquely designed small RNAs (~22 nt) with sequences distinct from endogenous miRNAs. It has been extensively tested and is compatible with various isolation methods, including phenol/chloroform, phenol-free, membrane, bead and precipitation-based methods, provided the method retains the small RNA fraction. The ID3EAL™ Panel RNA Spike-in is used to monitor and normalize experimental variations in sample RNA isolation, reverse transcription and qPCR.

6. Reconstitution Protocol

The ID3EAL™ Panel RNA Spike-in is shipped lyophilized to improve its stability and performance. Follow the reconstitution protocol below to prepare it for use.

Important: Always keep reagents on ice (or at 4˚C) during set up.

Steps:

1. Centrifuge the tube(s) at 1000 x g for 30 sec to ensure contents are at the bottom of the tube.
2. Add 66 µl nuclease free water to a tube of ID3EAL™ Panel RNA Spike-in.
3. Vortex well and spin down the solution.
4. Use immediately or store at - 80˚C.

7. ID3EAL™ Panel RNA Spike-in Protocol

The ID3EAL™ Panel RNA Spike-in can be used in either of two modes:

1. RNA Isolation Control (Mode 1)

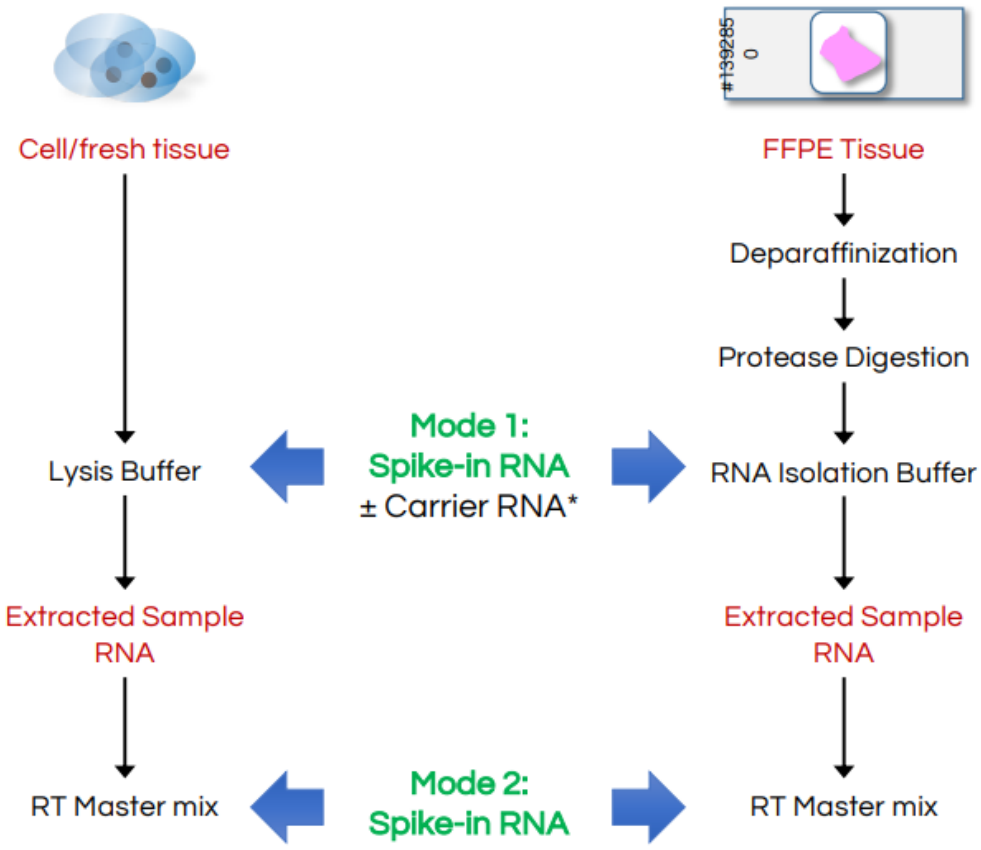
Add 5 µl of the reconstituted ID3EAL™ Panel RNA Spike-in per sample to the sample lysis buffer or RNA isolation buffer before mixing with biological sample. A difference in the measured RNA Spike-in C_T values between samples indicates varying RNA isolation yield and/or RNA purity.

Caution: ID3EAL™ Panel RNA Spike-in should never be added directly into biological samples as it can be rapidly degraded by nucleases present in the samples.

2. Reverse Transcription Control (Mode 2)

Add 1 µl of the reconstituted ID3EAL™ Panel RNA Spike-in to each RT reaction mix. RNA Spike-in C_T values allow normalization of technical variations between RT reactions.

Note: The ID3EAL™ Panel RNA Spike-in cannot be used for both Mode 1 and Mode 2.



*Addition of carrier RNA, such as bacteriophage MS2 total RNA, is recommended to improve RNA isolation yield, when the biological sample is expected to yield only small amounts of RNA. Select carrier RNA that is guaranteed to be free from microRNAs.

8. Reverse Transcription Protocol

Important: Always keep reagents on ice (or at 4˚C) during set up.

Stage I: Reverse Transcription

For the analysis of 352 cancer miRNAs, 4 RT reactions are required per sample, using RT primer pool A, B, C and D. These four types of Primer Pools are prepared and shipped in 24 µl of nuclease free water.

Note: Performing RT reactions for all samples at the same time minimizes batch to batch technical variations.

Steps:

1. Gently thaw template RNA on ice. Use up to 1 µg per 20 µl RT reaction. (For most sample types, 100 ng is sufficient).
2. Gently thaw ID3EAL™ RT Buffer and RT Primer Pool A, B, C and D on ice. Mix by vortexing and spin down by centrifugation. Incubate ID3EAL™ RT Buffer at 37˚C and vortex to dissolve any precipitate.
3. Assemble RT reaction according to Table 1. Reverse Transcriptase should be kept at -20˚C and added to the master mix last.

Table 1: Reverse Transcription reaction setup (per sample)

Reagents	RT_A	RT_B	RT_C	RT_D
Template RNA (up to 1 µg)	X µl	X µl	X µl	X µl
ID3EAL™ miRNA RT Buffer (4x)	5 µl	5 µl	5 µl	5 µl
RT Primer Pool A	2 µl	-	-	-
RT Primer Pool B	-	2 µl	-	-
RT Primer Pool C	-	-	2 µl	-
RT Primer Pool D	-	-	-	2 µl
Reverse Transcriptase	1 µl	1 µl	1 µl	1 µl
Nuclease free water (Optional: include 1 µl Panel Spike-in)	12 - X µl	12 - X µl	12 - X µl	12 - X µl
Total volume	20 µl	20 µl	20 µl	20 µl

4. Mix assembled reagents thoroughly and spin briefly.
5. Incubate reaction at 42˚C for 30 min followed by heat-inactivation at 95˚C for 5 min.

PAUSE POINT: Undiluted cDNA can be stored at -20 ˚C for up to 4 weeks. Avoid repeated freeze-thaw cycles.

9. qPCR Protocol

Important: Always keep reagents on ice (or at 4˚C) during set up.

Notes on Setting Up Reactions
○ Before preparing any reagents, turn on the thermal cycler.
○ If possible, Pre-heat the thermal cycler lid to 105 ˚C.
○ Keep all reagents and RNA on ice.
○ Set up all reactions on a cool block or on ice to minimize RNA degradation.
○ Check to ensure that the thermal cycling program is correct before placing samples into the thermal cyclers.
○ Always check that the thermal cycling program has correctly started before leaving the thermal cycler.

Stage II: Real-time qPCR amplification and detection

With consideration to the number of qPCR machines available, plan the following steps such that each cDNA is thawed only once.

6. Thaw cDNA from Step 5 and ID3EAL™ miRNA qPCR Master Mix on ice. Mix by vortexing and spin down by centrifugation. cDNA A, B, C and D are to be used for PCR plates A, B, C and D respectively.
7. Remove the PCR plate from the blue foil bag and centrifuge briefly (30 s at 200 g). Carefully peel off the carrier seal.
8. Place the PCR plate on a cold block in an ice bucket. Ensure PCR plate is sufficiently chilled before adding cDNA: PCR master mix.
9. Assemble cDNA: PCR master mix on ice according to Table 2.1 or Table 2.2.
10. Seal the plate using a qPCR compatible seal. Centrifuge the PCR plate briefly (30 s at 200 g).
11. Perform Real-time PCR amplification with the following cycling parameters.

Table 2: real-time qPCR thermos-cycling protocol

Cycles	Temperature	Time	Notes
1x	95˚C	10 min	Polymerase activation
	40˚C	5 min	
4x	95˚C	10 s	Denaturation
	60˚C	30 s	Annealing/extension (acquire fluorescence reading at end of step)

12. Data analysis.

10. Plate Layout

qPCR Plate Map (96 well plate)

All the necessary qPCR primers and inter-plate controls have been pre-loaded in the ID3EAL™ Panel plates. Use cDNA from each RT Primer Pool with its respective plate, i.e., cDNA from RT Primer Pool A should be used with Plate A. The reaction volume in each well is 20 µl.

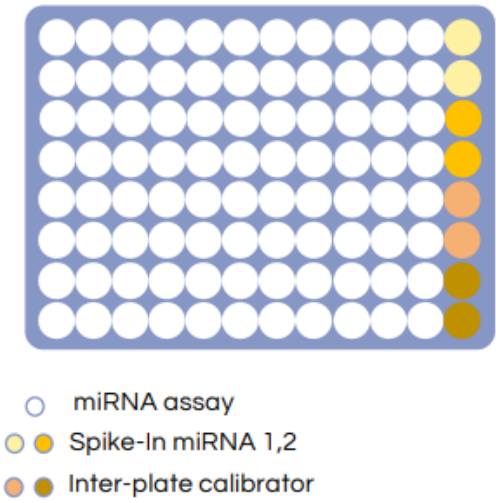


Table 2.1: qPCR reaction setup (96 well format)

Reagents	Plate_A	Plate_B	Plate_C	Plate_D
ID3EAL™ miRNA qPCR Master Mix	1000 µl	1000 µl	1000 µl	1000 µl
Nuclease free water	980 µl	980 µl	980 µl	980 µl
cDNA A	20 µl	-	-	-
cDNA B	-	20 µl	-	-
cDNA C	-	-	20 µl	-
cDNA D	-	-	-	20 µl
Total volume	2000 µl	2000 µl	2000 µl	2000 µl

qPCR Plate Map (384 well plate)

All the necessary qPCR primers and inter-plate controls have been pre-loaded in the ID3EAL™ Panel plates. Use cDNA from each RT Primer Pool with its respective wells, i.e., cDNA from RT Primer Pool A should be used with wells indicated "A" below. The reaction volume in each well is 10 µl.

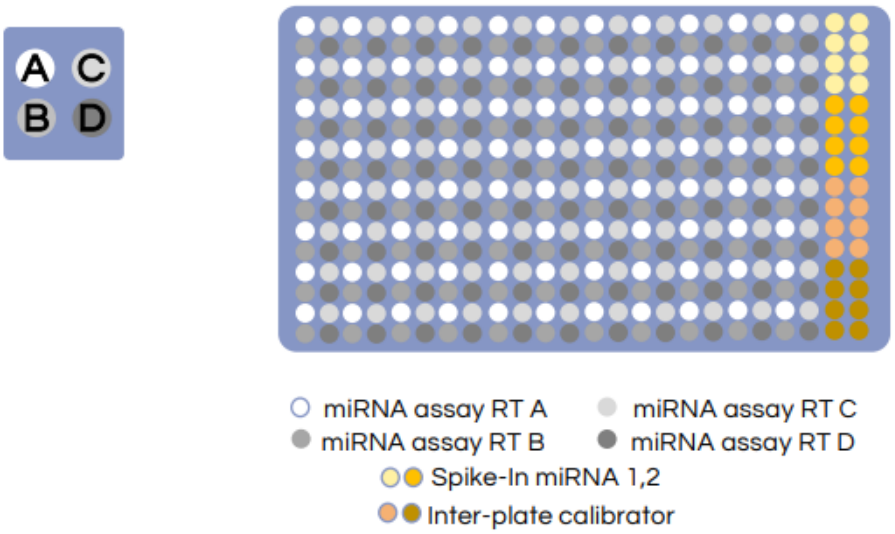


Table 2.2: qPCR reaction setup (96 well format)

Reagents	RT_A	RT_B	RT_C	RT_D
ID3EAL™ miRNA qPCR Master Mix	500 µl	500 µl	500 µl	500 µl
Nuclease free water	480 µl	480 µl	480 µl	480 µl
cDNA A	20 µl	-	-	-
cDNA B	-	20 µl	-	-
cDNA C	-	-	20 µl	-
cDNA D	-	-	-	20 µl
Total volume	1000 µl	1000 µl	1000 µl	1000 µl

11. Data Analysis

Analysis Template

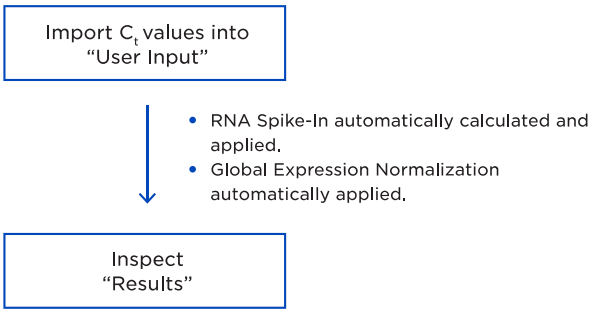
Analysis of up to 24 samples can be carried out using the Microsoft Excel template supplied. The template can carry out simple automated analyses including fold change plots and volcano plots. Basic to advanced workflows can be used based on user comfort level. More advanced analysis (e.g., analysis of sample groups, heat maps) can be carried out with specialized software packages or through our data analysis services.

a. Workflow

This tab shows the different workflows that may be used to analyse the miRNA expression data.

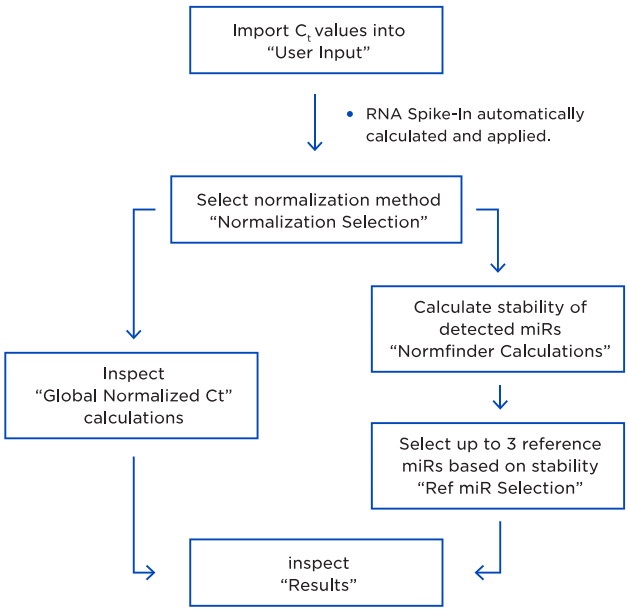
i. Basic workflow

This workflow only requires the input of raw data from the qPCR software.



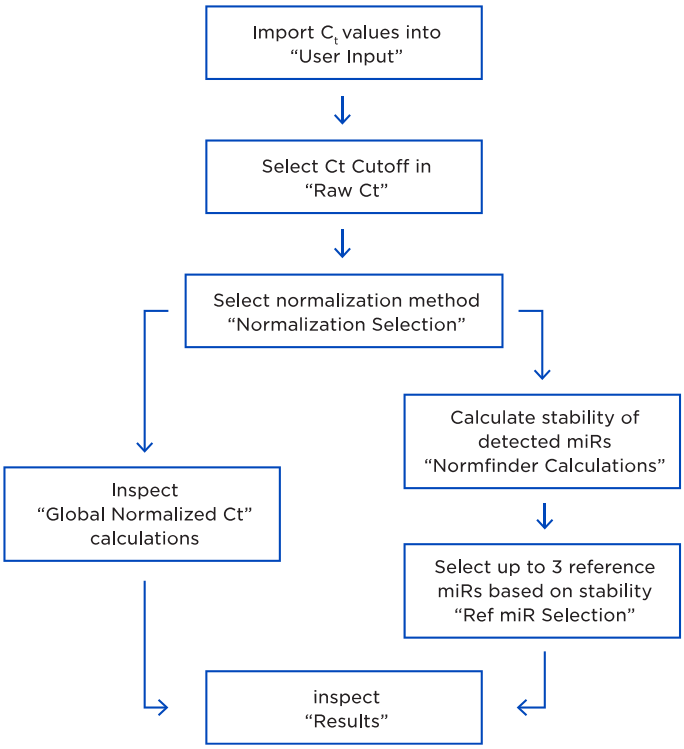
ii. Intermediate workflow

This workflow requires the user to select the desired type of normalization for their studies: Global Normalization or Reference miRNA Normalization using reference miRNAs identified by Normfinder (https://moma.dk/normfinder-software).



iii. Advanced workflow

This workflow requires the user to understand their experiment well enough to identify and specify their desired C_T Cut-off based on their miRNA of interest.



Verification of Spike-in

Spike-in 1 and 2 miRNAs are tested in duplicates for each cDNA pool. If Spike-ins have been added during isolation (Mode 1), Spike-in C_T values captures technical variations from isolation until qPCR. If Spike-ins have been added during cDNA synthesis (Mode 2), they only capture technical variation during cDNA synthesis onwards. Check to ensure that all Spike-ins from all samples have C_T value lower than 30. C_T value higher than 25 indicates poor isolation efficiency or cDNA synthesis efficiency. Increasing RNA input may improve results. Spike-in 1 and 2 will have different C_T values, but for the same sample, the difference of C_T values for each Spike-in duplicates should be within 0.5 C_T.

Verification of Inter Plate Control (IPC)

IPCs are DNA template that are tested 4 times for each cDNA pool. First check the standard deviation of IPCs C_T for each cDNA pool; Standard deviation greater than 0.5 indicates pipetting error, and caution should be taken when interpreting results. IPC serves two purposes. First, IPC can be used as positive controls for qPCR process. If IPC has C_T value greater than 25, it indicates qPCR reaction has failed. Secondly, IPC can be used to normalize plate to plate variation. Due to machine variations, C_T values from different machines are not directly comparable. Use IPCs to normalize the machine-to-machine variation as IPCs will have the same C_T value across machines if the machine variation is eliminated.

Relative Abundance Quantification

When multiple samples are tested with miRNA panels, the relative abundance of hundreds miRNAs in different samples can be simultaneously determined with $\Delta\Delta C_T$ method. User only need to export C_T values and input the values into respective positions of supplied Excel sheet. We recommend user treat C_T value less than 33 as positive values; and treat C_T greater than 33 as negative values.

Absolute Copy Number Quantification

The ID3EAL™ Cancer miRNA Knowledge Panel only supports relative quantification analysis. The format of the panels does not support absolute copy number analysis.

Should users require absolute copy number quantification, MiRXES provides the ID3EAL™ Premium 700 Profiling Service. For more information, please contact sales@mirxes.com.

12. Safety Notes

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13. Product Use Limitations

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The technology employed in this product is covered by Patent No: 185776, SG; ZL 201180038333.8, CN; 5851496, JP. Patents pending in other nations.

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Any questions related to the product specifications and performances can be answered by contacting the MiRXES Technical Support, your distributor or by visiting www.mirxes.com.

MiRXES
TO KNOW IS TO ACT

Visit our website or simply send an email:

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