ID3EAL miRNA Knowledge Panel 384 Targets

Principle and Protocol

MiRXES

To Know is to Act

Table of Contents

384 Target Assay Panel
Kit Content

ID3EAL miRNA Knowledge Panel 384 Targets (96 well format)

Component	(2)	(6)	(12)	(24)	Storage Temperature
	1105251 (96 well)	1105252 (96 well)	1105253 (96 well)	1105254 (96 well)	
ID3EAL miRNA RT Primers 96-plex	4 x 24 µl	4 x 24 µl	4 x 24 µl	8 x 24 µl	-20 °C
ID3EAL Panel Spike-in (lyophilized)	1 tube	1 tube	1 tube	2 tubes	-20 °C dry -80 °C reconstituted
*96-well plate	8 x 96 well plate	24 x 96 well plate	48 x 96 well plate	96 x 96 well plate	-20 °C
Additional Reagents Required					
ID3EAL cDNA Synthesis System	1 x (20) 1103101	1 x (60) 1103103	1 x (60) 1103103	2 x (60) 1103103	-20 °C
ID3EAL miRNA qPCR Master Mix (2X)	1 x (800) 1104204 /1104214	1 x (2400) 1104206 /1104216	2 x (2400) 1104206 /1104216	4 x (2400) 1104206 /1104216	-20 °C

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

Additional Reagents

Component	(20)	(60)	Storage Temperature
	1103101	1103103	
ID3EAL Reverse Transcriptase (20x)	1 x 20 µl	3 x 20 µl	-20 °C
ID3EAL miRNA RT Buffer (4x)	1 x 100 µl	3 x 100 µl	-20 °C

Component	(200)	(800)	(1200)	(2400)	Storage Temperature
	1104202	1104204	1104205	1104206	
ID3EAL miRNA qPCR Master Mix (2X)	2 x 1000 µl	8 x 1000 µl	12 x 1000 µl	24 x 1000 µl	-20 °C

Component	(200)	(800)	(1200)	(2400)	Storage Temperature
	1104212	1104214	1104215	1104216	
ID3EAL miRNA qPCR Master Mix – HR (2X)*	2 x 1000 µl	8 x 1000 µl	12 x 1000 µl	24 x 1000 µl	-20 °C

*For use in machines requiring Hi-ROX

384 Target Assay Panel
Kit Content

ID3EAL miRNA Knowledge Panel 384 Targets (384 well format)

Component	(2)	(6)	(12)	(24)	Storage Temperature
	1105261 (384 well)	1105262 (384 well)	1105263 (384 well)	1105264 (384 well)	
ID3EAL miRNA RT Primers 96-plex	4 x 24 µl	4 x 24 µl	4 x 24 µl	8 x 24 µl	-20 °C
ID3EAL Panel Spike-in (lyophilized)	1 tube	1 tube	1 tube	2 tubes	-20 °C dry -80 °C reconstituted
*384-well plate	2 x 384 well plate	6 x 384 well plate	12 x 384 well plate	24 x 384 well plate	-20 °C
Additional Reagents Required					
ID3EAL cDNA Synthesis System	1 x (20) 1103101	1 x (20) 1103101	1 x (20) 1103101	2 x (60) 1103103	-20 °C
ID3EAL miRNA qPCR Master Mix (2X)	2 x (200) 1104202 /1104212	1 x (1200) 1104205 /1104215	1 x (2400) 1104206 /1104216	2 x (2400) 1104206 /1104216	-20 °C

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

Additional Reagents

Component	(20)	(60)	Storage Temperature
	1103101	1103103	
ID3EAL Reverse Transcriptase (20x)	1 x 20 µl	3 x 20 µl	-20 °C
ID3EAL miRNA RT Buffer (4x)	1 x 100 µl	3 x 100 µl	-20 °C

Component	(200)	(800)	(1200)	(2400)	Storage Temperature
	1104202	1104204	1104205	1104206	
ID3EAL miRNA qPCR Master Mix (2X)	2 x 1000 µl	8 x 1000 µl	12 x 1000 µl	24 x 1000 µl	-20 °C

Component	(200)	(800)	(1200)	(2400)	Storage Temperature
	1104212	1104214	1104215	1104216	
ID3EAL miRNA qPCR Master Mix – HR (2X)*	2 x 1000 µl	8 x 1000 µl	12 x 1000 µl	24 x 1000 µl	-20 °C

*For use in machines requiring Hi-ROX

Cancer miRNA Assay Panel
Product Description

MicroRNAs (miRNAs) are small non-coding RNAs prevalent in many species of eukaryotes. MiRNAs play important regulatory functions within the cell and act as novel means of inter-cellular communication when secreted into local milieu or in circulation. Dysregulation of miRNAs have been observed in every hallmark of cancer. Several miRNAs are currently being evaluated as targets of therapeutic intervention for various forms of cancers. 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.

Sensitive and robust detection of miRNA is challenging due to its small size (~22nt) and high degree of sequence homology among family members. RT-qPCR remains as the gold standard for miRNA detection for its sensitivity, specificity and dynamic range. Methods relying on sequence-dependent probes and chemically modified primers can be time consuming, labor intensive and can suffer from sample-to-sample variability. The unique design features of the ID3EAL miRNA assays (Figure 1) offer several advantages that allows improved sensitivity, specificity and reliability while reducing reaction time and setup complexity.

The IDEAL™ Cancer miRNA assay panel consists of 352 miRNAs highly associated with various cancer types and regulate key onco- and tumor suppressor genes and pathways. The panel is selected through in-depth survey of in-house research data and published literature. Each assay has been extensively wet-lab validated using synthetic miRNA template and human sample RNA. These assays are formatted in 96-well plates and supplied with proportional amount of optimized RT and qPCR reagent in a complete and ready-to-use kit to minimize set-up time and maximize performance. Unique spike-in RNAs and inter-plate calibrators are built in to monitor and normalize technical variations from RNA isolation to qPCR. These kits are compatible with all major real-time qPCR platforms and are available in various pack sizes.

The Cancer miRNA assay Panel is meant to enable researchers to quickly and reliably screen large number of cancer related miRNAs to identify candidates of interest in their respective cell, animal models or in clinical specimen. Individual miRNA assays and customized panels are available to validate the dysregulation and interrogate the functions of candidates in greater details.

ID3EAL miRNA Assay Technology and Principle

Re-defining miRNA Quantification with Sensitivity, Specificity and Speed

Key Benefits

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

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

Speedy Detection
RNA to Ct in less than 2 hours for faster turnaround and improved throughput.

Reliable Data
Assays optimized by MIRXES' proprietary algorithm and wet-lab validated with synthetic miRNA templates and RNA from biological samples.

Convenience
Complete kit to minimize set-up time. Compatible with all major qPCR instruments.

Assay Principle

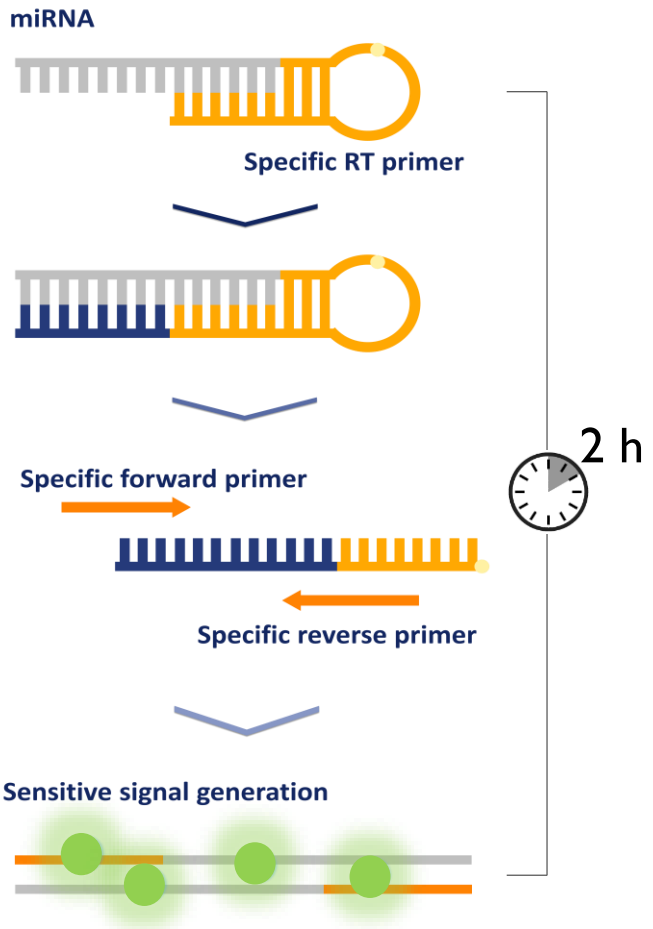


Figure 1. miRNA Assay Principle

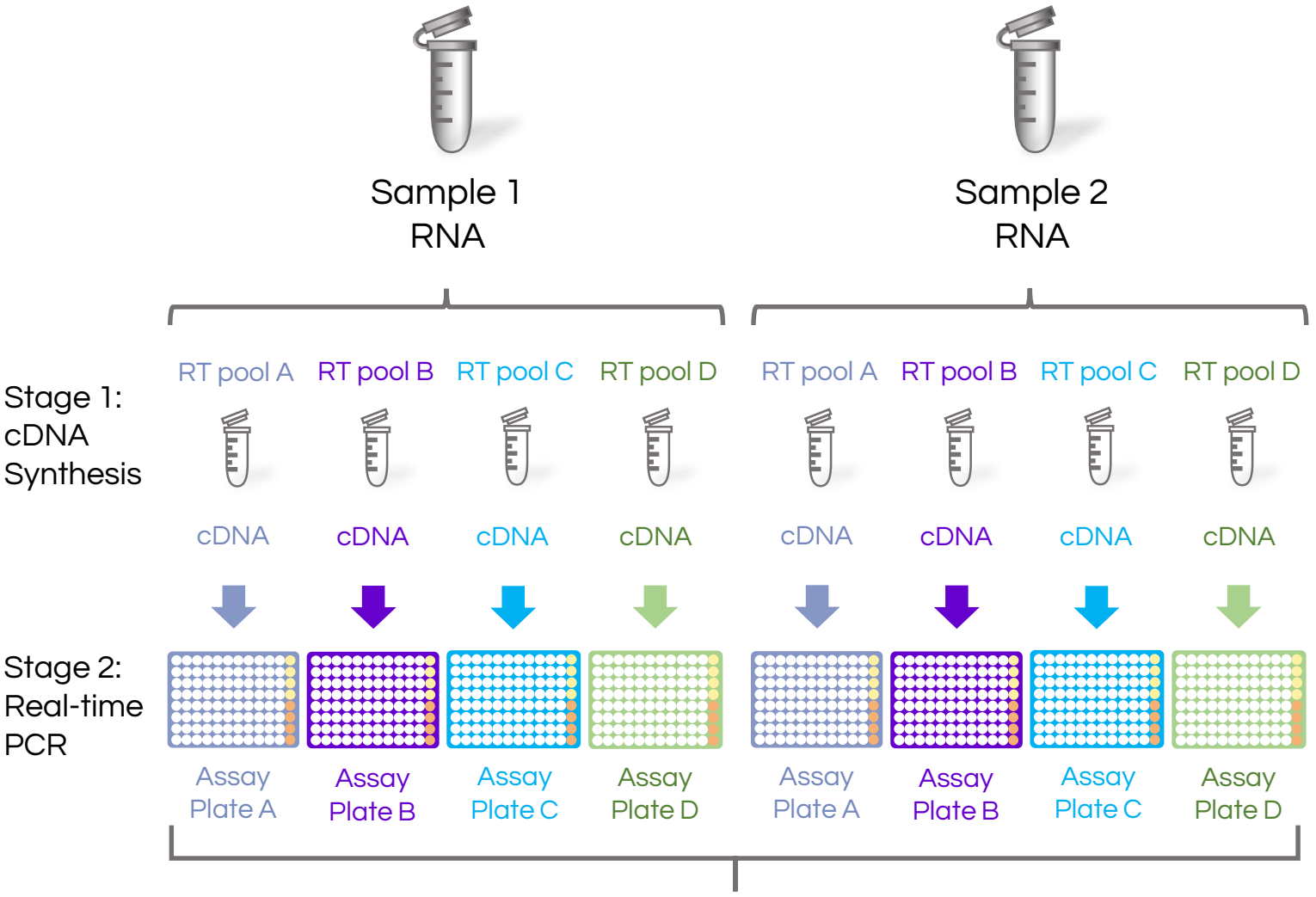
Unique Features

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.

384 Target Assay Panel
Workflow Overview



Stage 3:
Data
Analysis

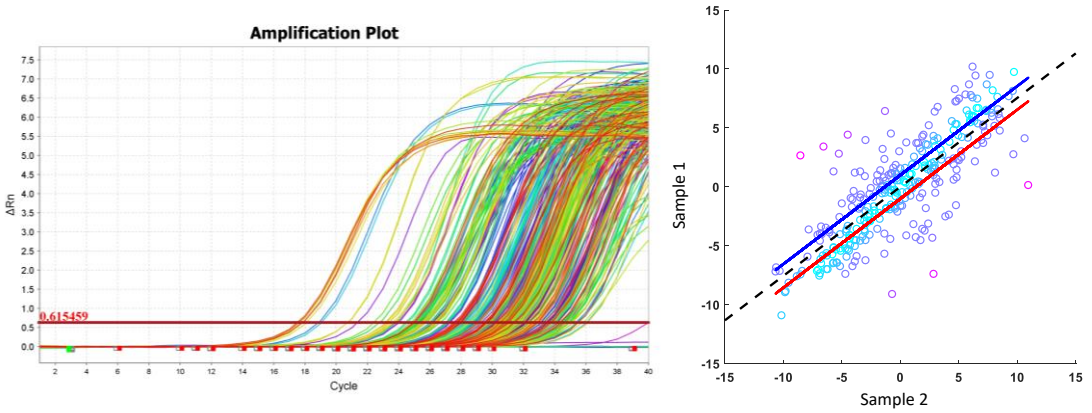


Figure 2. Cancer miRNA Assay Panel Workflow

RNA Isolation and Spike-ins

RNA Isolation

ID3EAL miRNA Assays are agnostic to type of biological samples and methods of RNA isolation. 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.

ID3EAL™ RNA Spike-Ins are uniquely designed small RNAs (~22 nt) with sequences distinct from endogenous miRNAs. These miRNA spike-ins have been extensively tested and are compatible with various isolation methods, including phenol/chloroform, phenol-free, membrane, bead and precipitation-based methods, provided the method retains the small RNA fraction. MiRXES recommends the use of miRNA spike-ins to monitor and normalize experimental variations in sample RNA isolation, reverse transcription and qPCR.

Reconstitution Protocol

ID3EAL Panel Spike-Ins and RT Primer Pools are shipped dry to improve their stability and performance. Follow the reconstitution protocol below to prepare them for use.

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

Step 1: Centrifuge the tube(s) at 1000 x g for 30 sec to ensure contents are at the bottom of the tube.

Step 2: Add nuclease free water to the tube as follows:

Product	Volume of nuclease free water	Storage after Reconstitution
ID3EAL Panel RNA Spike-In	66 µl	-80 °C
ID3EAL Panel RT Primer Pool	28 µl	-20 °C

Step 3: Vortex well and spin down the solution.

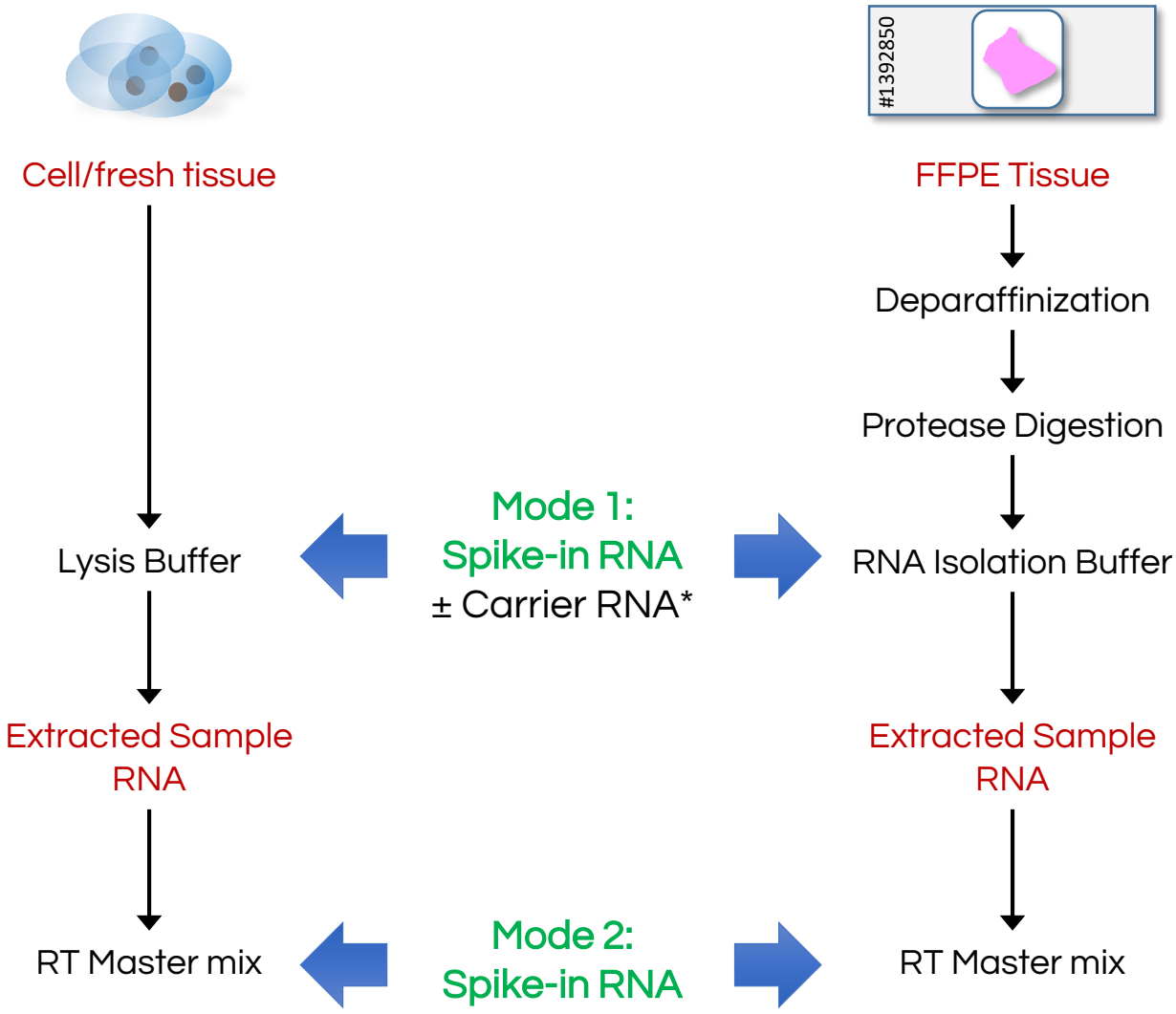
Step 4: Store in aliquots or use immediately.

miRNA Spike-in Protocol

The miRNA spike-ins can be used in two modes:

1. as RNA isolation control. Add 5 µl of the reconstituted spike-ins per sample to sample lysis buffer or RNA isolation buffer before mixing with biological sample. A difference in the measured levels of miRNA spike-ins indicates varying RNA isolation yield and/or RNA purity. Caution: miRNA spike-in mix should never be mix with biological samples directly as it can be rapidly degraded by nucleases present in the samples.
2. as reverse transcription control. Add 1 µl of the reconstituted spike-ins per reaction to RT master mix. Measured levels of miRNA spike-ins allow normalization between RT reactions.

Note: it is not possible to use the same miRNA spike-ins for both modes in the same experiment.



* 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.

384 Target Assay Panel
Reverse Transcription Protocol

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

Stage 1: 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.

Step 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).

Step 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.

Step 3: Assemble RT reaction according to Table 1. Reverse Transcriptase should be kept at -20˚C and added to the master mix last.

Note: Perform RT reactions for all samples under comparison at the same time to minimize experimental variations.

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
Nuclease free water (Optional: incl. 1 µl Panel Spike-In)	12 - X µl	12 - X µl	12 - X µl	12 - 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
Total volume	**20 µl**	**20 µl**	**20 µl**	**20 µl**

Step 4: Mix assembled reactions thoroughly and spin briefly.

Step 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 2 weeks.
Avoid repeated freeze-thaw cycles.

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

Stage 2: 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.

Step 6: Thaw **ID3EAL miRNA qPCR Master Mix** and **cDNA** from step 5 on ice. Mix by vortexing and spin down by centrifugation.
cDNA A, B, C, D are to be used for PCR plate A, B, C and D respectively.

Step 7: Remove the PCR plate from the blue foil bag and centrifuge briefly (30 s at 200 g). Carefully peel off the carrier seal.

Step 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.

Step 9: Assemble cDNA:PCR master mix on ice according to Table 2.1 or Table 2.2 according to plate type.

Step 10: Seal the plate using a qPCR compatible seal. Centrifuge the PCR plate briefly (30 s at 200 g).

Step 11: Perform Real-time PCR amplification with the following cycling parameters.

Table 3 – real-time qPCR thermo-cycling protocol

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

Step 12: Data analysis.

qPCR Plate Map (96 well plate)

All the necessary qPCR primers and inter-plate controls have been pre-loaded in the IDEAL™ Panel plates. Use cDNA from each RT Primer Pool with its respective plate. E.g. cDNA from RT Primer Pool A should be used with Plate A.

In a single PCR well, the reaction volume is 20 µl.

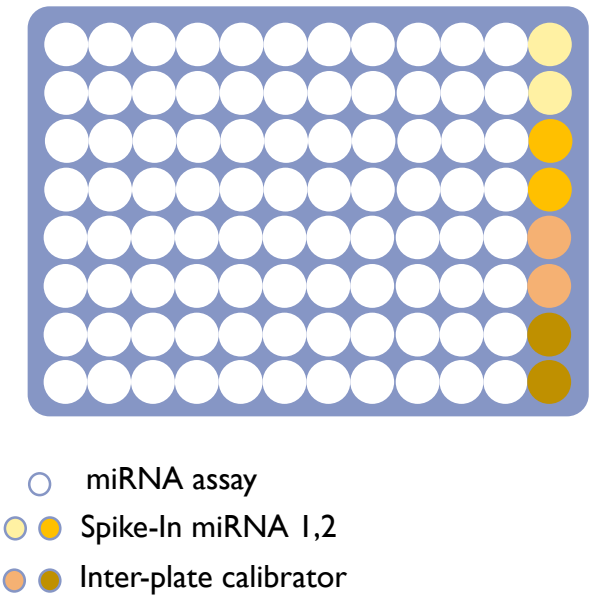


Table 2.1 – qPCR reaction setup (96 well format)

Reagents	Plate_A	Plate_B	Plate_C	Plate_D
Nuclease free water	980 µl	980 µl	980 µl	980 µl
ID3EAL miRNA qPCR Master Mix	1000 µl	1000 µl	1000 µl	1000 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 IDEAL™ Panel plates. Use cDNA from each RT Primer Pool with its respective plate. E.g. cDNA from RT Primer Pool A should be used with wells indicated "A" below.

In a single PCR well, the reaction volume is 10 µl.

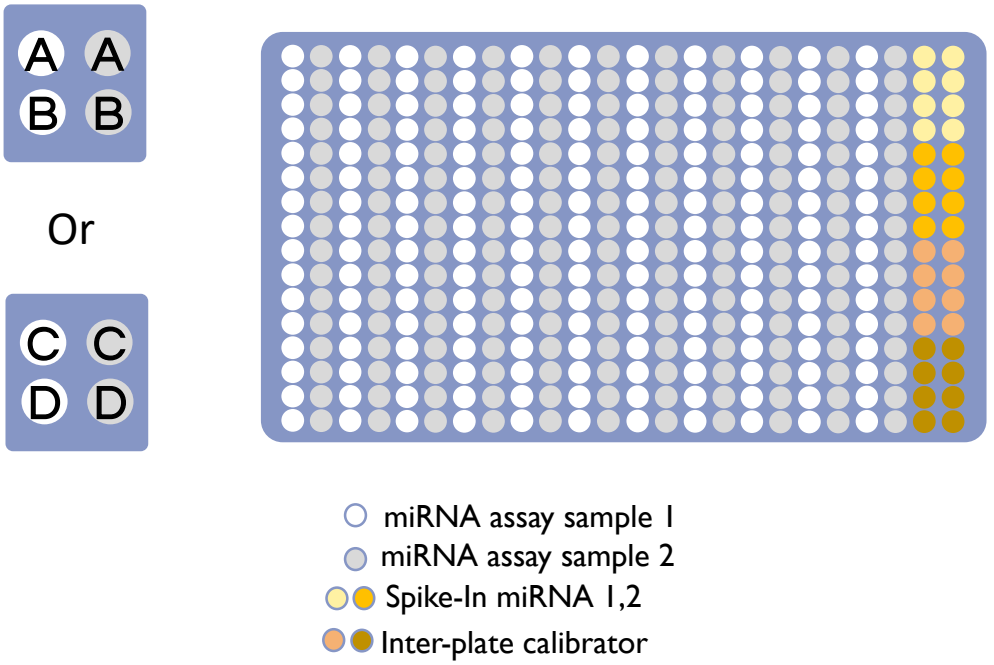


Table 2.2 – qPCR reaction setup (96 well format)

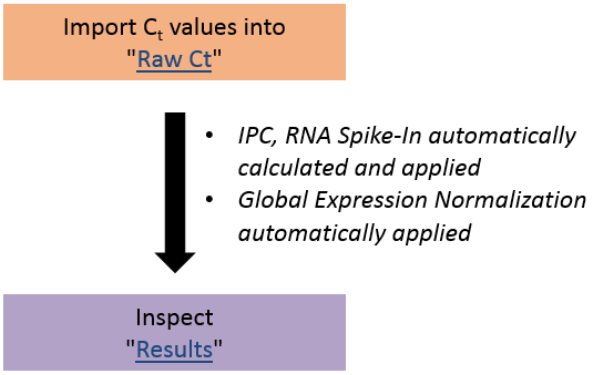
Reagents	RT_A	RT_B	RT_C	RT_D
Nuclease free water	480 µl	480 µl	480 µl	480 µl
ID3EAL miRNA qPCR Master Mix	500 µl	500 µl	500 µl	500 µl
cDNA A	20 µl	-	-	-
cDNA B	-	20 µl	-	-
cDNA C	-	-	20 µl	-
cDNA D	-	-	-	20 µl
Total volume	**1000 µl**	**1000 µl**	**1000 µl**	**1000 µl**

Data Analysis

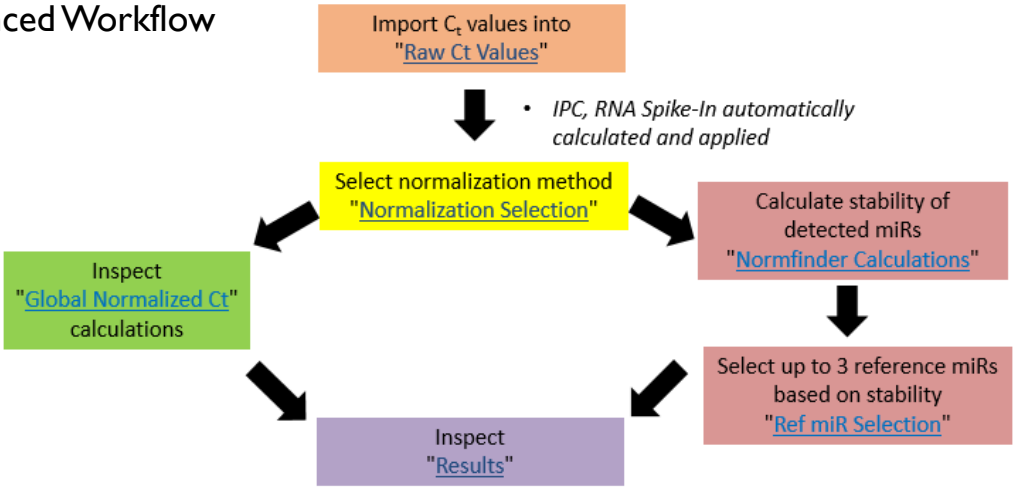
Analysis Template

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.

Basic Workflow



Advanced Workflow



Absolute Copy Number Quantification

The miRNA knowledge panel are only suitable for determining relative abundance. MiRXES provides catalogued single assays that can be used with synthetic miRNA standards to quantify absolute copy numbers. MiRXES' assays are also compatible with Bio-Rad ddPCR system.

MiRXES also provides a premium biomarker profiling service with absolute copy number quantification. For more information, please contact info@mirxes.com.

Stage 3 : Data Analysis

3.1 Verification of Spike-In

Spike-In 1 and 2 miRNAs are tested in duplicates for each cDNA pool. If Spike-Ins have been added in isolation process, Spike-In recovery captures workflow information from isolation until qPCR. If Spike-Ins have been added in cDNA synthesis, they only captures workflow information from cDNA synthesis onwards. Check to ensure that all Spike-Ins from all samples have Ct value lower than 30. Ct value higher than 25 indicates poor isolation efficiency or cDNA synthesis efficiency. Or RNA elution from sample isolation may need to be concentrated. Spike-In 1 and 2 will have different Ct values, but for the same sample, the difference of Ct values for each Spike-In duplicates should be within 0.5 Ct cycle. larger difference is usually a sign of pipetting error.

3.2 Verification of Inter Plate Control (IPC)

IPCs are DNA template that is run 4 times for each cDNA pool. First check the standard deviation of Cts of IPCs 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 Ct 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, Ct values from different machines are not directly comparable. Use IPCs to normalize the machine to machine variation as IPCs will have the same Ct value across machines if the machine variation is eliminated.

3.3 Relative Abundance Quantification

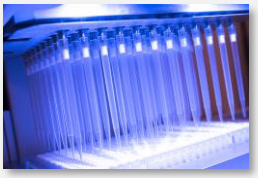
When multiple samples are tested with miRNA panels, the relative abundance of hundreds miRNAs in different samples can be simultaneously determined with ΔΔCt method. User only need to export Ct values and input the values into respective positions of supplied Excel sheet. We recommend user treat Ct value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

3.4 Absolute Copy Number Quantification

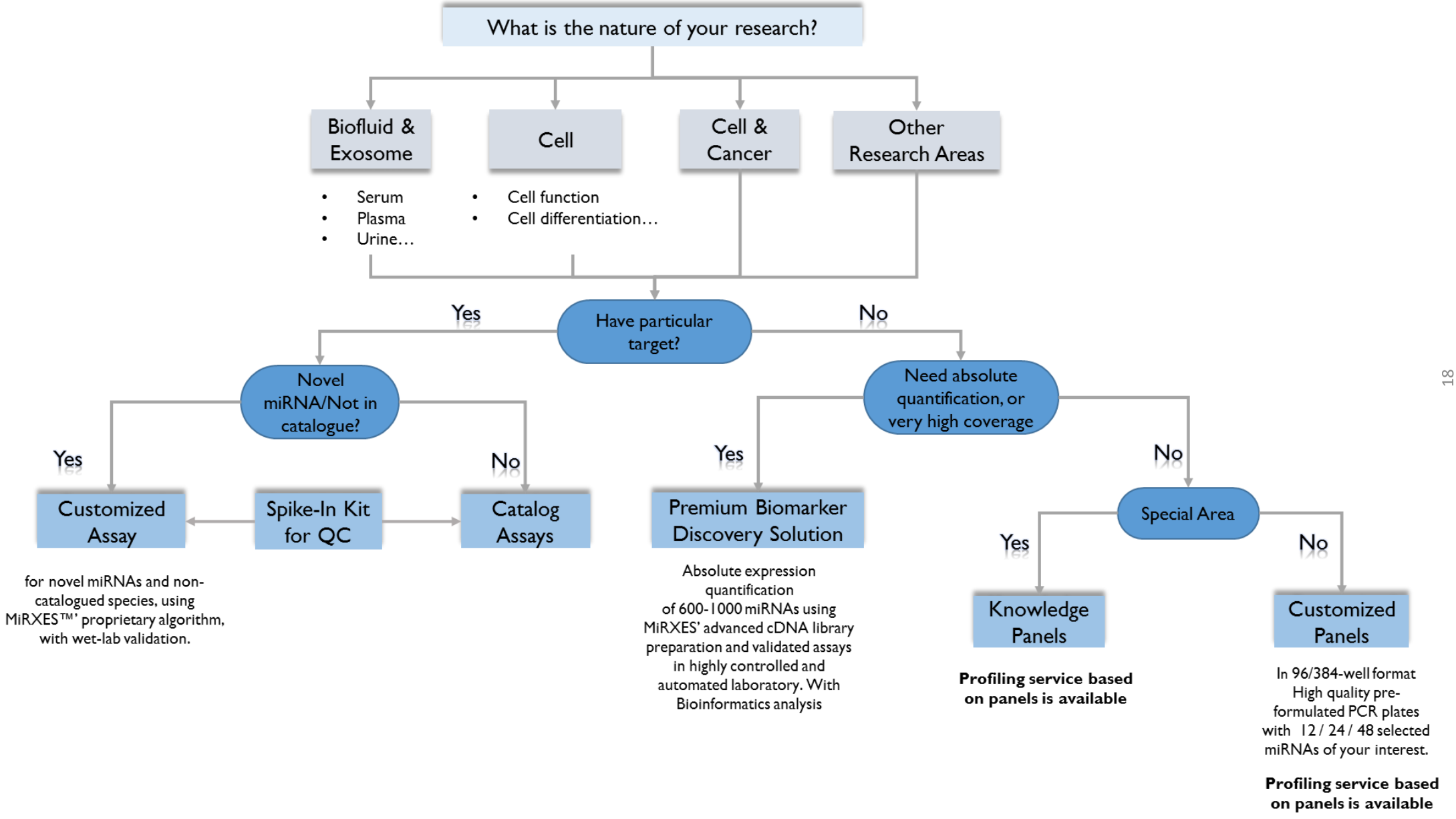
The miRNA knowledge panel are only suitable for determining relative abundance. The format of the panels does not allow an analysis of absolute copy number of miRNA molecule in samples. However, should users be interested in absolute copy number quantification for a limited number of miRNAs, MiRXES provides catalogued single assays. When user has synthetic miRNA standards available, these single assays can be used to quantify absolute copy number with standard curve. MiRXES' assays are also compatible with Bio-Rad ddPCR system. MiRXES also provides premium biomarker profiling service with absolute copy number quantification. For more information, please contact info@mirxes.com

Related miRNA Research Products and Services

Tailored Solutions For Different Needs

	MiRNA Target Identification	MiRNA Target Validation
Product	**Knowledge Panels** **ID3EAL™ miRNA Knowledge Panels** Ready-to-use assay panels for miRNAs relevant to specific areas of research. **Cancer Panel** 352 miRNA **Stem Cell Panel** 176 miRNA **Biofluid Panel** 176 miRNA • From RNA to answer, including spike-inRNA controls and data analysis template • In 96/384 well format, compatible with all major qPCR instruments 	**Catalogued Assays** **ID3EAL™ miRNA qPCR Assays** Complete kit comprising of individual miRNA RT-qPCR assays with optimized RT-qPCR master mixes. Ready-to-ship human, mouse, rat and virus assays (please refer to our assay list). • Sufficient for 50 RT and 100 qPCR reactions • Reverse transcription can be multiplexed 
Service	**Profiling Services** **Knowledge Panel Profiling Service** sample to answer screening service with miRNA Knowledge Panels, including sample isolation and RNA quality control services. 2-4 weeks turnaround. **Premium Biomarker Discovery Solution** **Absolute expression quantification** of 600-1000 miRNAs using MiRXES' advanced cDNA library preparation and validated assays in highly controlled and automated laboratory. **State-of-the-art data processing,** normalization and multi-variant statistical analysis.  8-12 weeks turnaround	**Customized Assays** **Customized Assay Design** Assay design and wet-lab validation for novel miRNAs and non-catalogued species. 2-4 weeks turnaround. **Customized miRNA Panels** MiRNAs of interest pre-formatted in 96 or 384-well PCR plates, with optimized miRNA RT-qPCR master mixes provided in proportion. One time customization charges with standard pricing for subsequent assay & panel orders  2-6 weeks turnaround

How To Choose A Product/Service



for novel miRNAs and non-catalogued species, using MiRXES™' proprietary algorithm, with wet-lab validation.

Absolute expression quantification of 600-1000 miRNAs using MiRXES' advanced cDNA library preparation and validated assays in highly controlled and automated laboratory. With Bioinformatics analysis

Profiling service based on panels is available

In 96/384-well format High quality pre-formulated PCR plates with 12 / 24 / 48 selected miRNAs of your interest.

Profiling service based on panels is available

Safety Notes

At MiRXES, we regard the safety of our customers and users of utmost importance.
Appropriate personal protective equipment should be worn at all times 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 authorities that is relevant to 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 particular 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 also provided at the back of our invoices.
Any questions related to the product specifications and performances can be answered by contacting the MiRXES Technical Services, your distributor or by visiting www.mirxes.com.

MiRXES

To Know is to Act

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