MiRXES
TO KNOW IS TO ACT

ID3EAL™ PanoramiR miRNA Knowledge Panel Manual

The fast track to impactful miRNA discoveries

The ID3EAL™ PanoramiR panel is an optimal research tool for studying miRNA regulation of signaling pathways, disease mechanisms and biomarkers discovery in disease diagnosis and subtyping.

PanoramiR targets 376 miRNAs curated from miRbase 22, HMDD 3.2, The Cancer Genome Atlas and cross-referenced with high-impact publications and in-house research data, providing valuable insights into miRNA-target interactions of over 14,000 disease associated genes.

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

1. Kit Contents

Reagent	Pack size	Quantity
ID3EAL™ PanoramiR RT Buffer* (A/B/C/D)	130 µl/tube	1 tube each
ID3EAL™ PanoramiR AUG Buffer 4X‡ (A1/A2/B1/B2/C1/C2/D1/D2)	160 µl/tube	1 tube each
ID3EAL™ PanoramiR miRNA PCR Buffer 4X	1800 µl/tube	12 tubes
ID3EAL™ PanoramiR RT Enzyme	60 µl/tube	1 tube
ID3EAL™ PanoramiR PCR Enzyme	700 µl/tube	1 tube
ID3EAL™ PanoramiR Spike-in RNA Template	20 µl/tube	1 tube
ID3EAL™ PanoramiR qPCR Reaction Plate (384-well)	1 pc	12 pcs

* ID3EAL™ PanoramiR RT Buffer includes RT multiplex primers.

‡ ID3EAL™ PanoramiR AUG Buffer includes AUG multiplex primers.

2. Instruments and Materials Recommended or Required (not included in the kit)

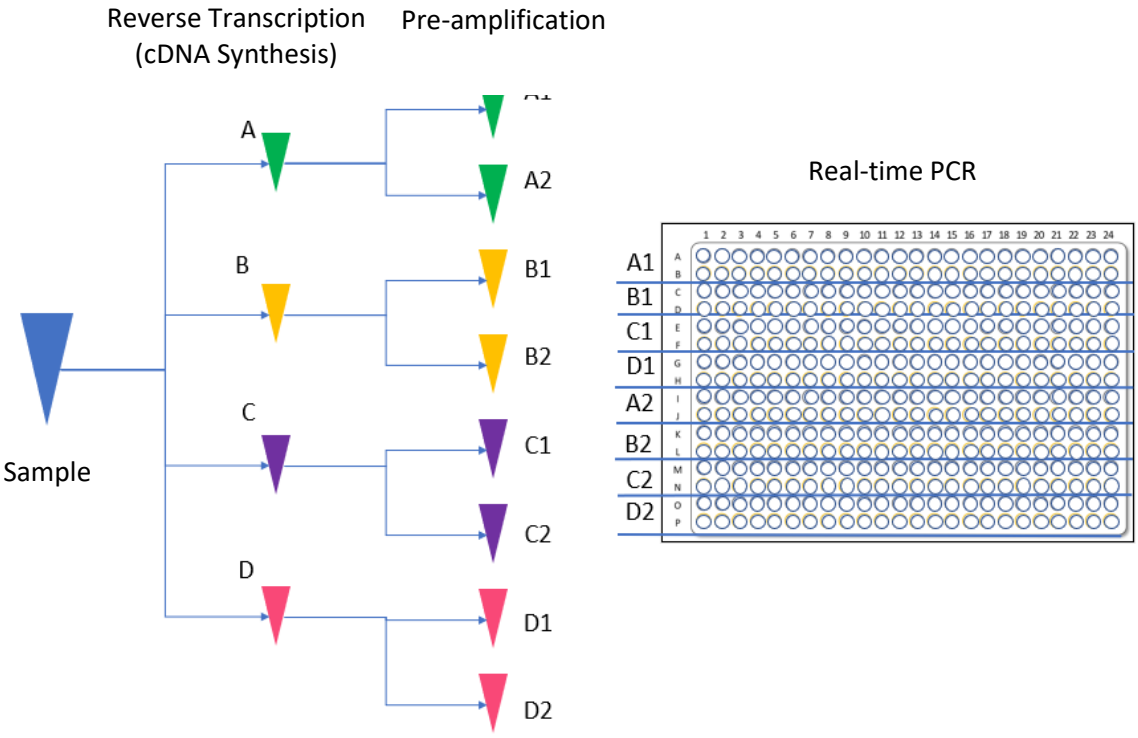
1. qPCR instruments (SYBR and ROX calibrated; low ROX compatibility)
2. Thermal cycler
3. Multi-channel Pipette (Manual or electronic)
4. 8-tube strips and 8-cap strips or 8-tube strips with attached caps
5. Nuclease-Free Water
6. Plate vortex
7. Tube-strip mini-centrifuge
8. Centrifuge for microplate
9. 96-well deep well plates
10. 8-channel reservoirs
11. RNA extraction / isolation systems

3. Workflow Overview

The workflow will take 3 to 4 hours, including 1 hour of total hands-on time, from RNA isolation to the end of the qPCR step.

Instrument run-times:

Experimental workflow:



4. RNA Isolation

The ID3EAL™ PanoramiR 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 RNA, 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.

5. Spike-in Protocol for RNA Extraction Step

The ID3EAL™ PanoramiR RNA Spike-in template 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. MiRXES recommends the use of miRNA spike-ins to monitor and normalize experimental variations in sample RNA isolation, reverse transcription and qPCR.

1. Prepare the ID3EAL™ PanoramiR RNA Spike-in template immediately before use during isolation by diluting it 30x with Nuclease-Free Water. For 12 samples, add 145 µl of Nuclease-Free Water into 5 µl of the RNA Spike-in concentrate.
2. Do not subject the diluted RNA Spike-in to multiple freeze thaws cycles. If necessary, make aliquots upon reconstitution.
3. Mix thoroughly and keep on ice.
4. Use the diluted RNA Spike-in template immediately by adding 10 µl of diluted RNA Spike-in template to the lysis buffer of each sample.
5. Prepare sufficient mixture of the RNA Spike-in and lysis buffer to account for pipetting error.

Spike-in Reaction Set-Up Examples

Number of Samples	Lysis Buffer / Sample (µL)	Diluted PanoramiR Spike-in RNA Template (µL)	Volume of Lysis Buffer + Spike-in per Sample (µL)
1	400	10	410
6	2400	60	2460
12	4800	120	4920

6. cDNA Synthesis Protocol

Notes on RT Procedure

- Remove RT Enzyme from the freezer ONLY when ready to set up the reaction.

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.

1. Add 5 µl of the isolated RNA to the bottom of each reaction tube according to the RT plate layout (Figure 1). Each 8-well strip corresponds to 1 sample.
2. Thaw 1 tube of each RT Buffer (RT Buffers A, B, C, D) on ice. Take out the RT Enzyme from the freezer when all the RT Buffers are thawed. Briefly spin down the tubes.
3. Add 10 µl of the RT master mix set-up indicated in Table 1.1 to each reaction tube according to the RT plate layout (Figure 1). Repeat dispense can be used at this step if such a feature is available on the pipettor. Avoid touching the RNA to prevent carry-over and contamination.

Table 1.1: RT Master Mix Set-Up (Prepare on ice)

Reagent	Single reaction set-up	12 samples set-up
PanoramiR RT Buffer A/B/C/D	9.25 µl	120 µl
ID3EAL™ PanoramiR RT Enzyme	0.75 µl	9.75 µl

4. Seal the tubes tightly. Centrifuge briefly.
5. Vortex the 8-well strips or 96-well plate on a PCR plate mixer at 1800 rpm for 20 s. Centrifuge briefly.
6. Immediately place the tubes in the thermocycler. Run the RT protocol according to Table 1.2.
7. Keep the cDNA on ice or in a 4 °C fridge. Proceed to the pre-amplification step within 60 min.

Notes on cDNA storage

- cDNA can be stored frozen at -20 °C or below for 1 month after transferring to microtubes.
- Do not thaw cDNA until ready to proceed to the pre-amplification step.

Table 1.2: RT Thermal Cycling Parameters

Cycles	Temperature	Duration	Notes
1x	25 °C	10 mins	Incubation
	30 °C	10 mins	Incubation
	35 °C	10 mins	Incubation
	40 °C	10 mins	Incubation
	95 °C	5 mins	Heat inactivation
	25 °C	Hold	Hold

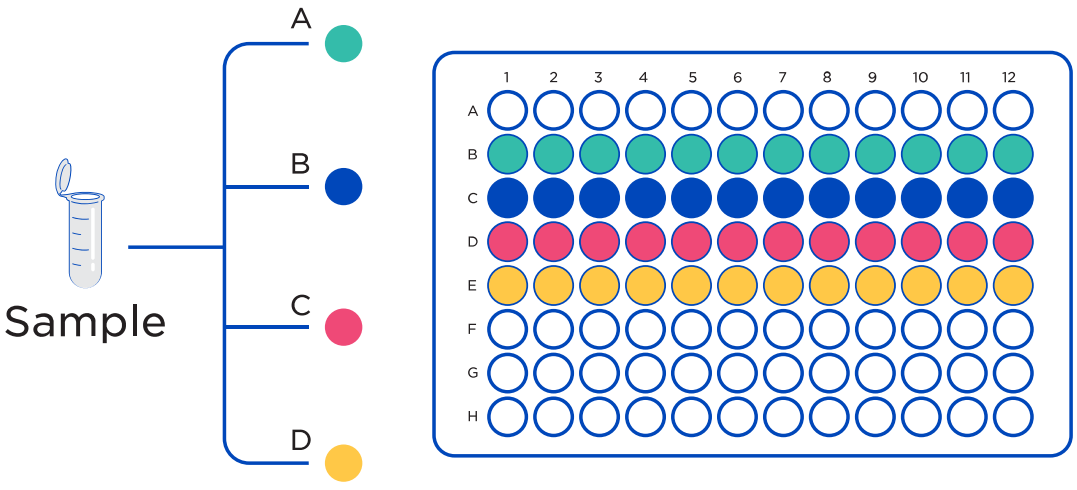


Figure 1: RT plate layout. The RT reaction can be set-up in 12 x 8-well strips or in one 96-well plate. Each column corresponds to 1 RNA sample.

7. Pre-Amplification (Augmentation) Protocol

1. Thaw 1 tube of 4X AUG Buffer from each group (A1, A2, B1, B2, C1, C2, D1, D2) on ice.
2. Ensure that the thermocycler lid is preheated to 105°C.
3. Only remove PCR Enzyme from the freezer when all 4X AUG Buffers are thawed.
4. Briefly spin down all tubes.
5. Dilute PCR Enzyme with nuclease-free water in a ratio of 1:20. Vortex well and spin down.

Notes on PCR Enzyme
○ Do not dilute the PCR Enzyme directly into the tube. ○ Please aliquot the desired amount into another sterile microfuge tube to make a small bulk for pre-amplification. ○ The stock PCR enzyme is still required for the qPCR step.

6. Add 41 µl of the pre-amplification (PA) master mix (master mix set-up as indicated in Table 2.1) to each reaction tube according to the PA sample layout (Figure 2). Each 8-well strip corresponds to 1 sample. Avoid touching the RNA to prevent carry-over and contamination. Repeat dispense can be used at this step if such a feature is available on the pipettor.

Table 2.1: PA Master Mix Set-Up (Prepare on ice)

Reagent	Single reaction set-up (µl)	12-sample set-up (µl)
PanoramiR AUG Buffer 4X (A1/A2/B1/B2/C1/C2/D1/D2)	11.2	146
ID3EAL™ PanoramiR PCR Enzyme (diluted 1:20)	1	13
Nuclease-Free Water (not provided)	28.8	374
Total	41	533

7. Transfer 4 µl of cDNA sample to wells according to the PA sample layout (Figure 2). Each cDNA sample is dispensed into duplicate tubes within an 8-well strip. Repeat dispense can be used at this step if such a feature is available on the pipettor.
8. Seal the sample with either a cap-strip / PCR seal as appropriate and centrifuge briefly. Vortex the PA samples on a PCR plate mixer at 1400 rpm for 20 s. Centrifuge briefly to collect all reaction mixture at the bottom of the tubes/wells.
9. Run the PA thermal cycling protocol according to Table 2.3.

Notes on qPCR Procedure
○ 5 min before the PA thermal cycling protocol is completed, start preparing qPCR master mix and plate. ○ Once the PA protocol is completed, place the augmented cDNA on ice and proceed to qPCR immediately. ○ Delaying qPCR may result in non-specific amplification which may affect the results.

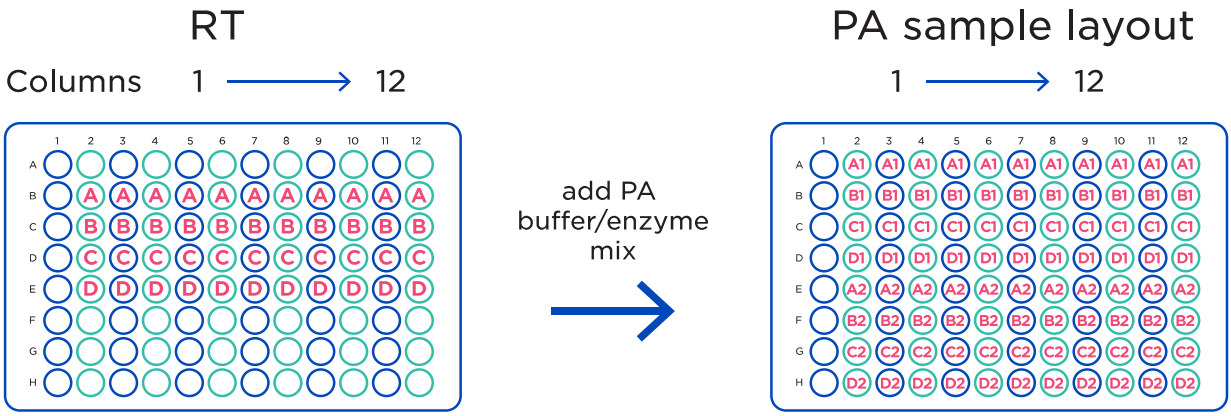


Figure 2: PA sample layout.

Table 2.3: PA Thermal Cycling Parameters

Cycles	Temperature	Duration	Notes
1x	25 °C	10 sec	Polymerase Activation
	95 °C	10 mins	
	40 °C	5 mins	
11x	95 °C	10 sec	Denaturation
	60 °C	30 sec	Annealing
1x	4 °C	Hold	Hold

8. Quantitative PCR (qPCR) Protocol

Notes on qPCR Procedure

- Keep all reagents and cDNA on ice.
- Set up all reactions on a cool block or on ice.
- Take out the PCR Enzyme from the freezer ONLY when ready to set up the reaction.
- 1 AUG strip corresponds to 1 qPCR plate.

1. Add 5350 µl of nuclease free water (not supplied) to one tube of 4X PCR Buffer and thaw on ice. PCR Buffer should be used within 60 min. DO NOT freeze and reuse PCR buffer after addition of water.
2. Add 48 µl of PCR Enzyme to the PCR Buffer to generate 1X qPCR Master Mix sufficient for 1 column of the deep-well plate or 1 qPCR plate (i.e. 1 sample) as indicated in Table 3. Mix by vortexing.

Table 3: qPCR Master Mix Set-Up (Prepare on ice)

Reagent	1X qPCR Master Mix (per column of deep-well plate or 1 qPCR plate/1 sample)
4X PCR Buffer	1800 µl
PCR Enzyme	48 µl
Nuclease Free Water	5350 µl

3. Chill a 2 mL deep-well plate on ice. Aliquot 864 µl of 1X qPCR Master Mix to each well in column 1 of the deep-well plate. 1 column corresponds to 1 qPCR plate (1 sample).
4. Centrifuge the PA samples briefly. Transfer 36 µl of augmented cDNA to the corresponding wells in the deep-well plate according to the layout in Figure 3. Seal the deep-well plate securely.
5. Vortex the deep-well plate at 1400 rpm for 20 s. Centrifuge briefly.
6. Remove qPCR plate from the sealed pouch. Place the plate on a 384-well cool block on ice.
7. Use a multi-channel pipette, dispense 15 µl cDNA/Master Mix into each PCR well of the qPCR plate according to Figure 4.
8. Seal the qPCR plate with an optical qPCR seal. Centrifuge the plate at 500 x g for 20s.
9. Immediately place the plate into the qPCR machine and run the thermocycling protocol according to Table 3.1.

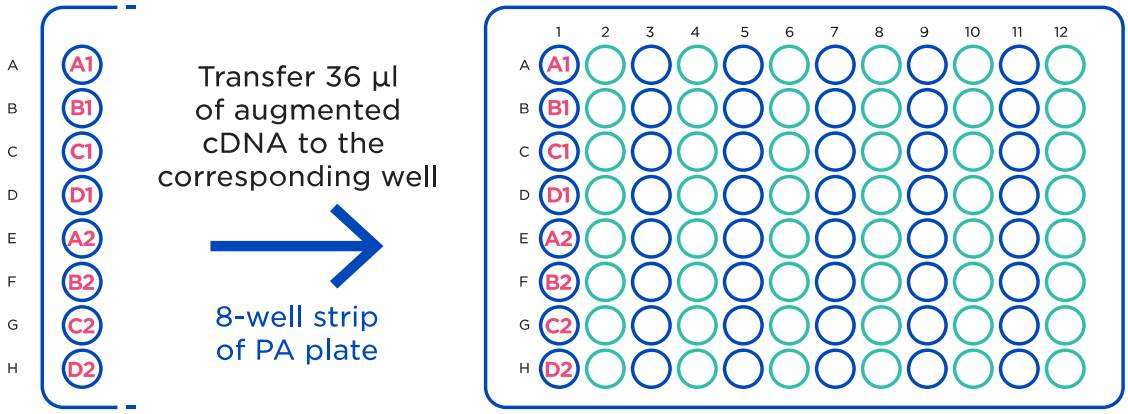


Figure 3: Deep-well plate layout

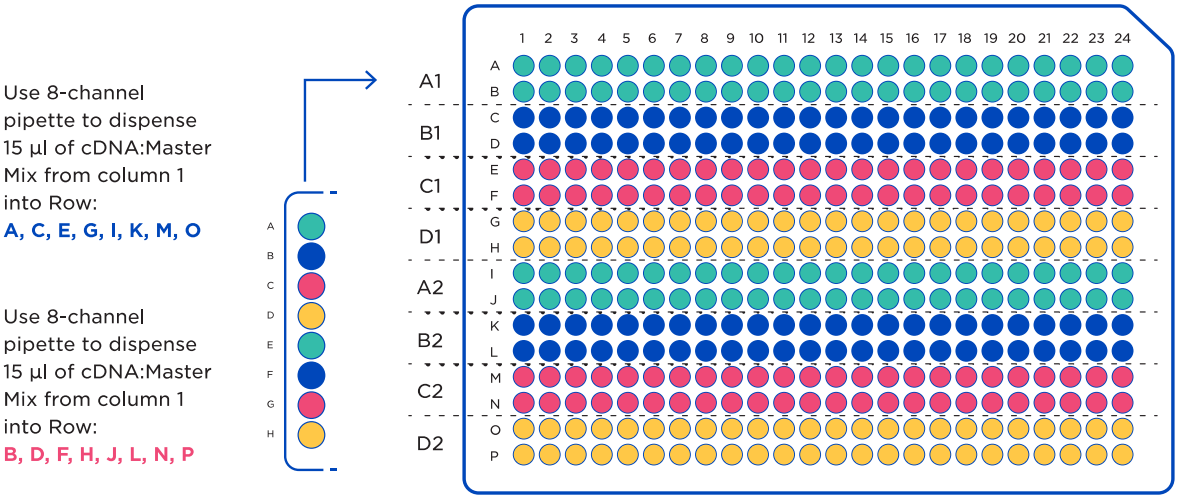


Figure 4: qPCR plate layout. Details of miRNA target in each well can be found in the PanoramiR Analysis Template.

Table 3.1: qPCR Thermal Cycling Parameters

Cycles	Temperature	Duration	Notes
1x	95 °C	10 minutes	Polymerase Activation
40x	95 °C	10 seconds	Denaturation
	60 °C	40 seconds	Annealing and extension

9. Data Analysis Protocol

The PanoramiR Analysis Template is an Excel file (.xlsx) that assists users in performing data analysis of the samples processed using the ID3EAL™ PanoramiR miRNA Knowledge Panel. It is intended to support laboratories that are studying miRNA expression changes.

The template contains several tabs that users may access for data analysis:

Excel tabs	Tab No.	Descriptions	User Input
Workflow	1	Instructions on the different data analysis workflows	Select data analysis workflow
miRNA Table	2	List of miRNAs for analysis	-
Plate Layout	3	Position of miRNA assays in the 384-well plate format	-
User Input	4	For user input of raw C_T values	Raw C_T values
Threshold Ct	5	C_T values with excluded data based on user predefined C_T Cut-off	C_T threshold
RNA Spike-in Normalized Selection	6	Normalized C_T value using Spike-in. Generation of Global Normalization Factor (GNF)	-
Normalization Selection	7	Selection of Normalization method - Global Mean Normalization or Ref miRNA Normalization	Select Normalization method
Results	8	Fold-change data and p-values	-
Fold-Change Plots	9	Bar charts displaying fold-changes for the results	-
Scatter Plot	10	Scatter plots for the results	-
3D plot	11	3D bar charts for the results	-
Volcano Plot	12	Shows the Log_2 of the fold-changes in each miRNA expression by plot	-
Global Normalized Ct	13	Global Normalized C_T values by using Spike-in normalized C_T values and Global Normalization Factor. For Global Mean Normalization method.	-
Global Normalized Data	14	Gene expression values using Global Normalized C_T. For Global Mean Normalization method.	-
Sample Normfinder Output	15	Sample output file from Normfinder. For Reference miRNA Normalization method.	-
Normfinder Calculations	16	Working sheet for Normfinder analysis. For Reference miRNA Normalization method.	Input working sheet for Normfinder analysis
Ref miR Selection	17	Selection of Reference miRNAs for normalization. For Reference miRNA Normalization method.	Filter and select reference miRNAs
Ref miR Normalization Data	18	Normalized fold change data using Normfinder workflow. For Reference miRNA Normalization method.	-

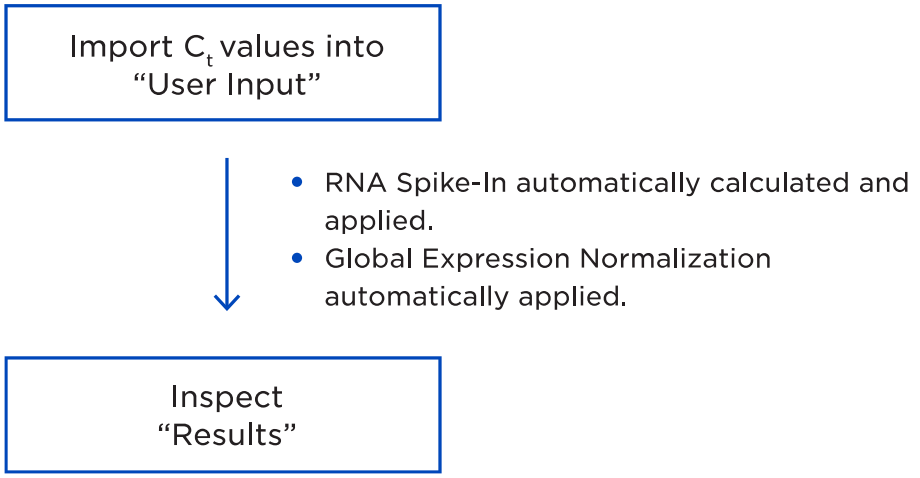
10. Performing Data Analysis

a. Workflow

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

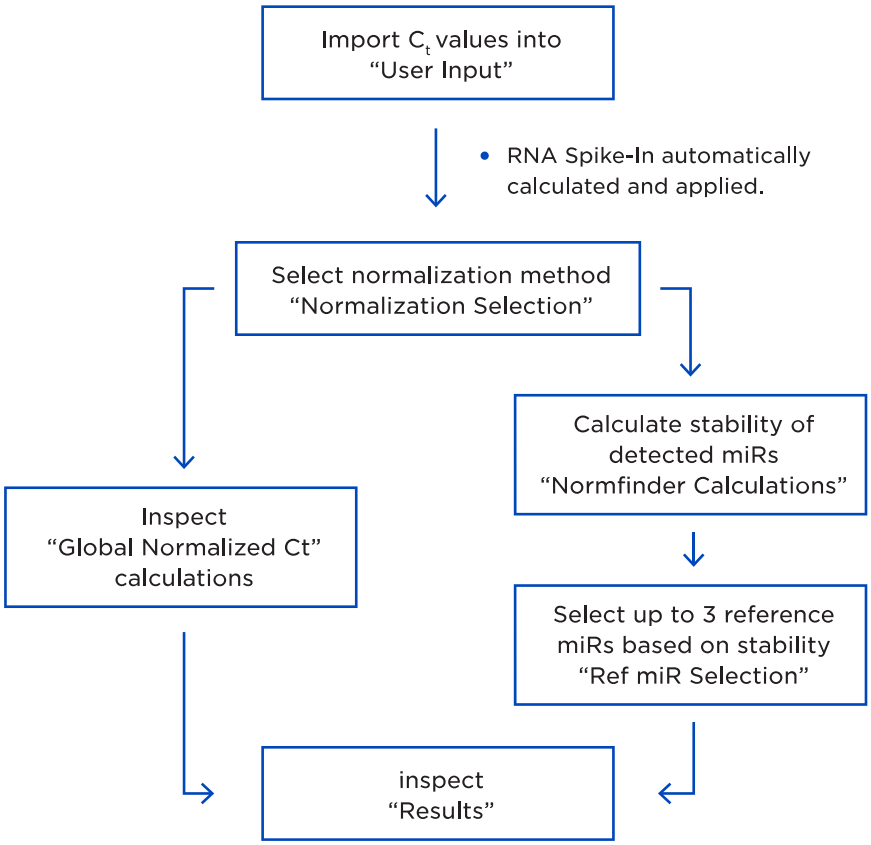
i. Basic workflow

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



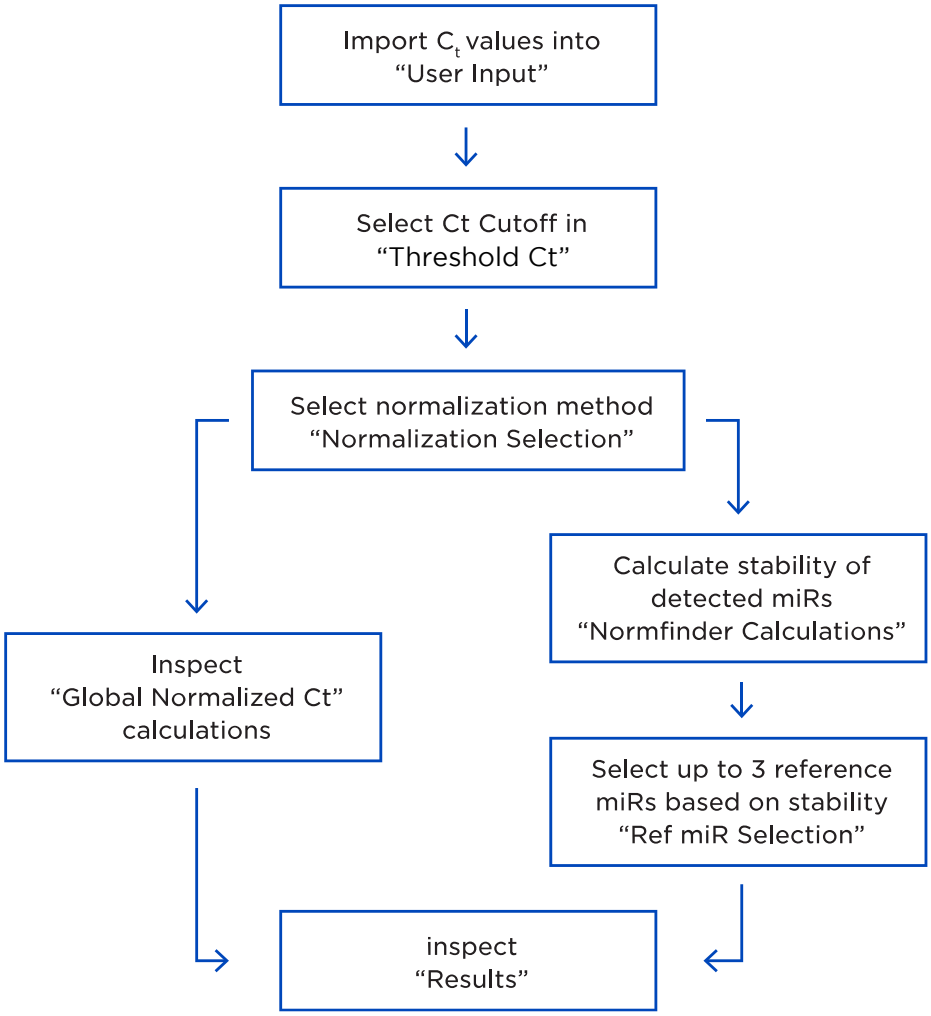
ii. Intermediate workflow

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.



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.



b. Excel tabs

i. miRNA Table

This tab shows the list of all miRNAs that are included for analysis in the PanoramiR kit, together with the miRNA sequences and the miRBase v22 Accession numbers.

ii. Plate Layout

This tab shows the plate layout of the miRNA assays in a 384-well plate format. It also shows the locations of the Spike-ins: A1.SP, B1.SP, C1.SP, D1.SP, A2.SP, B2.SP, C2.SP and D2.SP, all located in Column N. Each Spike-in is grouped together with 47 miRNAs, as shown in the below table:

Spike-In	Group	miRNA well group
A1.SP	A1	Well A1 – B24
B1.SP	B1	Well C1 – D24
C1.SP	C1	Well E1 – F24
D1.SP	D1	Well G1 – H24
A2.SP	A2	Well I1 – J24
B2.SP	B2	Well K1 – L24
C2.SP	C2	Well M1 – N24
D2.SP	D2	Well O1 – P24

iii. User Input Tab

This tab requires the user to input raw data exported from the qPCR software into the PanoramiR Analysis Template, “User Input” Tab.

Notes on Compatible Software

- Compatible software include:
  - ✓ QuantStudio Real-Time Analysis Software
  - ✓ QuantStudio Design and Analysis Software
  - ✓ Bio-rad CFX Maestro Software

- Import raw C_T data from the qPCR software to the PanoramiR Analysis Template, “User Input” Tab. There are 12 columns for 12 samples. For example, if you are using 3 samples each for control and treatment groups, please only use the first three columns.

→ RNA Spike Use : RT

Shortcuts
Basic Intermediate Advanced
Results Normalization Selection Threshold Ct Workflow

ParanomiR Panel		Control Sample									
RNA Input Volume →		1	1	1	1	1	1	1	1	1	1
Group	Position	miRNA ID	S01	S24	S26	S40	S44	S47	S62	S68	S77	S80
A1	A01	hsa-let-7i-5p	9.44	10.56	9.76	9.44	9.86	12.13	10.96	10.69	9.66	10.29
A1	A02	hsa-miR-10a-5p	14.31	15.00	15.11	14.88	15.91	18.09	16.64	15.76	15.24	15.65
A1	A03	hsa-miR-122-5p	18.35	19.42	15.36	15.80	16.88	17.37	18.95	19.14	16.67	17.16
A1	A04	hsa-miR-410-3p	16.90	17.69	16.77	17.72	17.18	19.06	17.99	19.37	16.42	17.92
A1	A05	hsa-miR-129-5p	22.29	24.59	22.80	22.57	23.12	24.27	24.57	24.60	22.93	23.42
A1	A06	hsa-miR-132-3p	17.29	18.01	18.26	17.57	17.65	20.42	18.43	18.10	17.87	18.18
A1	A07	hsa-miR-377-3p	17.25	17.43	17.13	17.88	17.79	19.11	18.31	19.78	16.66	18.23
A1	A08	hsa-miR-146a-5p	9.76	11.90	10.60	9.30	11.28	13.26	12.16	11.83	10.17	11.21
A1	A09	hsa-miR-4306	14.45	15.76	14.14	14.76	14.72	16.27	15.85	15.88	14.20	14.88
A1	A10	hsa-miR-494-3p	15.52	16.96	15.40	16.37	16.17	18.18	17.35	18.73	15.37	16.88
A1	A11	hsa-miR-151a-3p	14.29	14.11	14.23	14.57	14.90	16.01	15.46	15.90	14.14	14.67
A1	A12	hsa-miR-152-3p	14.21	14.63	14.89	14.50	15.55	17.24	15.74	15.68	14.92	15.09
A1	A13	hsa-miR-374b-5p	10.18	10.71	10.29	9.94	10.15	12.25	11.13	11.43	10.17	10.52
A1	A14	hsa-miR-181d-5p	14.50	14.98	14.56	13.89	14.59	16.12	15.25	15.36	14.70	14.49
A1	A15	hsa-miR-190b-5p	17.21	18.66	18.13	17.66	17.92	20.34	17.82	18.76	18.10	18.02
A1	A16	hsa-miR-582-5p	14.00	16.56	16.21	14.66	15.89	18.44	14.99	16.00	16.11	16.13
A1	A17	hsa-miR-26b-5p	7.01	8.09	7.57	7.08	7.76	9.68	8.46	8.14	7.10	7.49
A1	A18	hsa-miR-20a-5p	8.59	10.51	9.45	9.11	9.63	11.78	10.57	10.45	9.18	9.60
A1	A19	hsa-miR-664a-3p	13.60	13.57	13.39	13.35	13.50	15.71	14.10	14.03	13.26	13.41
A1	A20	hsa-miR-221-3p	10.03	11.39	10.01	9.91	10.73	12.59	11.42	11.53	10.45	10.86
A1	A21	hsa-miR-222-3p	11.64	12.35	11.65	11.29	11.97	13.77	12.78	12.93	11.71	12.22
A1	A22	hsa-miR-22-3p	11.51	12.34	11.43	11.45	11.61	13.36	12.42	12.42	11.06	11.92
A1	A23	hsa-miR-25-3p	10.42	11.06	10.63	10.31	10.39	12.70	11.23	11.24	10.47	10.77
A1	A24	hsa-miR-324-5p	14.03	14.35	14.45	14.10	14.45	16.40	14.59	15.32	14.32	14.72
A1	B01	hsa-miR-32-5p	12.44	13.71	13.13	12.91	13.48	15.53	13.93	14.26	13.08	13.43
A1	B02	hsa-miR-15b-5p	8.66	9.86	8.99	9.03	9.05	11.40	9.60	9.95	8.71	9.37
A1	B03	hsa-miR-34c-5p	21.15	23.06	22.26	21.99	22.17	23.64	22.47	23.03	22.00	21.96
A1	B04	hsa-miR-365b-5p	21.15	22.76	21.49	21.17	22.16	23.84	22.70	22.93	21.96	22.48
A1	B05	hsa-miR-770-5p	22.05	24.62	23.19	22.71	23.79	25.98	24.59	25.56	22.89	23.91
A1	B06	hsa-miR-376c-3p	15.97	18.04	15.72	16.65	16.85	18.52	18.01	19.34	15.99	17.58

- Choose the correct RNA Spike-in method based on your experiment:

→ RNA Spike Use : Isolation ←

RNA Spike Use : Isolation
RNA Spike Use : RT
NO SPIKE

ParanomiR Panel			Control Sample	
		RNA Input Volume →		
RT Group	Position	miRNA ID	C01	C02

- If the RNA Spike-in is used during the isolation step, input the Spike-in volume for both Control and Test sample, as the analysis tool will use the Spike-in volume to calculate the Input Scaling Factor using sample RNA Input Scale – the mean RNA input scale for all samples, where it is calculated in the Threshold C_T Tab.
- The RNA input scale is calculated as follows:

$$\text{RNA Input Scale} = -\log_2(\text{RNA input volume})$$

- The Input Scaling Factor will be used to calculate the RNA Volume Normalized Spike-in Factor.
- If the RNA Spike-in is used during the Reverse Transcription step, there will be no need to input the RNA input volume. The raw C_T value will be used for the analysis.

→ RNA Spike Use : RT ←

Shortcuts
Basic
Results

ParanomiR Panel			Control Sample			Test Sample		
RNA Input Volume →			1	1	1	2	1	1
Group	Position	miRNA ID	S01	S24	S26	S04	S05	S09

- The template caters for up to 100 samples (50 Control Samples and 50 Test Samples)
  - The heading for Control Samples is coloured in Pink (**Control Sample**), whereas the heading for Test Samples is coloured in Blue (**Test Sample**)

iv. Threshold C_T Tab

This tab requires the user to adjust the C_T Cut-off if the user wishes to include / exclude miRNAs based on their C_T values.

- After placing the data in the User Input tab, the user may adjust the maximum and minimum C_T thresholds in Column DB of the Threshold C_T Tab. This will help to filter and exclude wells with raw C_T values that fall outside the specified minimum and the maximum C_T range.

Enter the max and min Cts to be analyzed.

	Threshold Ct
Maximum	33
Minimum	8

Cutoff
29.00
28.00
25.00
33.00
25.00
29.00
27.00
25.00
29.00
28.00
24.00
33.00
24.00

This tab also helps to calculate the Spike-in Normalized Factors and the RNA volume Normalized Spike-In Factors, which will be used to calculate the RNA Spike-in Normalized C_T in the next tab. Since there are 8 different subgroups used in the pre-amplification (A1, B1, C1, D1, A2, B2, C2, D2), the Spike-in value will be calculated individually and automatically by the analysis tool.

(i) If the user has chosen the isolation RNA Spike-in method, the RNA volume Normalized Spike-in Factor will be required to calculate the RNA Spike-in Normalized Ct. The formula for calculating the factor is as follows:

RNA volume Normalized Spike-in Factor = RNA volume Normalized Spike-in C_T value - $\bar{x}$ RNA volume Normalized Spike-in C_T value for all samples (treated and untreated)

Where RNA volume Normalized Spike-in C_T = Raw Spike-in C_T – Input Scaling Factor

(ii) If the user has chosen the Reverse Transcription RNA Spike-in method, the Spike-in Normalization factor will be used instead. The formula for calculating the factors is as follows:

Spike-in Normalized Factor = Raw Spike-in C_T - $\bar{x}$ Raw Spike-in C_T value for all samples (treated and untreated)

v. RNA Spike-in Normalized Ct

There is no need for user input in this tab. The normalized C_T values have been calculated with the RNA Spike-in Normalization Factors. Users would need to take note of the Mean Expression Check value, and the number of miRNAs that are included / excluded in the analysis.

- After placing the data in the RNA Spike-in Normalized Ct, the user is to take note of the Mean Expression value in cell AG8. If the absolute Mean Expression value is more than 2, the analysis tool will flag this out with a message, "Warning – Global Expression Difference". The default threshold value is set at 2 cycles. If there is a need to change the threshold, please contact MiRXES technical support.

- The Mean Expression Check formula is as shown below:

  Mean Expression Check = $\bar{x}$ C_T of Control – $\bar{x}$ C_T of Test samples

  Where Global Mean of control and test samples cannot be more than 2 cycles apart.

Mean Expression Check	
-0.6	OK

Mean Expression Check	
2.1	Warning - Global Expression

The Global Normalization Factors are displayed for each sample. These are calculated as shown:

Global Normalization Factors = $\bar{x}$ Spike-in normalized C_T of Sample – $\bar{x}$ Spike-in normalized C_T of all samples

	Control Sample					
	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6
	19.55	18.64	20.20	20.08	20.53	20.59
Global Normalization Factor	-0.69	-1.60	-0.04	-0.16	0.29	0.35

The number of miRNAs that are included / excluded based on the C_T Cut-off range are also displayed.

	Ct Cutoff
Ct cutoff (32 is recommended) →	32

miRNAs Excluded from Global Mean calculation	81
miRNAs Included in Global Mean calculation	289
Global Expression Check	-0.62

vi. Normalization Selection

There is no need for user input in this tab. The user would need to take note of and select the desired normalization method.

vii. Results

This tab calculates the fold change in d C_T values between the Test and Control samples by using the formula:

Fold-Change (dC_T) = (Geometric Mean of all Test Samples) – (Geometric Mean of all Control samples)

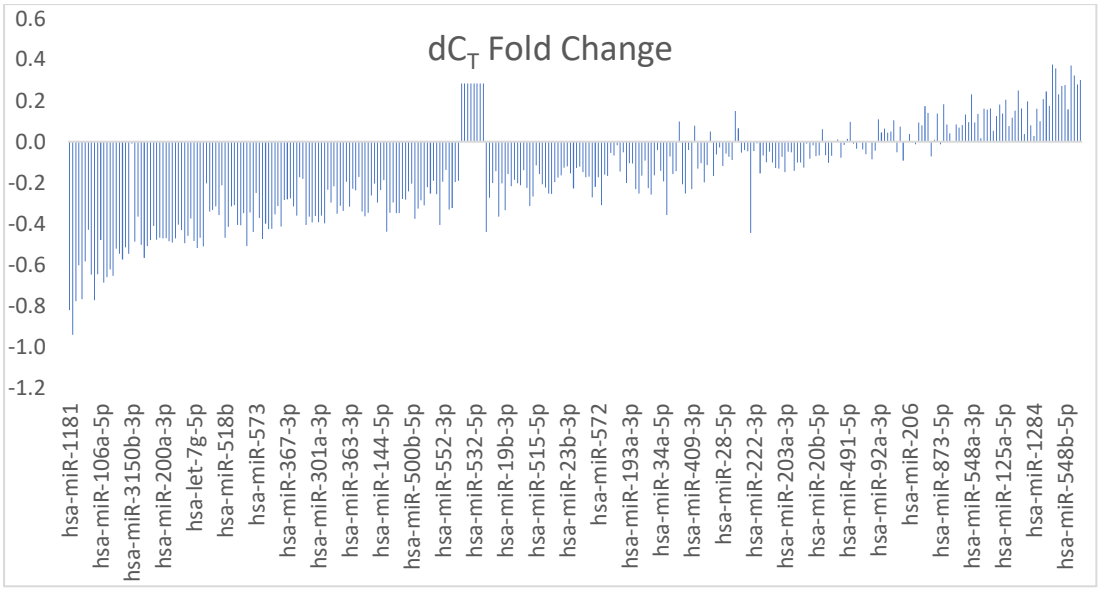
The fold-change results are tabulated in Column H. The column will also display up- and down-regulation of the fold-change differences in red and blue. The threshold is defined and may be adjusted in Cell L6 (up-regulation) and Cell L7 (down-regulation).

	A	B	C	D	E	F	G	H	I	J	K	L
1	ParanomiR Panel			Control Sample		Test Sample			T-TEST			
2	Group	Position	miRNA ID	Average Expression [C1-C12]	SD	Average Expression [T1-T12]	SD	(dCt)	p value	Comments		
3	A1	A01	hsa-let-7i-5p	10.51	0.83	10.88	2.49	0.37	0.38594		T-Test Type	3
4	A1	A02	hsa-miR-10a-5p	15.91	1.07	16.26	1.61	0.35	0.35496			
5	A1	A03	hsa-miR-122-5p	17.76	1.08	17.58	1.34	-0.18	0.65500			Highlight
6	A1	A04	hsa-miR-410-3p	17.97	1.15	18.15	1.09	0.17	0.60017		Upregulation	1
7	A1	A05	hsa-miR-129-5p	23.62	0.85	23.79	1.11	0.17	0.55761		Downregulation	-1
8	A1	A06	hsa-miR-132-3p	18.47	0.99	18.24	1.00	-0.23	0.44056			

The statistical p-values calculated by the Student's t-test are also displayed and calculated in Column H, where a p-value of ≤ 0.05 means that the data is statistically significant. A p-value of ≤ 0.05 will be highlighted in red.

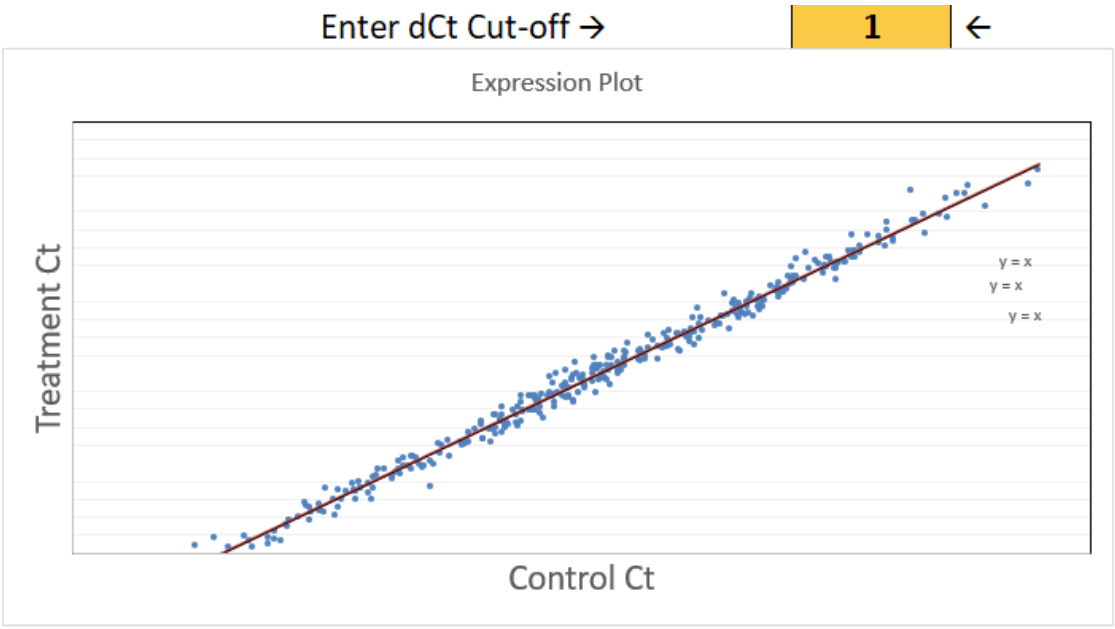
viii. dC_T Fold-Change Plot

This tab shows the graphs using the dC_T fold change data from the Result tabs. The results can be sorted in an ascending order for better visualization.



ix. Scatter Plot

The red line indicates the equivalence line. The two black lines bounding the equivalence line indicate a dC$_T$ fold-change defined by the user with the entry in Cell F1 (The default dC$_T$ fold-change cut-off is 1, i.e., fold-change = 2). Users may adjust the values of the x and y-axis to have better visualization of the experimental data.

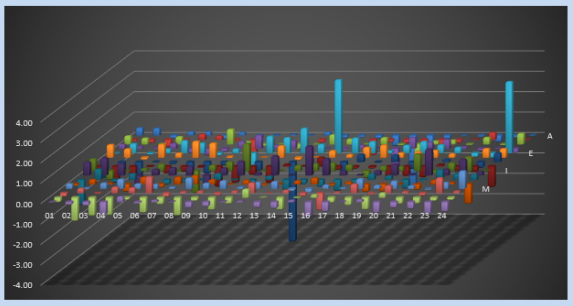


Recommended X-Axis Minimum:	6.98	Recommended Y-Axis Minimum:	4.02
Recommended X-Axis Max:	30.65	Recommended Y-Axis Max:	30.37
Change the X and Y axes to the values recommended above			

x. 3D Plot

The XY plane represents the 384-well format, while the Z-axis columns plot the fold-change difference in miRNA expression between the Test and Control samples. Columns pointing up (with Z-axis values

> 1) indicate an up-regulation of miRNA expression, and columns pointing down (with Z-axis values < 1) indicate a down-regulation of miRNA expression in the Test Groups relative to the Control Group.



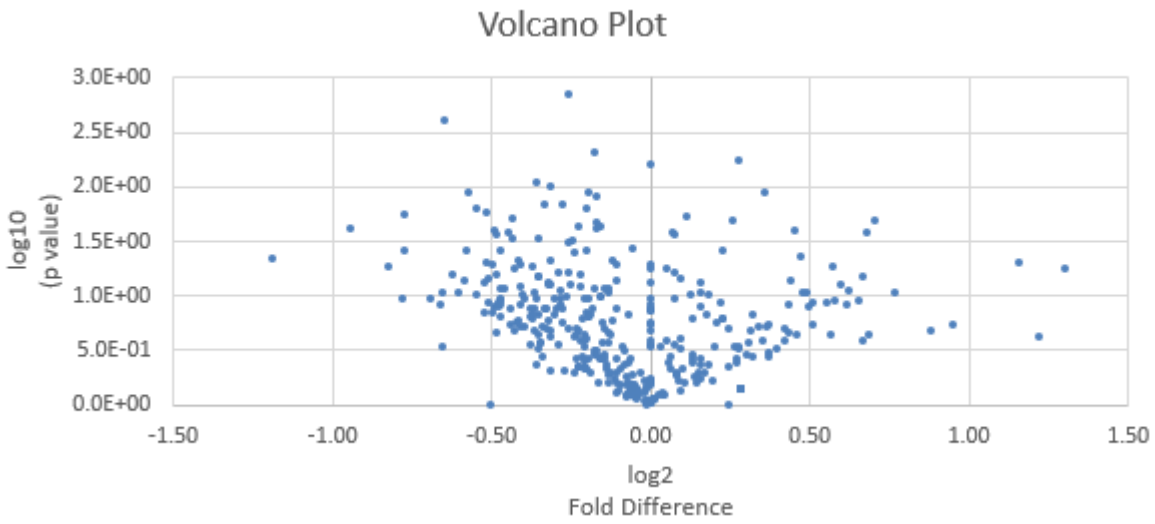
Shortcuts
Workflow
Results

Data Visualization
dCt Plot
Scatter Plot
Volcano Plot

		01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1	A	0.37	0.35	-0.18	0.17	0.17	-0.23	0.15	-0.24	-0.03	0.05	-0.03	0.15	-0.16	-0.11	-0.18	-0.54	-2.63	-0.83	-0.20	0.02	-0.08	-0.34	-0.16	-0.02
A1	B	-0.10	-0.19	-0.38	-0.42	0.28	0.19	-0.07	-0.85	-0.40	0.13	-0.29	-0.16		-0.03	-0.77	-0.15	-0.19	-0.52	-0.48	-0.19	0.01	0.36	0.18	0.00
B1	C	0.41	0.30	-0.09	0.37	-0.30	-0.08	0.74	0.13	-0.10		-0.26	0.00	0.47	0.25	0.00	0.32	-0.05	-0.84	0.16	0.20	-0.04	0.34	-0.05	0.53
B1	D	0.22	0.10	0.05	0.25	-0.24	-0.35	-0.19	0.36	0.66	0.17	0.39	-1.32		-0.09	0.17	0.00	-1.09	0.55	0.32	0.44	0.10	-0.12	0.03	-0.23
C1	E	-0.03	0.47	-0.07	0.20	0.61	0.49	-0.15	0.20	-0.82	0.83	0.77	1.22	0.44	3.60	0.74	0.57	0.12	0.38	0.57	0.41	0.28	-0.22	-0.26	3.50
C1	F	0.64	0.43	-0.13	0.66	0.22	0.80	0.71	0.08	0.31		0.58	-0.15		0.34	0.15	0.51	0.62	0.23	0.29	0.36	0.18	-0.25	0.44	0.44
D1	G	-0.79	-0.53	-0.84	-0.01	-0.13	-0.61	-0.59	-0.18	-0.57	-0.47	-0.62	-3.92	-0.01	-0.38	-0.52	0.38	-0.05	0.36	-0.35	0.51	0.13	-0.13	-0.32	0.45
D1	H	-0.43	-0.80	-0.52	-0.04	-0.36	-0.28	-0.08	-1.01	-0.83	-0.43	-0.83	0.11		0.45	-0.19	-0.13	-0.12	-0.56	-0.49	-0.55	0.10	-0.25	-0.29	-1.04
A2	I	0.59	-0.47	-0.02	0.11	0.33	-0.05	-1.11	0.28	0.45	1.34	0.00	0.00	0.26	0.35	0.39	0.30	-0.37	0.22	0.20	0.83	-0.14	0.39	0.68	
A2	J	0.66	0.86	0.58	-0.47	0.29	0.37	0.43	-0.55	0.35	0.69	0.12	0.95		1.43	0.81	0.21	0.01	0.22	0.54	0.07	1.28	0.29	0.78	0.18
B2	K	-1.25	0.52	-0.12	0.31	-0.66	-0.26	-0.13	-0.04	0.31	-0.03	-0.43	0.18	-0.52	-0.24	-0.36	-0.56	-0.45	0.30	0.20	-0.44	-0.07	0.50	0.30	0.26
B2	L	-0.10	0.23	0.06	-0.39	0.35	-0.18	0.35	0.42	0.22	-0.04	0.19	-0.08		0.17	0.31	-0.10	0.11	-0.40	-0.37	0.00	-0.30	0.19	0.08	-0.98
C2	M	0.24	0.05	0.28	0.47	0.24	0.21	0.08	0.53	0.27	0.39	0.08	0.32	0.35	-0.15	-0.24	0.18	0.06	0.49	0.16	0.37	0.14	-0.06	0.30	0.87
C2	N	-0.21	0.24	-0.03	0.30	0.26	0.81	-0.21	0.07	0.10	-0.13	0.11	0.51		0.15	-0.17	-0.86	0.06	0.45	-0.08	0.37	0.08	-0.15	0.76	0.10
D2	O	-0.26	-1.17	-0.92	-0.89	-0.14	-0.77	-0.36	-0.91	-0.19	-0.63	-0.33	0.41		-0.62	-0.03	0.14	-0.28	-0.41	-0.61	0.23	-0.34	-0.32	-0.19	-0.14
D2	P	0.01	-0.17	-0.20	-0.66	0.27	-0.09	0.09	0.00	-0.32	-0.25	0.03	0.06	-0.30	-0.34	0.08	-0.76	-0.49		0.12	-0.56	-0.31	-0.36	-0.61	-0.50

xi. Volcano Plot

The volcano plot visualizes the statistical significance (t-test p-value) of the differential expression patterns between Test and Control Samples. This plot graphs the $\log_2$ of the fold-change in each miRNA's expression between the Groups on the x-axis versus the $-\log_{10}$ of each miRNA expression change's p-value on the y-axis.



xii. Global Normalized C_T

This tab calculates the normalized C_T found in the RNA Spike-in Normalized C_T samples using the formula:

Global Normalized C_T = RNA Spike-in normalized Data - Global Normalization Factor

xiii. Global Normalized Data

This tab calculates the normalized expression values of all samples by using the formula:

Global Normalized Data = 2^-(Global Normalized Ct)

xiv. Sample Normfinder Output

This tab shows a sample output from Normfinder after analysing gene expression data. The tab should show the "Stability Value" of all the miRNAs and identifies the best reference gene(s) to be used for normalization.

Normfinder is an algorithm for identifying the optimal normalization gene among a set of candidates. It ranks the set of candidate normalization genes according to their expression stability in a sample set and experimental design. It can analyse expression data obtained through any quantitative method, for e.g. RT-qPCR or microarray-based expression analysis.

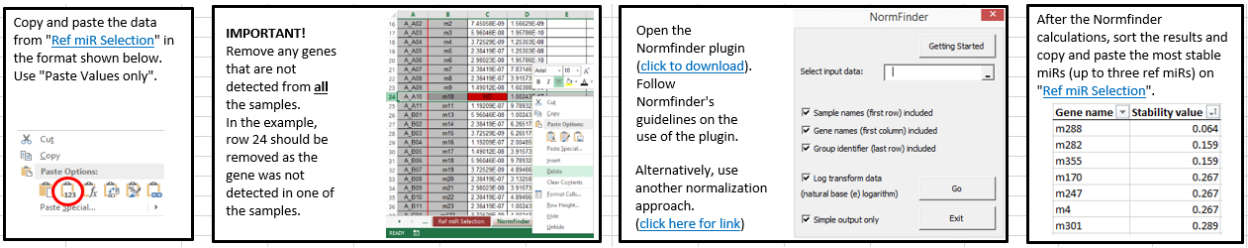
"NormFinder.xla" adds the Normfinder functionality directly to Excel. The instructions to install the add-in is stated in this link. [https://moma.dk/9-services/14-normfinder-faq]

Notes on NormFinder

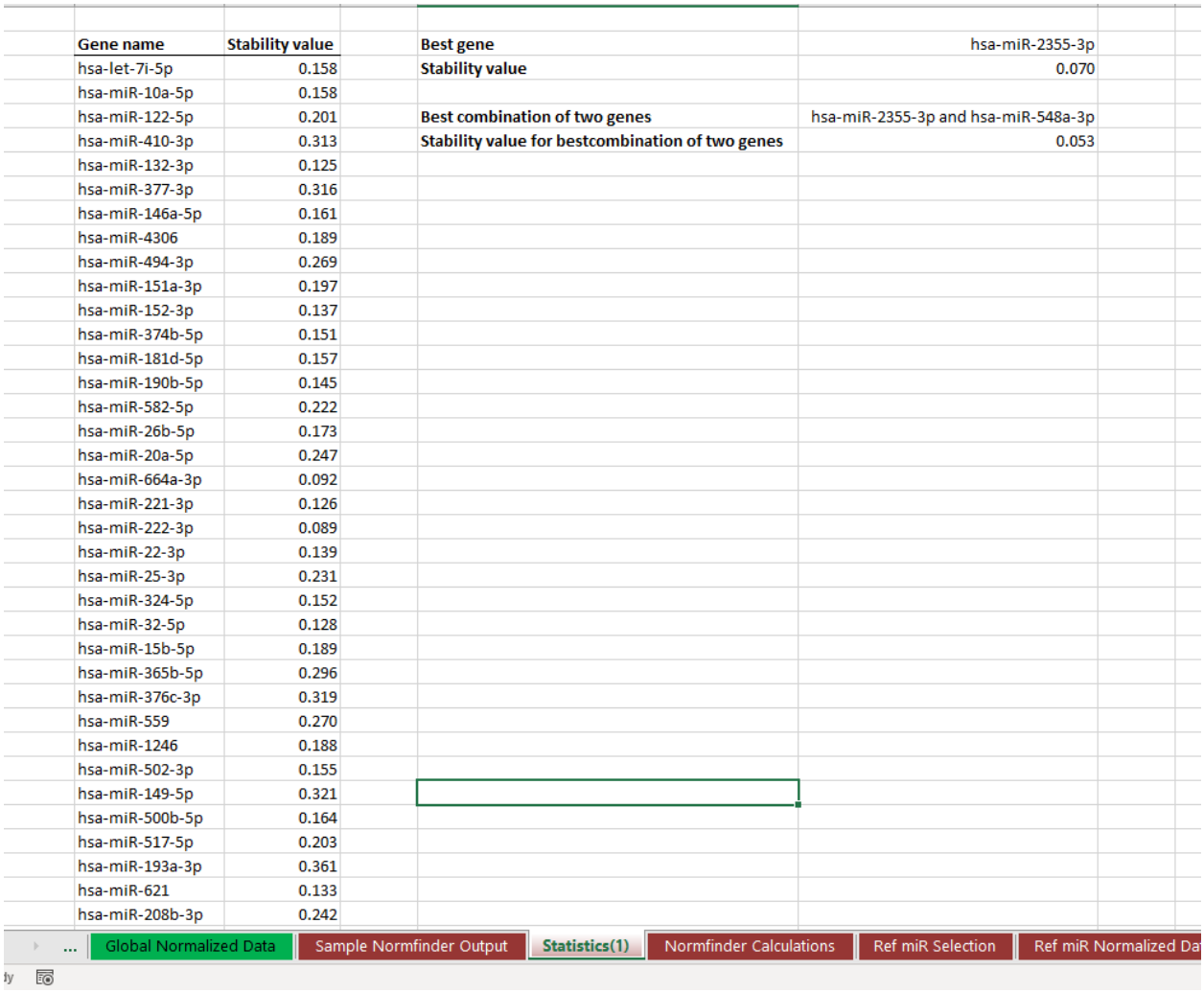
- The input data for Normfinder must be on a linear scale.
- Thus, raw Ct-values from real-time RT-PCR should not be used directly.
- Ct values is required to be transformed to linear scale expression quantities (without any negative values) by using a standard curve of assays amplifying at ~100% efficiency by 2-Ct.
- The NormFinder software log(N) transforms your data when you upload them if they are not log-transformed already.
- Do not use delta-Ct as input data.

xv. Normfinder Calculations

This tab shows instructions on how to use the Normfinder software to analyse the experiment data.



After analysing the data using Normfinder, a separate excel tab will be generated (Statistics). This tab shows the best reference miRNA candidate for normalization and the stability value of this miRNA. It also shows the best combinations of two stable miRNAs, and the stability value of the pairing.



Gene name	Stability value
hsa-let-7i-5p	0.158
hsa-miR-10a-5p	0.158
hsa-miR-122-5p	0.201
hsa-miR-410-3p	0.313
hsa-miR-132-3p	0.125
hsa-miR-377-3p	0.316
hsa-miR-146a-5p	0.161
hsa-miR-4306	0.189
hsa-miR-494-3p	0.269
hsa-miR-151a-3p	0.197
hsa-miR-152-3p	0.137
hsa-miR-374b-5p	0.151
hsa-miR-181d-5p	0.157
hsa-miR-190b-5p	0.145
hsa-miR-582-5p	0.222
hsa-miR-26b-5p	0.173
hsa-miR-20a-5p	0.247
hsa-miR-664a-3p	0.092
hsa-miR-221-3p	0.126
hsa-miR-222-3p	0.089
hsa-miR-22-3p	0.139
hsa-miR-25-3p	0.231
hsa-miR-324-5p	0.152
hsa-miR-32-5p	0.128
hsa-miR-15b-5p	0.189
hsa-miR-365b-5p	0.296
hsa-miR-376c-3p	0.319
hsa-miR-559	0.270
hsa-miR-1246	0.188
hsa-miR-502-3p	0.155
hsa-miR-149-5p	0.321
hsa-miR-500b-5p	0.164
hsa-miR-517-5p	0.203
hsa-miR-193a-3p	0.361
hsa-miR-621	0.133
hsa-miR-208b-3p	0.242

Best gene	hsa-miR-2355-3p
Stability value	0.070
Best combination of two genes	hsa-miR-2355-3p and hsa-miR-548a-3p
Stability value for bestcombination of two genes	0.053

xvi. Ref miR Selection

This tab shows log transformed data from the RNA Spike-in Normalized Tab, using the formula 2^{-Ct}. Based on the instructions defined in the Normfinder Calculations tab, any miRNA in the samples that are not detected / excluded is required to be filtered away.

After filtering, copy the “Position” and “miRNA ID” column into the Normfinder Calculations tab followed by the values from the Test and Control samples into the Normfinder Calculations tab.

RT Grou	Positio	miRNA ID	Replicate C1	Replicate C2	Replicate C3	Replicate C4	Replicate C5	Replicate C6	Replicate T1	Replicate T2	Replicate T3	Replicate T4	Replicate T5	Replicate T6
A1	A01	hsa-let-7i-5p	7.12E-05	1.65E-04	2.54E-05	8.39E-05	1.46E-05	2.04E-05	2.79E-05	4.85E-05	4.46E-05	1.73E-05	1.33E-05	2.30E-05
A1	A02	hsa-miR-10a-5p	7.27E-06	1.22E-05	6.90E-06	5.86E-06	1.03E-05	5.75E-06	5.01E-06	8.22E-06	6.01E-06	5.37E-06	5.58E-06	2.76E-06
A1	A03	hsa-miR-122-5p	1.56E-04	1.80E-04	8.04E-05	1.20E-04	9.55E-05	9.74E-05	1.04E-04	1.49E-04	3.34E-05	1.03E-04	4.36E-05	5.01E-05
A1	A04	hsa-miR-410-3p	2.30E-06	1.06E-06	6.98E-07	4.37E-07	3.48E-07	3.22E-07	8.64E-08	1.57E-06	2.42E-06	2.24E-07	9.79E-08	1.41E-07
A1	A05	hsa-miR-129-5p	Excluded	6.52E-08	3.73E-08	Excluded	5.34E-08	3.36E-08	2.72E-08	4.51E-08	2.66E-08	2.79E-08	Excluded	Excluded
A1	A06	hsa-miR-132-3p	1.30E-06	1.98E-06	4.11E-07	6.97E-07	6.21E-07	2.01E-07	3.69E-07	7.87E-07	5.41E-07	2.60E-07	2.15E-07	2.44E-07
A1	A07	hsa-miR-377-3p	4.94E-06	1.66E-06	1.07E-06	4.94E-07	6.00E-07	5.07E-07	1.88E-07	2.35E-06	2.95E-06	2.88E-07	1.67E-07	2.12E-07
A1	A08	hsa-miR-146a-5p	5.25E-05	5.90E-05	2.29E-05	1.62E-05	1.29E-05	1.03E-05	8.67E-06	3.31E-05	6.56E-05	1.14E-05	9.81E-06	7.73E-06
A1	A09	hsa-miR-4306	1.20E-05	6.71E-05	9.80E-06	4.02E-05	6.26E-06	5.83E-06	8.39E-06	2.33E-05	1.64E-05	9.74E-06	4.02E-06	8.18E-06
A1	A10	hsa-miR-494-3p	3.66E-06	1.54E-06	9.84E-07	5.42E-07	5.63E-07	4.77E-07	2.18E-07	1.97E-06	2.65E-06	4.23E-07	1.63E-07	2.31E-07
A1	A11	hsa-miR-151a-3p	1.00E-05	1.27E-05	3.70E-06	5.88E-06	2.06E-06	1.52E-06	2.39E-06	5.41E-06	1.16E-05	1.47E-06	2.10E-06	1.06E-06
A1	A12	hsa-miR-152-3p	4.87E-06	4.94E-06	1.64E-06	2.48E-06	1.27E-06	1.33E-06	1.66E-06	2.73E-06	4.77E-06	8.28E-07	1.07E-06	6.93E-07
A1	A13	hsa-miR-374b-5p	4.13E-05	7.75E-05	1.78E-05	3.39E-05	7.35E-06	1.65E-05	1.11E-05	3.36E-05	3.42E-05	9.07E-06	6.69E-06	1.50E-05
A1	A14	hsa-miR-181d-5p	1.87E-07	4.74E-07	1.23E-07	1.57E-07	1.36E-07	1.39E-07	4.48E-08	3.36E-07	4.60E-07	4.64E-08	7.79E-08	7.27E-08
A1	A15	hsa-miR-190b-5p	2.41E-07	7.59E-07	1.47E-07	3.23E-07	1.32E-07	9.50E-08	1.35E-07	1.72E-07	1.79E-07	6.38E-08	5.38E-08	1.26E-07
A1	A16	hsa-miR-582-5p	4.99E-07	7.69E-07	2.53E-07	5.09E-07	1.73E-07	3.92E-07	7.11E-07	5.09E-07	1.96E-07	2.01E-07	1.85E-07	9.56E-08
A1	A17	hsa-miR-26b-5p	1.64E-04	3.74E-04	6.31E-05	1.44E-04	3.42E-05	8.44E-05	4.98E-05	1.34E-04	1.20E-04	3.67E-05	3.10E-05	9.50E-05
A1	A18	hsa-miR-20a-5p	3.37E-04	1.60E-03	2.53E-04	9.36E-04	9.26E-05	2.32E-04	1.44E-04	4.21E-04	1.97E-04	1.51E-04	1.05E-04	3.49E-04
A1	A19	hsa-miR-664a-3p	1.68E-06	1.75E-06	8.06E-07	9.78E-07	5.14E-07	6.15E-07	5.77E-07	1.03E-06	1.55E-06	3.82E-07	4.24E-07	3.83E-07
A1	A20	hsa-miR-221-3p	7.83E-05	1.05E-04	3.70E-05	3.84E-05	2.70E-05	1.93E-05	2.47E-05	4.44E-05	8.45E-05	1.73E-05	1.96E-05	1.06E-05
A1	A21	hsa-miR-222-3p	1.05E-05	2.24E-05	5.24E-06	1.19E-05	5.06E-06	4.61E-06	5.34E-06	1.15E-05	9.47E-06	4.15E-06	3.94E-06	2.79E-06
A1	A22	hsa-miR-22-3p	5.79E-05	2.13E-04	5.31E-05	1.43E-04	4.61E-05	4.00E-05	3.63E-05	6.46E-05	6.03E-05	5.33E-05	3.09E-05	3.61E-05
A1	A23	hsa-miR-25-3p	1.91E-04	8.05E-04	1.08E-04	5.78E-04	4.99E-05	7.89E-05	1.33E-04	1.53E-04	1.41E-04	9.24E-05	5.81E-05	1.18E-04
A1	A24	hsa-miR-324-5p	3.13E-06	8.30E-06	1.08E-06	2.59E-06	8.92E-07	1.31E-06	9.99E-07	2.47E-06	2.57E-06	4.73E-07	5.32E-07	8.33E-07
A1	B01	hsa-miR-32-5p	5.49E-06	1.55E-05	2.89E-06	5.93E-06	1.64E-06	1.86E-06	2.75E-06	4.59E-06	3.87E-06	1.68E-06	1.16E-06	1.79E-06
A1	B02	hsa-miR-15b-5p	1.97E-04	2.07E-04	5.05E-05	1.40E-04	2.36E-05	2.97E-05	6.15E-05	6.46E-05	8.44E-05	3.34E-05	2.27E-05	2.73E-05
A1	B03	hsa-miR-34c-5p	Excluded	1.04E-08	Excluded	Excluded	Excluded	1.56E-08	Excluded	9.43E-09	6.69E-09	Excluded	Excluded	Excluded
A1	B04	hsa-miR-365b-5p	1.53E-08	3.33E-08	3.34E-08	3.58E-08	4.30E-08	3.20E-08	4.13E-08	2.94E-08	1.44E-08	3.46E-08	1.41E-08	7.26E-09
A1	B05	hsa-miR-770-5p	1.41E-08	1.50E-08	1.04E-08	6.94E-09	1.73E-08	6.38E-09	3.54E-09	1.54E-08	8.02E-09	4.98E-09	Excluded	Excluded
A1	B06	hsa-miR-376c-3p	3.00E-06	2.20E-06	1.31E-06	6.73E-07	6.84E-07	7.75E-07	1.07E-07	3.21E-06	4.86E-06	2.71E-07	1.79E-07	2.72E-07
A1	B07	hsa-miR-559	6.50E-08	2.42E-07	1.31E-07	1.64E-07	4.12E-07	1.30E-07	1.47E-07	1.74E-07	8.68E-08	1.44E-07	7.62E-08	8.21E-08
A1	B08	hsa-miR-1246	1.91E-06	8.74E-06	5.46E-06	4.02E-06	3.51E-06	3.12E-06	1.46E-06	3.40E-06	1.82E-06	1.90E-06	1.65E-06	2.31E-06
A1	B09	hsa-miR-502-3p	4.74E-06	1.49E-05	3.13E-06	1.08E-05	2.55E-06	2.22E-06	2.78E-06	4.27E-06	3.06E-06	2.64E-06	1.86E-06	2.16E-06
A1	B10	hsa-miR-149-5p	2.32E-08	6.39E-08	8.49E-08	5.30E-08	1.20E-07	6.09E-08	7.80E-08	4.11E-08	7.35E-08	8.80E-08	2.66E-08	8.44E-08
A1	B11	hsa-miR-500b-5p	5.57E-06	1.30E-05	2.54E-06	9.20E-06	2.23E-06	1.87E-06	3.13E-06	3.86E-06	2.93E-06	2.41E-06	1.40E-06	2.21E-06
A1	B12	hsa-miR-147b-3p	1.86E-09	5.64E-09	2.93E-09	1.97E-09	2.75E-09	Excluded	4.94E-09	Excluded	Excluded	1.96E-09	2.94E-09	1.64E-09
A1	B14	hsa-miR-517-5p	5.29E-06	5.29E-06	5.29E-06	5.29E-06	5.29E-06	5.29E-06	5.29E-06	5.29E-06	5.29E-06	5.29E-06	5.29E-06	5.29E-06
A1	B15	hsa-miR-522-3p	Excluded	5.83E-09	3.57E-09	Excluded	4.41E-09	5.14E-09	4.44E-09	7.86E-09	3.06E-09	2.12E-09	2.02E-09	2.76E-09
A1	B16	hsa-miR-193a-3p	6.42E-09	3.56E-09	2.42E-09	4.53E-09	7.89E-09	2.69E-09	3.22E-09	1.30E-08	5.66E-10	3.27E-09	1.63E-09	2.14E-09
A1	B17	hsa-miR-621	9.12E-08	2.73E-07	8.34E-08	1.72E-07	9.71E-08	8.24E-08	7.16E-08	1.01E-07	7.80E-08	7.40E-08	5.12E-08	6.16E-08
A1	B18	hsa-miR-648	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded	7.18E-09	Excluded	Excluded
A1	B19	hsa-miR-657	Excluded	2.18E-08	1.16E-08	Excluded	2.22E-08	1.10E-08	1.27E-08	1.26E-08	Excluded	1.73E-08	Excluded	8.20E-09
A1	B20	hsa-miR-658	Excluded	Excluded	Excluded	Excluded	2.74E-09	Excluded	Excluded	Excluded	2.81E-09	Excluded	Excluded	Excluded

It is advisable to add the group numbers (Group identifiers) at the back, as specified by Normfinder. Grouping allows the identification of stable reference genes in groups, e.g., 1-5 samples are from the 1st time point, and 6-10 are from the 2nd time point. [Refer to Normfinder documentation for more details.]

D2	P11	hsa-miR-134-5p	2.73E-06	2.83E-06	1.03E-06	2.09E-06	6.40E-07	7.96E-07	1.16E-06	9.97E-07	1.11E-06	6.50E-07	5.29E-07	6.45E-07
D2	P12	hsa-miR-103a-3p	5.76E-06	7.29E-06	3.03E-06	4.22E-06	2.92E-06	2.98E-06	4.20E-06	3.90E-06	1.93E-06	2.94E-06	1.90E-06	1.60E-06
D2	P13	hsa-miR-145-5p	1.41E-05	1.68E-05	4.92E-06	1.08E-05	2.29E-06	2.83E-06	6.42E-06	6.18E-06	1.75E-05	2.96E-06	3.15E-06	3.29E-06
D2	P14	hsa-miR-526b-3p	1.48E-05	2.77E-05	6.52E-06	1.24E-05	7.41E-06	4.97E-06	1.09E-05	8.85E-06	6.54E-06	6.13E-06	4.13E-06	4.57E-06
D2	P15	hsa-miR-551b-3p	1.12E-06	2.25E-07	1.97E-07	1.13E-07	1.13E-07	9.71E-08	1.24E-07	4.05E-07	3.68E-07	6.32E-08	2.83E-08	6.02E-08
D2	P16	hsa-miR-561-3p	2.97E-06	1.13E-06	8.22E-07	3.64E-07	3.62E-07	3.24E-07	1.08E-07	1.75E-06	2.36E-06	1.49E-07	1.22E-07	1.56E-07
D2	P17	hsa-miR-199a-3p	8.46E-05	1.81E-04	4.74E-05	9.42E-05	2.46E-05	2.75E-05	6.22E-05	4.51E-05	6.18E-05	3.59E-05	2.80E-05	4.15E-05
D2	P18	hsa-miR-223-3p	5.94E-07	1.24E-06	4.28E-07	1.51E-07	8.04E-07	1.83E-07	9.19E-08	2.84E-07	1.92E-07	1.03E-07	1.33E-07	9.99E-08
D2	P19	hsa-miR-584-5p	1.98E-06	6.80E-07	4.09E-07	2.07E-07	2.18E-07	2.00E-07	7.51E-08	5.89E-07	1.44E-06	1.81E-07	7.43E-08	9.35E-08
D2	P20	hsa-miR-761	8.44E-05	3.78E-04	4.41E-05	1.20E-04	2.19E-05	3.33E-05	4.23E-05	7.22E-05	4.48E-05	2.65E-05	1.62E-05	4.04E-05
D2	P21	hsa-miR-769-5p	1.55E-05	2.05E-05	2.25E-05	8.68E-06	1.51E-05	1.16E-05	8.08E-06	1.50E-05	1.16E-05	7.57E-06	8.11E-06	6.10E-06
D2	P22	hsa-miR-192-5p	1.62E-08	4.14E-08	1.73E-08	5.06E-09	5.05E-08	1.49E-08	2.44E-08	2.50E-08	5.19E-09	1.34E-08	1.02E-08	4.97E-09
D2	P23	hsa-miR-99b-5p	5.20E-07	3.31E-07	1.78E-07	8.04E-08	1.03E-07	6.77E-08	6.79E-08	1.86E-07	2.82E-07	7.81E-08	1.25E-07	6.76E-08
D2	P24	hsa-miR-181b-5p	3.06E-09	1.20E-08	Excluded	5.64E-09	3.97E-09	3.42E-09	3.18E-09	Excluded	Excluded	4.46E-09	Excluded	Excluded
			1	1	1	2	2	2	3	3	3	4	4	4

Users may select up to 3 miRNAs in cell AD10 to be used as reference miRNAs.

Reference miR Selection							Control
	miRNA ID	Replicate C1	Replicate C2	Replicate C3	Replicate C4	Replicate C5	Replicate C6
Reference miR 1	hsa-miR-122-5p	.000156254	0.000179777	8.03608E-05	0.000120312	9.5451E-05	9.74072E-05
Reference miR 2	hsa-miR-122-5p	.30451E-06	1.06246E-06	6.98201E-07	4.36792E-07	3.47719E-07	3.22272E-07
Reference miR 3	hsa-miR-410-3p						
	hsa-miR-129-5p						
Reference Expres	hsa-miR-132-3p	1.8976E-05	1.38205E-05	7.49053E-06	7.24923E-06	5.76108E-06	5.60282E-06
	hsa-miR-377-3p						
	hsa-miR-146a-5p						
	hsa-miR-4306						
	hsa-miR-494-3p						
NF Scaling Factor		2.378648	1.7324067	0.93894	0.908693	0.722153	0.702315

This tab then calculates the Reference Expression Factor and the NF Scaling Factor using the geometric mean.

xvii. Ref miR Normalized Data

This tab calculates the Normfinder normalized data using the expression values from Ref miR selection divided by the NF scaling factor. This set of data is used in the data plots to analyse miRNA expression.

11. FAQ

Q: Do we need to use a full 96-well plate for just 1 sample during pre-amplification?

A: A 96-well plate is useful for preparing sample batches of 12 or more samples. 8-tube PCR strips may be used for preparing less samples, whichever is convenient.

Q: How many samples can be analysed with the PanoramiR kit?

A: One kit has sufficient reagents for 12 samples. If you wish to run 12 samples each for control and test groups (a total of 24 samples), you will need 2 kits.

Q: Can the PanoramiR kit be used to detect diseases in patients?

A: The PanoramiR kit is a Research Use Only (RUO) product. The intended use is as a discovery tool for miRNA research. It is not intended for diagnostics use.

Q: Can the PanoramiR kit be used to determine absolute copy number?

A: The ID3EAL™ PanoramiR 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.

Q: Does the PanoramiR kit include the Spike-in template?

A: Yes, the Spike-in template is included.

Q: What is the RNA input volume that I should specify in the analysis template?

A: You will only need to specify your RNA input volume when you opt for the RNA isolation Spike-in method. After isolation, the volume of RNA used for reverse transcription would be the RNA input volume.

Q: The graph in Fold-Change Plot/Scatter Plot appears to be empty. What should I do?

A: Filter the data range to ensure blank cells or cells with error values are not selected. For the scatter plot, adjust XY settings of the plots based on the calculated minimum / maximum XY values. Use the Format Axis function to change the minimum / maximum values.

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