ab203485 – Multiplex miRNA Assay Core Reagent Kit - Circulating purified RNA

Instructions for Use

This product is for research use only and is not intended for diagnostic use.

Version 6 Last Updated 06 July 2016

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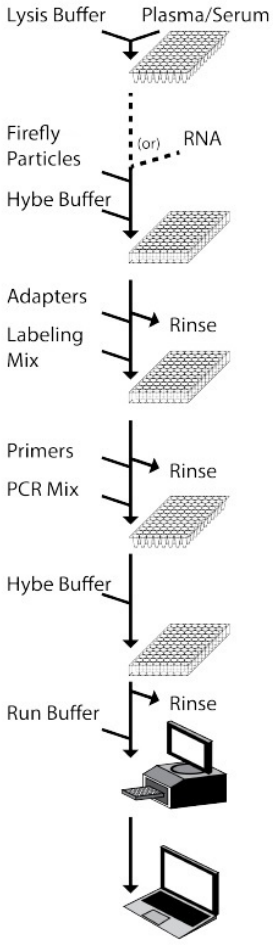
1. BACKGROUND

The Multiplex Circulating miRNA Assay enables high-throughput detection by flow cytometry of up to 68 miRNA targets and streamlined analysis with Firefly's® Analysis Workbench Software. The assay is performed in 96-well plate format such that users can detect up to 68 miRNA targets for each of 96 samples in less than a day's work.

The Multiplex Circulating miRNA Assay provides PCR sensitivity while eliminating the need for separate reverse transcription reactions and mitigating amplification biases introduced by target-specific qPCR. This is made possible by combining Firefly's uniquely encoded hydrogel particles with single-step RT-PCR amplification using universal primers. The assay reliably detects as few as 1000 miRNA copies per sample with a linear dynamic range of ~5 logs.

In addition to increased sensitivity, Abcam's multiplexed detection conserves precious sample by detecting multiple miRNA targets in less than 5 ng of purified RNA or by directly detecting miRNA in serum, plasma, and PAXgene™ samples.

2. ASSAY SUMMARY



Hybe: Add 35 µL Firefly particles then apply vacuum.
Add 25 µL Hybe Buffer and 25 µL Total RNA.
Shake at 37°C for 60 minutes. Rinse twice with 175 µL 1X Rinse A.

Labeling: Add 75 µL 1X Labeling Mix with Enzyme.
Shake at room temperature for 60 minutes.
Rinse twice with 165 µL 1X Rinse B.
Rinse once with 175 µL 1X Rinse A.

PCR: Elute with 110 µL RNase-free water.
Add 75 µL 1 X Rinse A to each well to store.
Combine 30 µL Eluent + 20 µL PCR Master Mix
PCR amplify.

Capture: Apply vacuum to clear Rinse A from Particles.
Combine 20 µL PCR Product + 60 µL Hybe Buffer.
Shake at 37ºC for 30 minutes. Rinse twice with 175 µL 1X Rinse A.

Report: Add 75 µL 1X Reporter Mix.
Shake at room temperature for 15 minutes.
Rinse twice with 175 µL 1X Rinse A.

Scan: Add 175 µL Run Buffer. Scan on appropriate flow cytometer.

Analyze: Flow cytometer files analyzed with the Firefly Analysis Workbench Software.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Upon receipt of this kit, please store the -20°C module at -20°C. The rest of the kit should be stored at +2-8°C.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in sections 9 and 10.

5. MATERIALS SUPPLIED

Item	Quantity	Storage Condition (Before Preparation)
1x Hybridization Buffer (Circulating Core)	25 mL	+2-8ºC
10x Rinse Buffer A (Circulating Core)	30 mL	+2-8ºC
Rinse Buffer B (Circulating Core)	33 mL	+2-8ºC
Labeling Diluent (Circulating Core)	30 mL	+2-8ºC
Run buffer (Circulating Core)	30 mL	+2-8ºC
Calibration beads (Cellular Core)	1 mL	+2-8ºC
Multiscreen HTS BV Dura 96x plate (Circulating Core)	1 X 96 Wells	RT
Catch Plate (Circulating Core)	1 Unit	RT
Primer Mix (Circulating Core)	250 µL	-20ºC
dNTP mix (Circulating Core)	120 µL	-20ºC
PCR Enzyme (Circulating Core)	60 µL	-20ºC
Labeling Buffer (Circulating Core)	200 µL	-20ºC
Labeling Enzyme (Circulating Core)	50 µL	-20ºC
PCR Buffer (Circulating Core)	2 mL	+2-8ºC
5x Reporter solution (Circulating Core)	4 mL	+2-8ºC
Circulating Control	50 µL	-20°C

6. MATERIALS REQUIRED, NOT SUPPLIED

This equipment is not included in the kit, but will be required to successfully utilize this assay:

- Shaking incubator(s).
- Vacuum manifold (ab204067 recommended).
- Thermocycler.
- Flow Cytometer.
- RNase-free water.
- 96 well PCR Plate compatible with your thermocycler.
- 25 mL Reagent Reservoirs.

NOTE: Mixing rates depend upon the orbital radius of your shaking incubator. Information about the recommended rate for your shaker can be found in the Technical Notes section.

NOTE: Firefly particles are designed to be read using a blue (488nm) laser with green, yellow, and red detectors. Additional information about supported cytometers and shaking incubators can be found on our website.

7. MATERIAL RE-ORDER INFORMATION

- Spare filter plates ab204280.

8. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

9. TECHNICAL HINTS

- For best results it is important to perform this protocol in a manner that avoids RNase contamination and that conforms to good PCR procedures. This includes use of aseptic conditions, wearing gloves at all times, keeping samples capped and on ice before use, and decontaminating work surfaces. Additionally, disposable polypropylene tubes and fresh filter tips should be used.
- Whenever possible steps should be performed using multi-channel pipettes and reagent reservoirs to minimize time between steps and wells.
- Excess amounts are included in each quantity/sample below, so when calculating mix volumes multiply the given volumes by the actual number of samples to be run (including controls).
- When applying vacuum to samples in the filter plate, turn off the vacuum as soon as the liquid is gone from each well to prevent over-drying.
- Steps to be performed on a heated shaker CANNOT be performed on a thermocycler instead; optimal mixing is critical.
- For optimal assay performance adequate mixing during incubation steps is critical, and depends upon both speed and orbital diameter. The mixing speed of 750 RPM recommended in this manual is for a shaking incubator with an orbital diameter of 3 mm. Customers should determine the orbital diameter of their shaking incubator prior to use. For shakers with a different orbital diameter, adjust the rpm according to the formula:

The speed you should use (in RPM) =

$$\sqrt{\frac{1687500}{orbital\ diameter\ (in\ mm)\ of\ your\ shaker}}$$

- Independently verify that the heated shakers, when set to 60°C and 37°C, both reach and maintain those temperatures. It is important that steps be performed at the recommended temperatures, for many heated shakers, the stated temperature and the actual temperature within the incubated area may vary.
- Set a heated shaker to the initial temperature needed. At various times during the protocol samples may shake at 60°C, 55°C, 37°C and room temperature. If available, pre-set multiple shakers to ease workflow, otherwise, remember to adjust temperatures early so that there is time for the shaker to equilibrate to its new temperature.
- Before running this assay on a given cytometer for the first time, we strongly recommend performing a test run of the particles through your flow cytometer to confirm that the settings are accurate and it is functional.
- If the assay will be repeated multiple times, it is recomended that a shaker be set aside for the final post-PCR hybridization step. It is important to prevent PCR amplicons from contaminating initial hybridization and labeling steps.
- Program your thermocycler with the recommended steps indicated in the Assay.
- Discard filter plate after use. Once a filter plate contains PCR product, it should not be used to run the assay again due to the risk of contamination. Use a fresh filter plate for subsequent runs if only a portion of the reagents were used.
- To prevent clogging, carefully follow the cleaning procedure recommended by the manufacturer of your flow cytometer.

10. REAGENT PREPARATION

Keep all reagents at room temperature during preparation. Reagents should only be used in their 1X working concentration.

10.1 **1X Rinse Buffer A**

Dilute 10X Rinse Buffer A by mixing the entire supplied volume (30 mL) with 270 mL RNase-free water in a clean container. Excess 1X Rinse Buffer can be stored at room temperature

11. SAMPLE PREPARATION

11.1 **Isolated Total RNA from plasma or serum**: For best results, isolate total RNA from plasma or serum using the TRIzol-LS® standard protocol as applied to 250 µL of sample, with 1 µL 15 mg/mL GlycoBlue™ added prior to precipitation. After precipitation with isopropanol, wash once with 75% ethanol then dry the pellet and resuspend in 50 µL RNase-free water. It is recomended that 40 µL sample-equivalents be run (i.e. 8 µL of the 50 µL + 17 µL RNase-free water = 25 µL sample for assay).

11.2 **Isolated Total RNA from cells & tissues:** For best results, isolate total RNA from cells or tissues using the TRIzol® standard protocol. It is recomended that RNA be diluted to 0.2 ng/µL in 25 µL RNase-free water (i.e. 5 ng in 25 µL).

Note: Other RNA isolation methodologies may be used. For a full list of supported isolation kits contact technical@abcam.com. Regardless of isolation method, it is important that the RNA be resuspended in RNAse-free water or 1X TE to keep salt to a minimum.

12. EXPERIMENTAL DESIGN

To obtain reliable miRNA expression data it is important that an experiment be designed to accurately subtract background signal, allow for appropriate normalization and assess inter-well variability. Every experiment performed with the Firefly Circulating miRNA Assay includes (1) **positive and blank controls in each well**, and ideally (2) **negative control wells**, and (3) **biological or technical replicates**.

12.1 Controls within each well

The assay utilizes both a positive control ("X-Control") and a blank ("Blank") by default in each custom or fixed panel. Endogenous controls may also be included.

Positive control particles contain probes for a miRNA-like target, X-Control, that is present in the Hybe Buffer. This control ensures that the assay was successfully implemented in every well.

BLANK particles contain no probe, generating a baseline level of the background fluorescence in every well.

Additional **Endogenous** controls are included in our fixed panels, and customers are free to select endogenous controls most appropriate for your experiments on custom panels.

12.2 **Negative control wells**

In order to obtain an accurate measurement of the background signal for each miRNA in a panel, it is necessary to run negative control wells where carrier buffer is used in place of a biological sample. Furthermore, the use of multiple negative control wells allows users to estimate inter-well variability, giving more confidence to the results obtained. It is strongly recommended that the use of at least three negative control wells per panel every time an assay is performed.

12.3 **Replicates**

The use of replicates gives statistical meaning to results by, for example, enabling the calculation of mean and standard deviation. Replicates can be performed at the stage of sample preparation (biological) or assay (technical).

12.4 **Circulating Control**

To use the Circulating Reference RNA, dilute 1 uL of RNA into 24 µL Rnase-free water, mixing well. The full 25 µL sample should then be added to a prepared well with Particles during step 14.2.7 of the assay

13. FLOW CYTOMETER SET UP

It is critical to complete flow cytometer set up prior to beginning the assay procedure as Firefly particles behave differently from beads and cells used in conventional flow cytometry or other bead-based multiplex assays.

Specific flow cytometer set up information can be found at the following links:

Millipore Guava EasyCyte™ 5, 8, 12, 5HT, 6HT, 8HT, 12HT and 6 2L

http://docs.abcam.com/pdf/protocols/guava-protocol.pdf

http://docs.abcam.com/pdf/protocols/millipore-guava-tube-handler-protocol.pdf

BD Biosciences AccuriTM C6

http://docs.abcam.com/pdf/protocols/accuri-protocol.pdf

http://docs.abcam.com/pdf/protocols/bd-accuri-c6-tube-loader.pdf

ThermoFishers Attune®

http://docs.abcam.com/pdf/protocols/attune-protocol.pdf

http://docs.abcam.com/pdf/protocols/life-technologies-attune-tube-handler-protocol.pdf

Please note that the assay is not validated for the ThermoFishers Attune NxT flow cytometer.

Detailed cytometer-specific instructions for scanning Firefly particles on your cytometer can be found at the following website: http://www.abcam.com/protocols/flow-cytometry-protocols-for-multiplex-mirna-assays

14. ASSAY PROCEDURE

14.1 **Hybridization**

14.1.1 Check that a heated shaker is at 37°C.

14.1.2 Record Firefly Particles Barcode #_____

Note: The barcode can be found on the outside of the Firefly Particles tube included with your kit. It is important to retain this number so that the appropriate .plx file can be used to analyze the results.

14.1.3 Peel backing off the seal film and apply over the filter plate (not the filter plate lid). Cut off the seal from the included filter plate to reveal one well for each sample and one well for each control.

Note: Do not reapply seal at any point (when covering plate, do so only with the supplied lid). If the plate seal is reapplied to the plate it may result in leakage during subsequent plate shaking.

14.1.4 Invert Firefly Particles end-over-end and vortex and add 35 µL to each well of filter plate, keeping particles mixed while distributing.

Note: Mixing is vital to ensure that each well receives an equal number of particles. Close and re-invert Firefly Particles tube every 5 wells.

If distributing with a multichannel pipette, add 4 mL 1X Rinse Buffer to a clean reagent reservoir (not supplied). Add 4 mL Firefly Particles to the reservoir, and mix by rocking 10 times. Using an 8-well multichannel pipette to transfer 70 µL of the particle mix, pipetting up and down 1x between each of columns 1-10. Remove 4 tips, tilt reservoir, and transfer the remaining particle mix 4 wells at a time, pipetting up and down 2 x between batches.

14.1.5 Apply vacuum to the filter plate to remove storage buffer and blot the underside of the plate dry with a Kimwipe™.
CAUTION: Excess buffer under the filter plate wells may result in assay failure so blot thoroughly

14.1.6 Add 25 µL Hybe Buffer to each well of the filter plate.
CAUTION: Hybe Buffer is viscous, take care during pipetting to ensure each well receives an equal volume.

14.1.7 Transfer 25 µL sample to each well of the filter plate.

14.1.8 Cover with lid and incubate the samples for 60 minutes at 37°C while shaking.
Note: Shake at speed appropriate for shaker (See Section 9 Technical Hints).

14.2 **Labeling**

14.2.1 Remove filter plate from shaker and adjust the temperature of the shaker to room temperature.
Note: Alternatively a second shaker may be used.

14.2.2 Prepare 1X Labeling Mix as indicated in the table below for each sample. Vortex to mix.

Component	Quantity/Sample	Total
Labeling Diluent	78.4 µL	
Labeling Buffer	1.6 µL	
Labeling Enzyme	0.4 µL	

(includes 10% excess for volume loss from pipetting)x sample #

14.2.3 Rinse wells by applying 175 µL 1X Rinse A on top of liquid in each well followed by application of vacuum.

14.2.4 Rinse wells a second time by applying 175 µL 1X Rinse A to each well followed by application of vacuum.
Note: Blot the underside of the plate dry with a Kimpwipe™.

14.2.5 Add 75 µL 1X Labeling Mix prepared above to each well.

14.2.6 Cover filter plate with lid and incubate the samples for 60 minutes at room temperature while shaking.
Note: Shake at speed appropriate for shaker (see Section 9 Technical Hints).

14.3 **PCR**

14.3.1 Adjust the temperature of the shaker to 55°C.
Note: While the same shaker used in previous steps may be reused, to limit PCR contamination a separate, post-PCR shaker is recommended for this and future steps.

14.3.2 Thaw -20°C reagents and store on ice.

14.3.3 Rinse wells by applying 165 µL RINSE B on top of liquid in each well followed by application of vacuum.
CAUTION: Be sure to use RINSE B at this step.

14.3.4 Rinse wells a second time by applying 165 µL RINSE B directly to particles in each well followed by application of vacuum.
CAUTION: Be sure to use RINSE B at this step.

14.3.5 Rinse wells once by applying 175 µL 1X RINSE A to the wells followed by application of vacuum.
CAUTION: Be sure to use RINSE A for this and future rinses. After final rinse, blot the underside of the plate to remove excess liquid.

14.3.6 Add 110 µL RNase-free water to each well

14.3.7 Cover filter plate with lid and incubate the samples for 30 minutes at 55°C while shaking.
Note: Shake at speed appropriate for shaker (see Section 9 Technical Hints).

14.3.8 Insert the catch plate into the vacuum manifold and place the filter plate atop the vacuum manifold, aligning carefully. Then apply suction, catching eluent in the filter plate.
Note the orientation of the catch plate so the proper samples get transferred to PCR.

14.3.9 Remove the filter plate from the vacuum manifold and add 175 µL 1X RINSE A to each well. Cover the filter plate with its lid and store at 4°C until it is needed after the PCR.

14.3.10 Prepare PCR Master Mix in order as follows in the table below.

Component	Quantity/Sample	Total
PCR Buffer	19.8 µL	
Primer Mix	2.4 µL	
dNTP Mix	1.2 µL	
PCR Enzyme	0.6 µL	

(includes 10% excess for volume loss from pipetting) x sample #

Note: Store Master Mix on ice until it is ready for use.

14.3.11 Using a fresh user-supplied PCR plate, first mix the eluent by pipetting up and down and then transfer 30 µL of the eluant from step 13.3.9 from the catch plate to the fresh PCR plate.

14.3.12 Add 20 µL PCR MASTER MIX to each well of the user-supplied PCR plate containing 30 µL of eluant in step 13.3.10, mixing well by pipetting up and down.
Note: Remember to change tips between pipetting different samples.

14.3.13 Transfer reaction mixtures to a thermal cycler.
Note: Leftover eluant may be stored at -20°C for future use if properly sealed with a plate seal. This is recommended in case mistakes are made during PCR sample prep or rehybridization. Fresh particles may be used and the process continued from here.

14.3.14 Thermal cycle using the following procedure:

Cycle	Temperature/Time
1 Cycle	93°C for 15 seconds
27 Cycles	93°C for 5 seconds
	59°C for 15 seconds
	72°C for 60 seconds
6 Cycles	93°C for 5 seconds
	63°C for 15 seconds
	72°C for 60 seconds
1 Cycle	72°C for 5 minutes
1 Cycle	94°C for 4 minutes
1 Cycle	4°C forever

14.4 **Capture**

14.4.1 Adjust the temperature of the shaker to 37°C.

14.4.2 Apply vacuum to the filter plate to remove the 1X RINSE A that kept the Firefly Particles stable and blot the underside dry.

14.4.3 Add 60 µL HYBE BUFFER to each well of the filter plate, then transfer 20 µL of the PCR Product from the PCR plate to the filter plate.
Note: Care should be taken to place the PCR Product in the well from which its corresponding Eluant was taken.

14.4.4 Cover with lid and incubate the samples for 30 minutes at 37°C while shaking.
Note: Shake at speed appropriate for shaker (see Section 9 Technical Hints).

14.5 **Report**

13.5.1 Adjust temperature of heated shaker to room temperature.

13.5.2 Prepare 1X Reporter Mix as indicated in the table below for each sample. Vortex to mix.

Component	Quantity/Sample	Total
RNase free water	64 µL	
5X Reporter	16 µL	

(includes 10% excess for volume loss from pipetting) x sample #

13.5.3 Remove filter plate from shaker.

13.5.4 Rinse wells by applying 175 µL 1X Rinse A on top of liquid in each well followed by application of vacuum.

13.5.5 Rinse wells a second time by applying 175 µL 1X Rinse A to each well followed by application of vacuum.

Note: Blot the underside of the plate dry with a Kimpwipe™.

13.5.6 Add 75 µL 1X Reporter Mix prepared above to each well.

13.5.7 Cover filter plate with lid and incubate the samples for 15 minutes at room temperature while shaking.

Note: Shake at speed appropriate for shaker (See section 9 Technical Hints).

14.6 **Scan**

14.6.1 Rinse wells by applying 175 µL 1X Rinse A to the top of each well followed by application of vacuum.

14.6.2 Rinse wells a second time by applying 175 µL 1X Rinse A to the top of each well followed by application of vacuum.
Note: Blot the underside of the plate with a Kimwipe™.

14.6.3 Add 175 µL Run Buffer to each well (do not mix).
Note: Ensure that the wells aren't leaking by setting the filter plate on a dry surface to see if there is liquid transfer after 30 seconds. If leakage occurred simply re-blot the underside and bring the volume of Run Buffer up to 175 µL in the leaky wells and reassess.

14.6.4 Scan on an approved flow cytometer.
Note: Detailed cytometer-specific instructions for scanning Firefly particles on your cytometer can be found at www.abcam.com/FireflyCytometerProtocols**.** *It is critical that researchers follow these instructions as Firefly particles behave differently from beads and cells used in conventional flow cytometry or bead-based multiplex assays.*

CAUTION: Discard filter plate after use. Once a filter plate contains PCR Product it should not be used to run the assay again, or one risks contamination.

14.7 Analysis

14.7.1 The Firefly Analysis Workbench Software enables easy *analysis of the data generated through this procedure.*

Note: Detailed instructions on how to use the Firefly Analysis Workbench Software can be found online at www.abcam.com/FireflyAnalysisSoftware*. Note: If you are using this assay with a Fixed Panel the necessary plex file is available with the Firefly Analysis Workbench Software. If you are using this assay with a Custom Panel the plex file can be found on the USB stick that accompanied the panel.*

15. SOFTWARE INSTALLATION GUIDE

15.1 First Time Use

- Go to www.abcam.com/FireflyAnalysisSoftware find the Software tab at the top. Choose 'Analysis' from pop up menu.
- Click the blue "Download the Firefly Analysis Workbench" to access additional instructions and a blue "Launch" button.
- Clicking the blue "Launch" button downloads a short Java web-start script and launches it.
- The Java program will automatically be copied to your desktop.

15.2 Subsequent Use

- Whenever the analysis workbench is updated, the application will download the new version, otherwise it will use the version it has already downloaded in order to save time.
- An internet connection is not needed for subsequent use, except for update purposes.

15.3 Troubleshooting

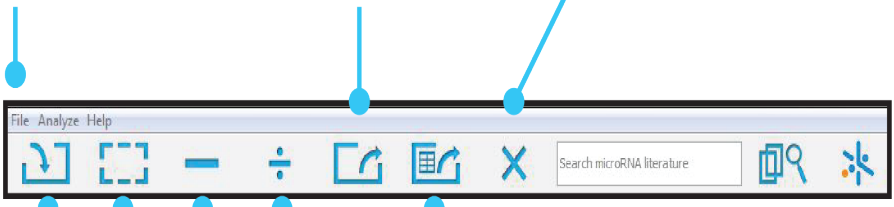
- Depending on your browser and system configuration, the web-start script (suffix .jnlp) may start automatically or may need to be manually started. If it does not start automatically, go to the downloads folder of your browser and double-click the "firecode.jnlp" file to download and launch the software.
- On some machines, system security may prevent the application from running with a double-click; proceed by right-clicking the application and selecting Open with Java Web Start.
- Java security may ask if you want to run the program either after the web-start program has been downloaded or after the Workbench has been downloaded. Click OK at the prompts.

- You may receive a warning that an application is requesting access to your system. If you do, check the details of the certificate and click "Allow." This is needed so the Analysis Workbench can open your data files.
- You may also be asked to allow a shortcut to be installed on your desktop. Accept to run the Workbench without an internet connection.
- On some systems, Java Web Start may ask for permission to access the Internet to check for a new version of Java. Although not required for the Analysis Workbench unless your Java is older than 2006, it is recommended to stay up to date for security purposes.

16. SOFTWARE QUICKSTART GUIDE

For more detailed information on using the Analysis Workbench please refer to the Analysis Workbench User Guide found at www.abcam.com/FireflyAnalysisSoftware

16.1 **Step-by-step instructions for uploading and naming your data.**



2
File>Rename Samples to optionally load a .csv file with sample names and group information.

7
Save Firecode Workspace so that you can review your results at a later time just where you left off.

8
Clear all data

6
Export all data into .csv files for additional analyses.

1
Load new .fcs files from the cytometer and included plex files for fixed panels or plex file on supplied flash drive for custom panels.
OR
Load saved .fws workspace to pick up where you left off.

5
Choose and apply/remove a normalization method

3
Select wells of interest (samples and controls) and join them together with the Experiment button. Multiple, independent experiments can be created on a plate.

4
Highlight and select wells that contain samples appropriate for background subtraction and define these as negatives.

16.2 **Analyzing your results**

Create and modify heat maps.

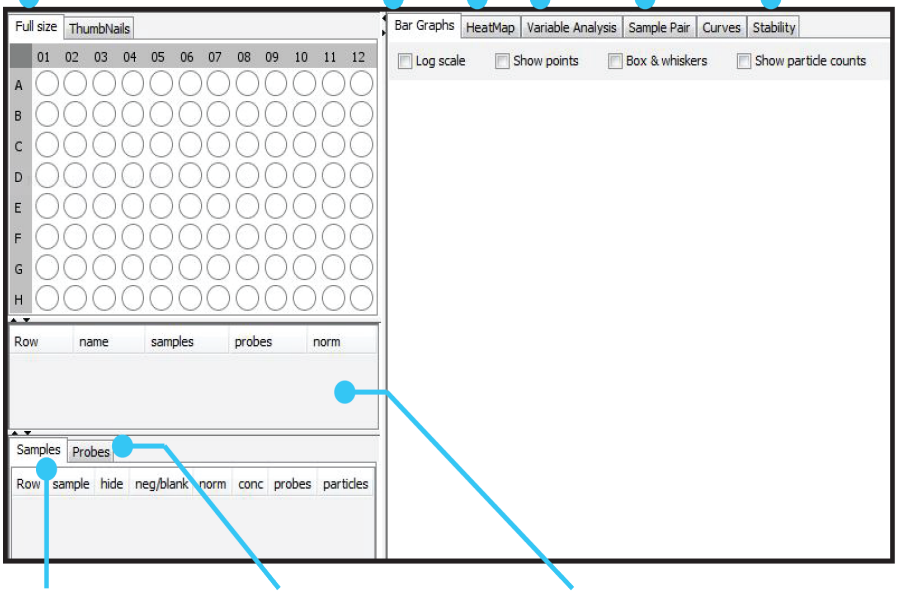
Perform Pearson correlations between any two samples.

Display expression levels of all targets in one chosen well and expression level of one chosen target in all wells.

Perform ANOVA statistics upon groups or subsets of groups.

Visualize stability of targets for selection as potential normalizers.

View and select samples in 96-well format and review well QC.



View and edit sample traits such as name, status as a negative well, group name and if the sample is hidden.

View and edit probe traits such as name, status as normalizers, and if the probe is hidden.

View details of each selected experiment.

17. TROUBLESHOOTING

Problem	Cause	Recommendation
Low throughput in a subset of wells	Low Run Buffer volume	Run Buffer volume can wick out of the well prior to scanning. To avoid this, ensure the bottom of the plate is dry at the end of filtration. If necessary, add additional Run Buffer and re-scan.
	Too-few Firefly Particles	Resuspend particles within 30 seconds of transferring. Use a mutli-channel pipette and reagent reservoirs for increased speed.
	Punctured filter membrane	When adding liquids to the filter plate wells, add to sides or just over the surface so as not to puncture the membrane.
Filtration isn't working well	Inappropriate pressure	Maintain vacuum pressure below 2 PSI during filtration and DO NOT OVER-FILTER particles. Bubbles are OK.
Leaking Filter Plate wells	Failure to blot plate	Be sure to blot plate after filtration.
	Wells tightly sealed	Cover the plate with its lid but do not use a plate seal on the wells during shaking incubation steps.
Signal in negative wells	PCR Contamination	Separate pre- and post-PCR work areas; use a different shaker for pre- and post-PCR. Do not reuse plates that have had post-PCR products hybridized to particles for fresh experiments. We

Problem	Cause	Recommendation
		suggest using a fresh filter plate.
	High background Probes	Probes for a few miRNAs are known to exhibit high background level. This is not a result of contamination and the only consequence is to limit the dynamic range of these rare probes. Please contact support@fireflybio.com if you are unclear if any probes used in your panel are high background.
Low Signal	Insufficient sample input	Re-verify sample quantification using an alternate method if possible.
	Thermocycler inaccuracy	Verify the program is correct and the block temperature on the thermocycler is calibrated.
	Insufficient PCR cycles	Cycle numbers used in this protocol have been well tested, but if signal is low, additional cycles may be used.
	Shaker at wrong temperature	Verify the shaker has accurately reached temperature before each step is begun.
	Insufficient reaction mixing	Labeling and PCR mixes must be thoroughly mixed.
	Incorrect cytometer settings	Check cytometer settings.
No Signal for any probe	Missing PCR component	Repeat PCR step on reserved eluant

18. NOTES

UK, EU and ROW
Email: technical@abcam.com | Tel: +44-(0)1223-696000

Austria
Email: wissenschaftlicherdienst@abcam.com | Tel: 019-288-259

France
Email: supportscientifique@abcam.com | Tel: 01-46-94-62-96

Germany
Email: wissenschaftlicherdienst@abcam.com | Tel: 030-896-779-154

Spain
Email: soportecientifico@abcam.com | Tel: 911-146-554

Switzerland
Email: technical@abcam.com
Tel (Deutsch): 0435-016-424 | Tel (Français): 0615-000-530

US and Latin America
Email: us.technical@abcam.com | Tel: 888-77-ABCAM (22226)

Canada
Email: ca.technical@abcam.com | Tel: 877-749-8807

China and Asia Pacific
Email: hk.technical@abcam.com | Tel: 108008523689 (中國聯通)

Japan
Email: technical@abcam.co.jp | Tel: +81-(0)3-6231-0940

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