ab217049 – FirePlex miRNA Assay V2 – Human Discovery Panels

Protocol Booklet Version 1.0 January 2020

Please note that this product replaces the previous Human Discovery Panels for Plasma/Serum (ab217048) and Immunology (ab217050).

Two significant changes have been implemented for this new product:

- the panels for ab217048 and 217050 have been combined together, and we are now providing a single product for Human miRNA Discovery Panel analysis.
- the miRNA panels are now utilizing sequences from V22 of miRBase

Please carefully review this protocol prior to running assays, as protocols are subject to updates and changes. This product is for research use only and is not intended for diagnostic use.

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1. Overview

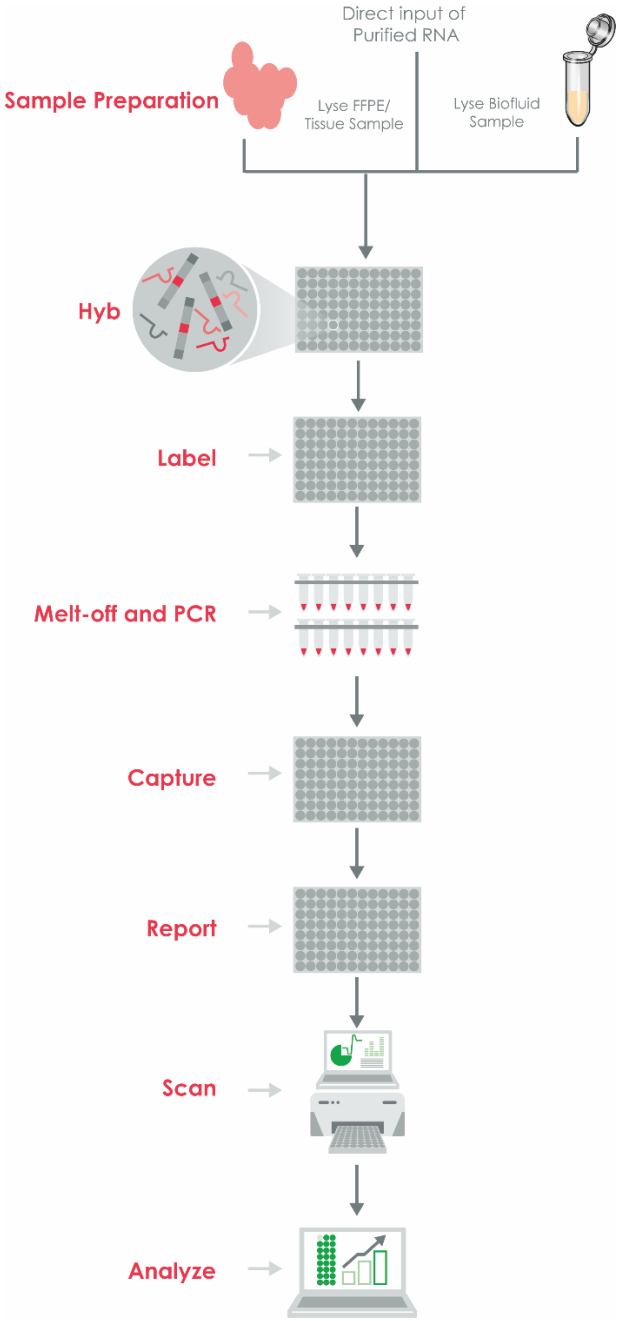
The FirePlex® miRNA Assay enables high-throughput detection by flow cytometry of 405 miRNA targets and streamlined analysis with the FirePlex® Analysis Workbench Software. The assay is performed in 96-well plate format such that users can detect 405 miRNA targets for 16 samples per plate.

The FirePlex® 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 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, multiplexed detection using the FirePlex® platform conserves precious sample by detecting multiple miRNA targets from: 6ng of purified RNA; directly from 100 µL serum, plasma, and PAXgene™ samples without RNA isolation; or from 5 µm sections of tissues and FFPE samples.

CAUTION! The assay protocol supplied in this document corresponds to running the FirePlex miRNA Assay Discovery Panels. The assay protocols for running a single panel product (maximum 65plex panel) can be accessed via the Abcam website, on the respective product pages (i.e. FirePlex miRNA Panel – Oncology V2, ab218367).

2. Protocol Summary



3. Precautions

Prior to running the assay, please familiarize yourself with the assay protocols and workflow.

CAUTION! Biofluid and FFPE/Tissues samples require an initial lysis step, as outlined in Sections 14.1 and 14.2, respectively. If using purified RNA as the sample input, proceed directly to Section 15.

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, 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 the supplied datasheet.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Kits are guaranteed for 6 months from the date of receipt, if stored and handled according to the instructions provided on each component label (i.e. protected from light and stored at the indicated temperatures).
- 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.

6. Materials Supplied

FirePlex Particle Mixes

Item	Volume (12 test/ 48 test)	Cap Color	Panel Lot #
FirePlex Particle Mix – Human miRNA Discovery Panel A	500 µL/2 mL	Dark Blue	
FirePlex Particle Mix – Human miRNA Discovery Panel B	500 µL/2 mL	Yellow	
FirePlex Particle Mix – Human miRNA Discovery Panel C	500 µL/2 mL	Green	
FirePlex Particle Mix – Human miRNA Discovery Panel D	500 µL/2 mL	Orange	
FirePlex Particle Mix – Human miRNA Discovery Panel E	500 µL/2 mL	Red	
FirePlex Particle Mix – Human miRNA Discovery Panel F	500 µL/2 mL	Black	
FirePlex Particle Mix – Human miRNA Discovery Panel H*	500 µL/2 mL	Light Blue	
*Panel H is a reference panel, to be run only with positive and negative controls			

7. Materials Required, Not Supplied

Below is a list of the reagents and equipment needed to successfully run the FirePlex miRNA Assay V2:

- FirePlex miRNA Assay – Core Reagent Kit V2 for specific sample types: Biofluid (ab218342); Purified RNA (ab218365); or FFPE/Tissues (ab235025)
- FirePlex Run Buffers (refer to Section 10 of this protocol booklet for additional details)
- Shaking incubator(s)
- Vacuum manifold (ab204067 recommended)
- Vacuum trap
- Thermocycler
- Flow Cytometer
- RNase-free water
- 96 well PCR Plates and caps/seal, compatible with your thermocycler
- 25 mL Reagent Reservoirs
- Optional – Additional Filter Plates (ab204280)

Δ **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 (Section 8).

Δ **Note:** FirePlex® 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.

8. 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.
- The volumes indicated for master mixes at each step include an overage of 10%. When calculating the required volume inputs, simply multiply the indicated volumes by the total number of samples being assayed (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; mixing is necessary.
- 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 heated shakers, when set to 60°C and 37°C, 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, users must first run the cytometer setup kit (ab245835) through their flow cytometer to identify the appropriate Run Buffer for your instrument, confirm that the settings are accurate, and that the cytometer is functional. It is strongly recommended to run two wells of the cytometer setup kit prior to each run of the assay, to ensure cytometer performance is meeting recommended specifications.
- If the assay will be repeated multiple times, it is recommended 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 Section 15.3.
- 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.

9. Experimental Design

We recommend that each experiment performed with the FirePlex® miRNA Assay includes (1) **positive and blank controls in each well**, and ideally (2) **negative control wells**, and (3) **biological or technical replicates**. These features assist estimation of background signal, allow for appropriate normalization, and assess inter-well variability.

9.1 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. Three **off-species** controls are included in each pre-designed and custom panel to help determine the limit of detection (LOD) for the assay.

Positive control particles contain probes for a miRNA-like target, X-Control, that is present in the Hyb 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.

9.2 Negative control wells

It is recommended that the user run two negative control wells, i.e. replacing the sample input with water, every time an assay is performed (refer to the plate layout in Section 9.5). Significant signal in negative control wells can indicate problems executing the assay, for example PCR cross-contamination between wells.

If running this assay for the first time, users may want to run negative controls both for the digest (by adding water to the Lysis Mix) and for the assay as a whole (by adding water to Hyb Buffer in the first hybridization step).

9.3 Replicates

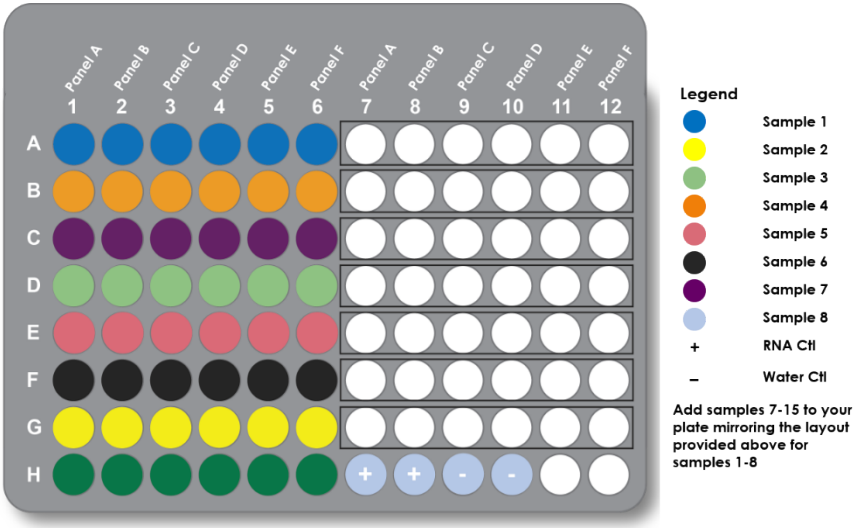
The use of replicates provides statistical meaning to results by, for example, enabling the calculation of mean and standard deviation. Replicates can be performed at the stage of sample preparation (biological) or assay (technical).

9.4 RNA Control

To use the RNA Control, dilute 4 µL of RNA into 96 µL RNase-free water, mixing well. Add 25 µL of the diluted stock RNA to each control well (Wells H1-H2, see Section 15.3.7).

9.5 Recommended Plate Layout

We recommend using the plate layout below for running the FirePlex Discovery Panels.



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 A can be stored at room temperature.

10.2 1X Rinse Buffer B

Dilute 2X Rinse Buffer B by mixing the entire supplied volume (33 mL) with 33 mL RNase-free water in a clean container. Excess 1X Rinse Buffer B can be stored at room temperature.

11. Flow Cytometer Set-up and Verification

It is critical to complete flow cytometer set up prior to starting the assay procedure to determine the optimal FirePlex Run Buffer for data acquisition on your flow cytometer.

- Using the Cytometer Setup Kit V2 (ab245835) and specified protocol, complete flow cytometer set up according to the instructions for your validated flow cytometer model.
- Performing the cytometer setup according to the specified protocol is critical for identifying the optimal FirePlex Run Buffer for your particular instrument.
- Please ensure that you use the flow cytometer settings file provided and have optimized and verified this protocol to work on your own cytometer.
- The appropriate Run Buffer to use for your particular instrument can be purchased as an accessory item from Abcam, using the part codes below:

Item	Quantity
Run Buffer I (ab245836)	1x96 tests
Run Buffer II (ab234450)	1x96 tests
Run Buffer III (ab245837)	1x96 tests
PBS (user supplied)	—

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

EMD 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 Accuri™ C6

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

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

ThermoFisher Original Attune® (first generation)

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

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

Δ **Note:** The assay is not validated for the ThermoFisher Attune NxT flow cytometer (second generation).

Detailed cytometer-specific instructions for scanning FirePlex® Particles on your cytometer can be found at the following website:

http://www.abcam.com/protocols/flow-cytometry-protocols-for-multiplex-mirna-assays

12. Sample Preparation

12.1 Biofluid Samples, Extracellular Vesicles, and Cell Suspensions

Biofluids:

For best results, store the sample at -80°C and limit the number of freeze/thaws that the samples undergo prior to quantification with the FirePlex® miRNA Assay.

*Optional: centrifuge samples at 2000 x g for 15 min to clarify and sediment cell debris.

Extracellular Vesicles

FirePlex miRNA Assays have been validated for use with a wide range of commercially available extracellular vesicle (ECV) isolation kits. We recommend referring to the manufacturer's instructions for the specific kit you're using for isolation methods, and to determine the required sample starting volume for ECV isolation. Refer to Section 13 for ECV input volumes into the FirePlex miRNA Assay.

Cell Suspensions

Cells should be rinsed twice with 1x PBS and then pelleted. Remove PBS and resuspend in an appropriate volume of fresh 1x PBS, such that the sample input volume corresponding to the required number of cells is 40 µL.

Refer to Section 14.1 for Biofluid/Cell Suspension Lysis Instructions.

12.2 FFPE and Tissue Samples

Use tissue less than 5mm thick and fix in 4-10% neutral-buffered formalin with a maximum fixation time of 24 hours. Yield will vary due to fixation and storage conditions. The section for analysis should be taken from an unexposed region of the block as oxidation can degrade RNA. Use a single 5-10 µm thick section for best results. Trim off any excess paraffin where possible.

Refer to Section 14.2 for FFPE/Tissue Lysis Instructions.

12.3 Isolated Total RNA

From cells & tissues:

For best results, isolate total RNA from cells or tissues using the TRIzol® standard protocol.

Isolated Total RNA from biofluids:

For best results, isolate total RNA from biofluids 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 recommended that 20 µL sample-equivalents be run (i.e. 4 µL of the 50 µL + 21 µL RNase-free water = 25 µL sample for assay).

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

If using Purified RNA as the sample input, proceed directly to Section 15 for the Assay Procedure.

13. Recommended Sample Dilutions

Sample Type	Recommended Starting Input*
Plasma/Serum	100 µl
Exosome Fractions	100 µl▲
Urine	100 µl
Cell Culture Supernatant	100 µl
FFPE or Tissue	1 x 5-10 µm curl
Purified RNA	6 ng
*May need to be adjusted based on specific sample types ▲Based upon exosomes isolated from 200 µl serum	

14. Sample lysis

Δ **Note:** Refer to Section 14.1 for Lysis of Biofluids/Cell suspensions; Section 14.2 for Lysis of FFPEs/Tissues. If using Purified RNA as the sample input, proceed directly to Section 15.

14.1 Lysis – Biofluids and Cell Suspensions

14.1.1 Prepare Lysis Mix as indicated in the table below for each sample.

Component	Quantity/Sample	Total
Protease Mix	14 µL	
Digest Buffer	140 µL µL	

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

14.1.2 In a clean tube combine 100 µL Plasma or Serum with 140 µLLysis Mix.

Δ **Note:** Mix the samples well by pipetting up and down repeatedly.

14.1.3 Carefully seal the tubes and incubate the samples for 45 minutes at 60°C while shaking.

Δ **Note:** Give sufficient time for the shaker to reach temperature before starting the assay (often 30 minutes). Shake at speed appropriate for shaker (See Section 8 Technical Hints).

14.1.4 Remove samples from shaker and store in freezer until needed.

Δ **Note:** Prior to starting the hybridization step (15.2), allow samples to thaw completely.

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

Δ **Note:** Alternatively, a second shaker pre-set at 37°C may be used.

14.1.6 Proceed to Section 15 to continue the Assay Procedure.

14.2 Lysis – FFPE and Tissues

14.2.1 Prepare Lysis Mix as indicated in the table below for each sample.

Component	Quantity/Sample	Total
Protease Mix	25 µL	
Digest Buffer	250 µL	

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

14.2.2 Transfer a single 5-10 µm FFPE curl to a 1.5 or 2 mL microfuge tube. Add 250 µL of the prepared Lysis Mix to each tube. For negative control well, use 250 µL Lysis Mix.

Δ **Note:** Take care to ensure that the FFPE curl is fully immersed in Lysis Mix.

14.2.3 Carefully seal the tubes and incubate the samples for a total of 45 minutes at 60°C while shaking.

Δ **Note:** Give sufficient time for the shaker to reach temperature before starting the assay (often 30 minutes). Shake at speed appropriate for shaker (See Section 8 Technical Hints).

14.2.4 After 10 minutes of shaking, pause the shaker and pulse vortex each tube to ensure that the FFPE curl becomes completely immersed in Lysis Mix. Return to the shaker the remaining 35 minutes of incubation.

14.2.5 Remove samples from shaker, pulse vortex to mix, then centrifuge samples for at 2000 x g for 1 minute.

14.2.6 Let sit at room temperature for 2 minutes.

14.2.7 Store the samples on ice for 5 minutes.

14.2.8 Transfer liquid to an included filter plate.

14.2.9 Insert one of the two Catch Plates into the vacuum manifold and place the Filter Plate atop the vacuum manifold, aligning carefully. Then apply suction, catching eluant in the Filter Plate.

Δ Note the orientation of the Catch Plate so the proper samples get transferred to hybridization.

14.2.10 Save filtrate for use in step 15.1.7. The filter plate can now be discarded and should not be used later in the assay.

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

Δ **Note:** Alternatively, a second shaker pre-set at 37°C may be used.

14.2.12 Proceed to Section 15 to continue the Assay Procedure.

15. Assay Procedure

15.1 Hybridization

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

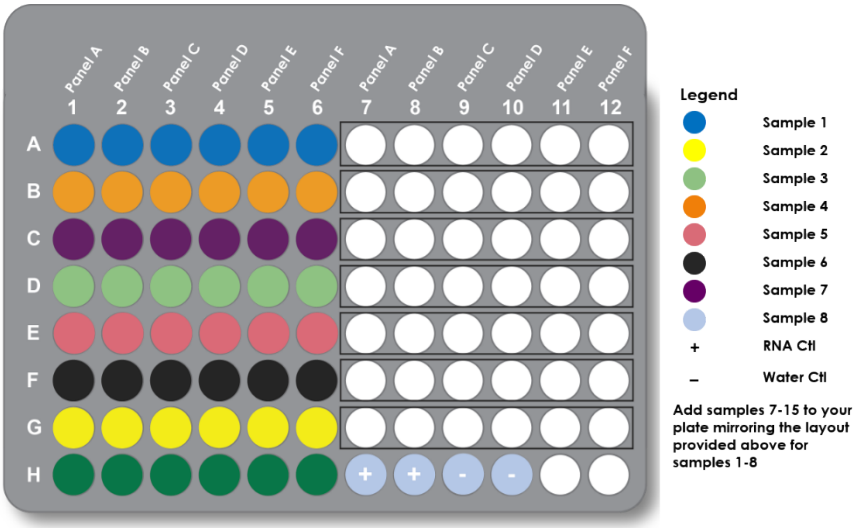
15.1.2 Record the lot numbers for your FirePlex® Particle Mix Panels A-F, and Panel H in the Table provided in Section 6.

Δ **Note:** The lot number can be found on the outside of the FirePlex® Particles tube included with your kit.

15.1.3 If running fewer than 15 samples, peel backing off the Plate Seal and apply over the Filter Plate (not the Filter Plate lid). Cut and remove sections of the Plate Seal to reveal one well for each sample and one well for each control.

Δ **Note:** After removal, do not reapply the Plate Seal at any point as it will result in leakage during subsequent plate shaking. Cover the plate only with the supplied lid.

15.1.4 To dispense FirePlex® Particle Mix – Human Discovery Panels A-F and Panel H into the Filter Plate, we recommend using the plate layout below (also provided earlier, in Section 9.5).



Briefly:

- Invert the tube containing Panel A end-over-end, and vortex to resuspend. Add 35 µL of Panel A to Wells A1-H1 and A7-G7 in the Filter Plate. Close and re-invert Particle Mix every 4 wells to keep particles mixed while distributing.
- Repeat this step for Panel B, first inverting to mix and then adding 35 µL of Panel B to Wells A2-H2 and A8-G8 in the Filter Plate.
- Continue this for Panels C-F, until all Panels have been added to the Filter Plate, as indicated in the plate layout.
- Add 35 µL of Panel H (reference panel) only to Wells H7-H10- for the positive control (supplied RNA control) and negative control (Water).

15.1.5 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 ensure that Filter Plate is blotted thoroughly.

15.1.6 Add 25 µL Hyb Buffer (Hybridization Buffer) to each well of the Filter Plate.
CAUTION! Hyb Buffer is viscous. Take care during pipetting to ensure each well receives an equal volume.

15.1.7 To add your samples and controls to the Filter Plate, again use the plate layout above.

Briefly:

- Transfer 25 µL of Sample 1 to Wells A1-A6, changing tips after addition to each well.
- Next, transfer 25 µL of Sample 2 to Wells B1-B6, again changing tips after addition to each well.
- Repeat this step until all 15 samples have been added to the Filter Plate.
- Transfer 25 µL of diluted RNA Control from Section 9.4 to Wells H7 and H8, and 25 µL of water to Wells H9 and H10.

15.1.8 Cover with lid and incubate the samples for 60 minutes at 37°C while shaking.

Δ **Note:** Shake at speed appropriate for shaker (See Section 8 Technical Hints).

15.2 Labeling

15.2.1 Remove Filter Plate from shaker and adjust the temperature of the shaker to room temperature.

Δ **Note:** Alternatively a second shaker may be used.

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

Component	Quantity/Well	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 well #

15.2.3 Rinse wells by applying 165 µL 1X Rinse Buffer A on top of liquid in each well followed by application of vacuum.

15.2.4 Rinse wells a second time by applying 165 µL 1X Rinse Buffer A to each well followed by application of vacuum.

Δ **Note:** Blot the underside of the plate dry with a Kimwipe™.

15.2.5 Add 75 µL of the 1X Labeling Mix prepared above to each well.

15.2.6 Cover Filter Plate with lid and incubate the samples for 60 minutes at room temperature while shaking.

Δ **Note:** Shake at speed appropriate for shaker (See Section 8 Technical Hints).

15.3 PCR

15.3.1 Remove plate from shaker, then adjust the temperature of the shaker to 55°C.

Δ **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.

15.3.2 Thaw -20 °C reagents and store on ice. Thoroughly invert and vortex PCR Buffer before use.

15.3.3 Rinse wells by applying 165 µL 1X Rinse Buffer B on top of liquid in each well followed by application of vacuum.

CAUTION! Be sure to use 1X Rinse Buffer B at this step.

15.3.4 Rinse wells a second time by applying 165 µL 1X Rinse Buffer B directly to particles in each well followed by application of vacuum.

CAUTION! Be sure to use 1X Rinse Buffer B at this step.

15.3.5 Rinse wells once by applying 165 µL 1X Rinse Buffer A to the wells followed by application of vacuum.

CAUTION! Be sure to use 1X Rinse Buffer A for this step. After final Rinse, blot the underside of the plate to remove excess liquid.

15.3.6 Add 110 µL RNase-free water to each well

15.3.7 Cover Filter Plate with lid and incubate the samples for 30 minutes at 55°C while shaking.

Δ **Note:** Shake at speed appropriate for shaker (See Section 8 Technical Hints).

15.3.8 Insert the Catch Plate into the vacuum manifold and place the Filter Plate atop the vacuum manifold, aligning carefully. Then apply suction, catching eluant in the Filter Plate.

Note the orientation of the Catch Plate so the proper samples get transferred to PCR.

15.3.9 Remove the Filter Plate from the vacuum manifold and add 165 µL 1X Rinse Buffer A to each well. Cover the Filter Plate with its lid and store at 4°C until it is needed after the PCR.

15.3.10 Pool 25 µL of Sample 1 eluant from Wells A1-A6 (150 µL L total) into a fresh, user supplied 0.2 mL microfuge tube. Repeat this for Samples 2-15, pooling each sample into a separate tube. DO NOT pool the positive and negative control samples (Wells H7-H10).

Δ **Note**: After addition, take care to mix by carefully by pipetting up and down 6-7 times.

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

Component	Quantity/Sample	Total
PCR Buffer	59.4 µL	
Primer Mix	7.2 µL	
dNTP Mix	3.6 µL	
PCR Enzyme	1.8 µL	

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

Δ **Note:** When preparing the PCR Master Mix, be sure to include both the number of individual samples, and controls being run.

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

15.3.12 Set up two PCR reactions, by adding 45 µL of the pooled eluant from step 15.4.10 to two wells of a fresh, user-supplied PCR plate for each sample. For control samples only one (1) PCR reaction need be set up, using the same volumes as indicated above.

15.3.13 Add 30 µL PCR Master Mix from step 15.4.11 to each well of the PCR plate, and mix well by pipetting up and down.

Δ **Note:** Remember to change tips between pipetting different samples.

15.3.14 Seal tubes and transfer reaction mixtures to a thermocycler.

Δ **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.

15.3.15 Thermal cycle using the following procedure

Cycle	Temperature/Time
1 Cycle	93°C for 15 seconds
32 Cycles	93°C for 5 seconds
	57°C for 30 seconds
	68°C for 60 seconds
1 Cycle	68°C for 5 minutes
1 Cycle	94°C for 4 minutes
1 Cycle	4°C forever

15.4 Capture

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

Δ **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.

15.4.2 Pool both 75 µL PCR reactions for Sample 1 into a fresh tube. Repeat this for Samples 2-15. DO NOT pool the positive and negative control samples (Wells H7-H10), nor add any additional PCR buffer to these samples.

Δ **Note**: After addition, take care to mix carefully by pipetting up and down 6-7 times.

15.4.3 Apply vacuum to the Filter Plate to remove the 1X Rinse Buffer A from the FirePlex® Particles and blot the underside dry.

15.4.4 Add 60 µL Hyb Buffer to each well of the Filter Plate.

15.4.5 Transfer 20 µL of the pooled PCR Product for Sample 1 (Step 15.5.2) to Wells A1-A6 of the Filter Plate. Repeat this step for Samples 2-15, taking care to add each sample to the corresponding wells of the Filter Plate. For the positive and negative control samples, transfer 20 µL of the unpooled PCR product to Wells H7-H10, respectively.

15.4.6 Cover with lid and incubate the samples for 30 minutes at 37°C while shaking.

Δ **Note:** Shake at speed appropriate for shaker (See Section 8 Technical Hints).

15.4.7 Remove Filter Plate from shaker.

15.4.8 Rinse wells by applying 165 µL 1X Rinse Buffer B on top of liquid in each well followed by application of vacuum.

15.4.9 Rinse wells a second time by applying 165 µL 1X Rinse Buffer B to each well followed by application of vacuum.

Δ **Note:** Blot the underside of the plate dry with a Kimwipe™.

15.4.10 Rinse wells by applying 165 µL 1X Rinse Buffer A on top of liquid in each well followed by application of vacuum.

Δ **Note:** Blot the underside of the plate dry with a Kimwipe™.

15.5 Report

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

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

(includes excess for volume loss from pipetting) x well #

15.5.2 Adjust temperature of heated shaker to room temperature.

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

15.5.4 Cover Filter Plate with lid and incubate the samples for 15 minutes at room temperature while shaking.

Δ **Note:** Shake at speed appropriate for shaker (See Section 8 Technical Hints).

15.5.5 Rinse wells by applying 165 µL 1X Rinse Buffer A to each well followed by application of vacuum.

15.5.6 Rinse wells a second time by applying 165 µL 1X Rinse Buffer A to each well followed by application of vacuum.

Δ **Note:** Blot the underside of the plate dry with a Kimwipe™.

15.6 Scan

15.6.1 Add 100-175 µL of the appropriate Run Buffer to each well Place plate on orbital shaker to mix.

Δ **Note:** The appropriate Run Buffer for your machine should be pre-determined **prior to starting the assay** using the FirePlex Cytometer Setup Kit, as outlined in Section 11.

Δ **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 in the leaky wells and reassess.

15.6.2 Scan on an approved flow cytometer.

Δ **Note:** Detailed cytometer-specific instructions for scanning FirePlex® Particles on your cytometer can be found at www.abcam.com/FirePlexCytometerProtocols. It is critical that researchers follow these instructions as FirePlex® Particles behave differently from beads and cells used in conventional flow cytometry or bead-based multiplex assays.

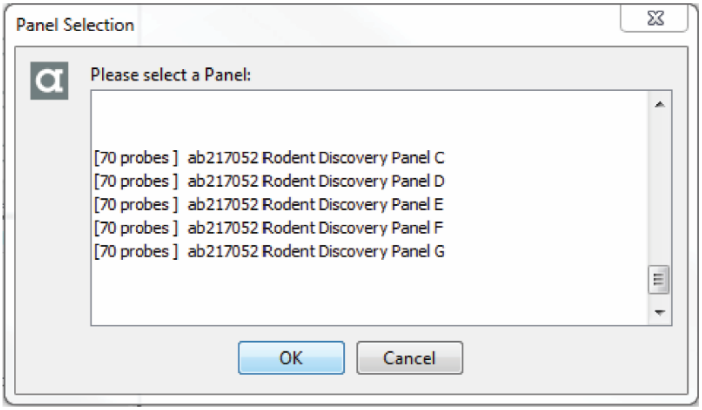
CAUTION! Discard Filter Plate after use. Once a Filter Plate contains PCR Product it should not be used to run the assay again, or one risks contamination.

15.7 Analysis

15.7.1 The FirePlex® Analysis Workbench Software enables easy analysis of the data generated through this procedure.

Δ **Note:** Detailed instructions on how to use the FirePlex® Analysis Workbench Software can be found online by referring to: https://www.abcam.com/Kits/fireplex-analysis-workbench-software-for-fireplex-mirna-assays

15.7.2 If you are using this assay with a Pre-designed Panel the necessary plex file is available within the FirePlex® Analysis Workbench Software, listed under the panel name and abID, as indicated in image below:



16. Software Installation Guide

16.1 First Time Use

- Go to the link provided in Step 14.7.1 and click on the link for the Analysis Workbench Software.
- Click the blue "Download now" button to download a short Java web-start script and launch it.
- The Java web-start script (file extension .jnlp) will automatically be copied to your Downloads folder. You may want to copy it somewhere convenient, such as your desktop. When this file is double-clicked, it downloads the Java application (file extension .jar).

16.2 Subsequent use

- Whenever the FirePlex® Analysis Workbench is updated, the script will download the new Java application, otherwise it will use the version it has already downloaded to save time.
- An internet connection is not needed for subsequent use, except for update purposes. If no connection is available, the script will use the last-downloaded copy.

16.3 Troubleshooting

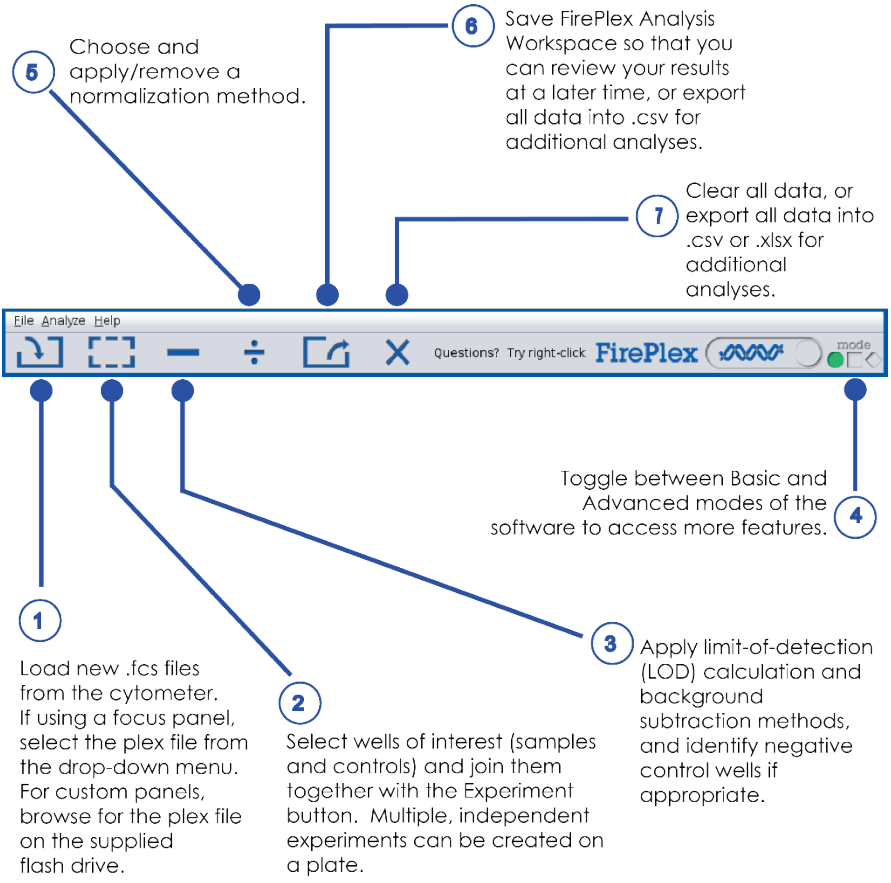
- Depending on your browser and system configuration, the web-start script (suffix. jnlp) may start automatically or may need to be manually started. If it does not start automatically, go to the downloads folder of your browser and double-click the "firecode.jnlp" file to download and launch the software.
- 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 software 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 software 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.

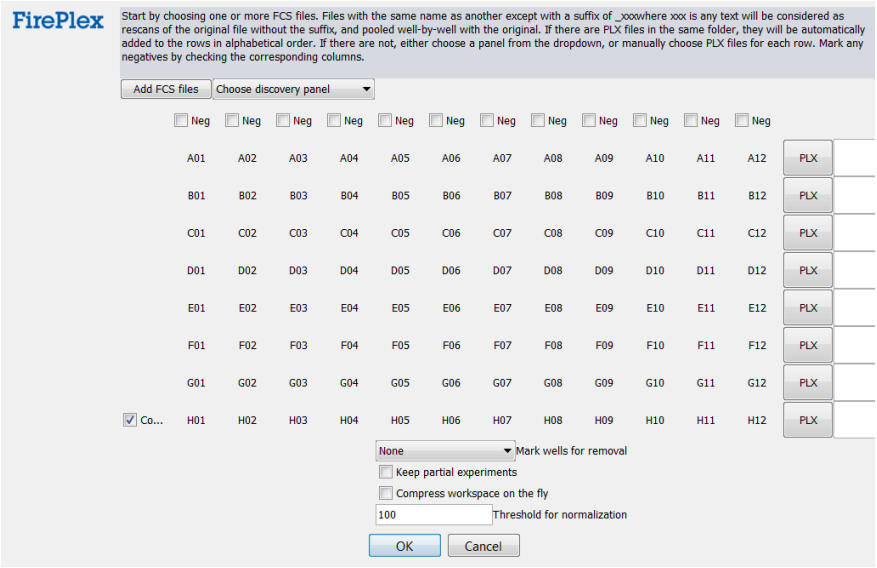
17. Software QuickStart Guide

For more detailed information on using the FirePlex Analysis Workbench please refer to the User Guide found at the link provided in Step 14.7.1 .

17.1 Step-by-step instructions for uploading your data



17.1.1 Discovery Panel analysis begins with a dropdown from the File Menu at the top of the control bar, named "Discovery GUI".



Discovery GUI

17.1.2 Each row corresponds to one plex file from the panel. Each column corresponds to a sample, with exception of the bottom row, containing the control samples.

17.1.3 Begin by adding FCS files (the top button). All files named will be loaded into the same experiment. Files whose names are an underscore extension of another filename, e.g. Plate24_rescan.fcs and Plate24.fcs, will be treated as rescans of a plate, and their wells will be pooled. Files with independent names will be treated as separate plates.

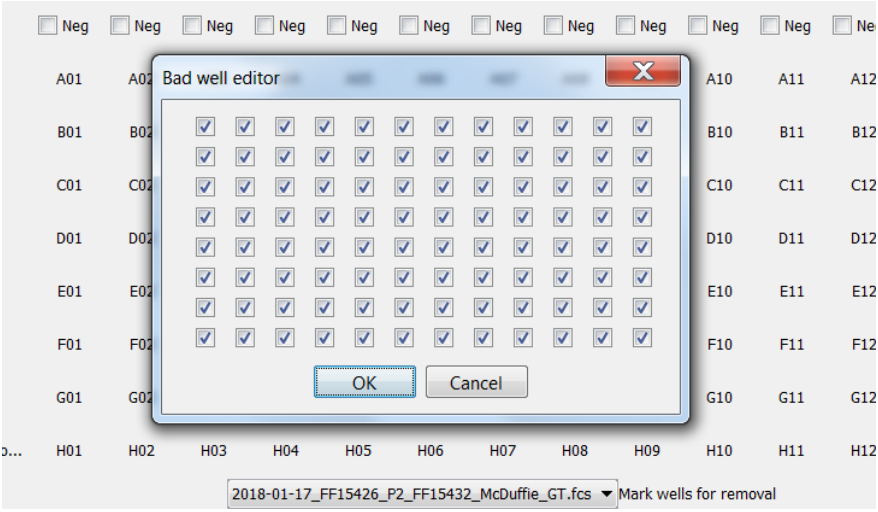
17.1.4 If there are already a set of PLX files in that folder, they will be loaded into the rows in alphabetical order, for instance Discovery-panel-A.plx will be loaded into row A, Discovery-panel-B.plx will be loaded into row B, and so on. More commonly, you should select a discovery panel from the panel drop down. In either case, a choice of PLX can be

overridden using the PLX button at the end of the row. The box to the right of the button lists the PLX file for that row.

17.1.5 If there are control samples in the bottom row using a PLX file that is not part of the discovery set, choose that PLX file and check the control box.

17.1.6 Assay negatives are designated using the Neg checkboxes at the top of the plate.

17.1.7 If there are wells that should be omitted, for instance because of known contamination, they can be removed using the bad well editor, brought up by selecting a plate on the plate selector:



17.1.8 Click OK and the plate(s) will be loaded and processed. When the analysis is finished, there will be two experiments, or three experiments if there is a control experiment.

17.1.9 The experiment "User Samples prior to joining" contains the samples prior to merging their probes, so each will have about 70 probes and there will be as many samples as there are wells used on the plate. The experiment has a block diagonal heatmap, because each sample only has a

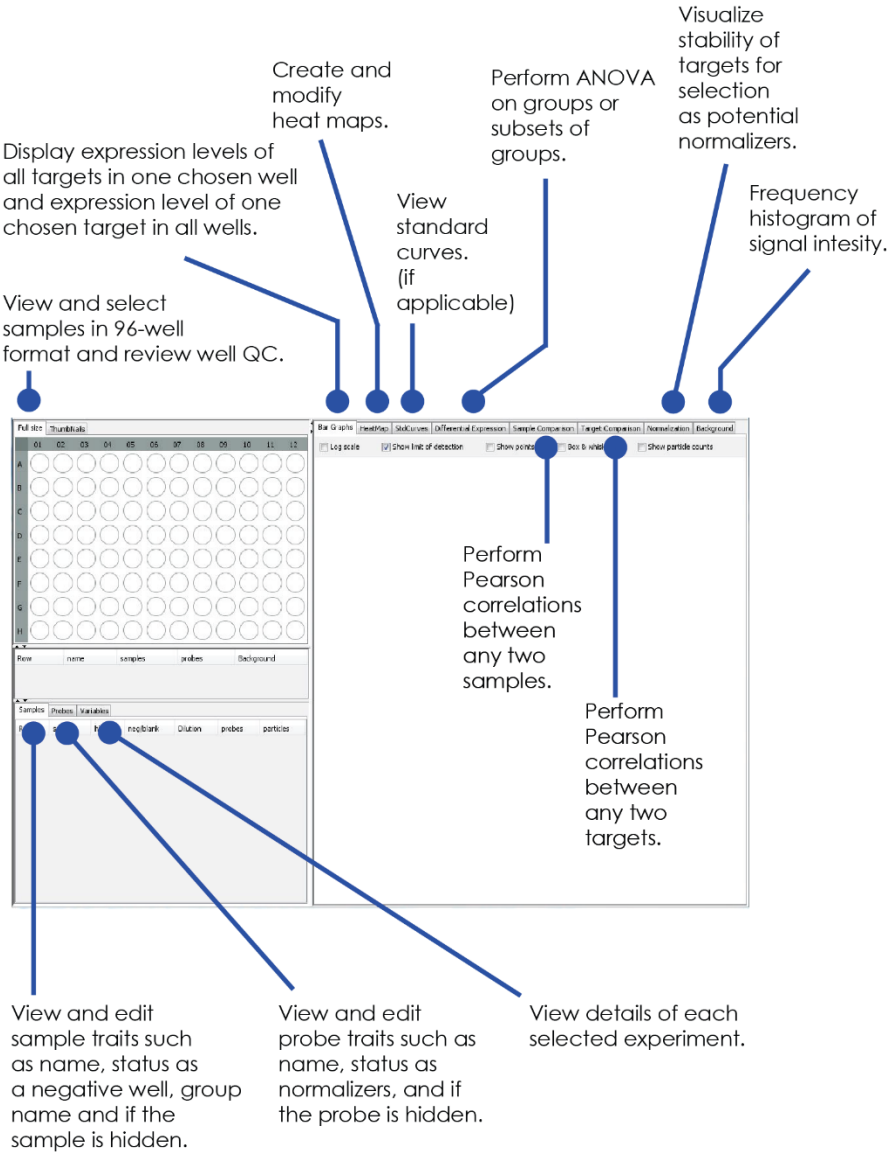
certain set of probes. The common probes appear as a vertical streak in the heatmap.

17.1.10 The experiment "User Samples after joining" contains the samples after negative subtraction, equalization and merging. It will have one sample for each column used on the plate, and about 400 probes.

17.1.11 For negative subtraction, the level of each probe in the negative wells is averaged and subtracted from the corresponding probe level in each well containing that probe.

17.1.12 After negative subtraction, a special normalization step called equalization is performed. The common probes in each row for a single biological sample are averaged and the average made the same between rows. After equalizing the row samples, they are merged to form a super-sample with 400 probes

17.2 Analyzing your results



17.2.1 Samples can be normalized using the division button on the top bar.

17.2.2 The bar graphs show all the probes in a selected sample, and all the samples for a selected probe. Samples are selected by clicking in the sample table. Probes are selected by clicking in the probe table.

17.2.3 The heatmap is a snapshot of all the probes and samples. Probes and samples can be clustered using the controls.

17.2.4 Any two samples can be correlated against each other using "Sample correlation" and any two probes can also be correlated against each other. A multi-bar graph of all the probes in selected samples can be visualized using "Sample comparison".

17.2.5 The normalization vector is shown in on the Normalization tab, and the stability of each probe, as defined by the geNorm algorithm, is presented.

17.3 Saving your results

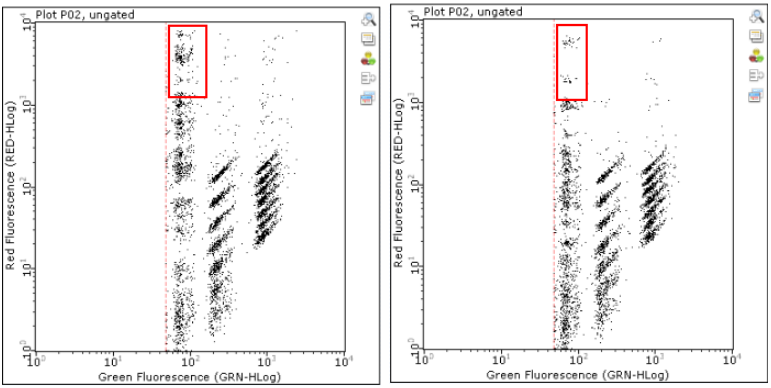
The export button  allows the data to be saved in either CSV or XLSX format. In both cases, there is one sample per row and one probe per column.

18. Troubleshooting

Problem	Reason	Solution
Low throughput in a subset of wells (i.e. less than 8 particles scanned per miRNA in a well)	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 FirePlex® Particles	Resuspend particles within 30 seconds of transferring. Use a multi-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.
	Too much Paraffin present in the sample	Ensure not to carry over excess Paraffin from the chilled FFPE sample after Lysis.
Leaking Filter Plate wells	Failure to blot plate	Be sure to blot plate after filtration.
	Wells tightly sealed	Cover the Filter 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; use a fresh Filter Plate.
	High background Probes	Probes for a few miRNAs may exhibit signal in negative controls. This is not a result of contamination and the only consequence is to limit the dynamic

		range of these rare probes. Please contact multiplex.fas@abcam.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 enhance signal.
	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 and continue with Fresh Particles
Incorrect resuspension/ acquisition volume	Insufficient particle concentrations in the well resulting from incorrect sample volume resuspensions/ different run buffers	Ensure that your machine can acquire and decode sufficient particles using resuspension and acquisition conditions that were determined using the Cytometer Setup Kit particles (ab211043).
High CoV values for Plasma/Serum samples	Debris in sample	Centrifuge samples at 2000 x g for 15min to clarify and sediment cell debris. This debris may contain additional RNAs that impact profiling of cell-free nucleic

		acids.
Precipitate is observed in Hyb Buffer	Hyb Buffer has frozen at 4°C	Warm the buffer to room temperature and mix thoroughly prior to proceeding with use.
Precipitate is observed in 2X Rinse Buffer B	2X Rinse Buffer B has frozen at 4°C	Warm the buffer to room temperature and mix thoroughly prior to proceeding with use.
Optimal Sample Input for Fold Change Discrimination:	Excess sample input	With excessive sample input, the fluorescent signals for some of the targets measured in an experiment may fall outside of the linear range of the assay. In cases where a large number of targets assayed in a digested sample show raw values in the top log of the cytometer acquisition range (see image below), it may be beneficial to dilute samples in step 14.1.7 of the 'Assay Procedure' 2 to 10-fold in RNase-free water.



On the left is an example Red vs. Green fluorescence scatter plot on a Millipore Guava EasyCyte™ 6HT for a serum sample, where several targets show raw values in the top log of the cytometer acquisition range. On the right is the same serum sample, where digest was diluted with RNase-free water.

19. Notes

Technical Support

For technical inquiries, contact FirePlexSupport@abcam.com