FirePlex®-384 Catalogue Panels Assay Protocol

1 x 384 test size

Protocol Booklet Version 4.1 September 2021

For quantitative measurement of human and mouse analytes in serum, plasma, and cell culture supernatant samples, using the FirePlex-384 platform.

This is a general FirePlex-384 assay protocol booklet. For panel-specific information, please refer to the Product Datasheets and Certificates of Analysis.

This product is for research use only and is not intended for diagnostic use. Please read entire protocol booklet prior to starting, as protocols are subject to updates.

Table of Contents

1. Overview

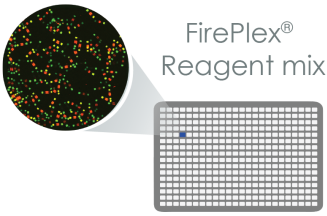
Abcam FirePlex-384® multiplex immunoassays utilize patented FirePlex particles for quantitation of up to 10 protein analytes from 6.25 µL of biological sample.

- Workflow format: two-step, no-wash
- Assay format: 384-well plate
- Assay readout: High-Content Imagers (for a list of all validated instruments, please refer to Section 18)
- Data analysis: FirePlex Analysis Workbench software

This booklet provides the recommended protocol for running the FirePlex-384 panels. For a full list of FirePlex-384 products, please refer to www.abcam.com

This kit is supplied with the required volumes of reagents to assay 1 x 384-well plate.

2. Protocol Summary

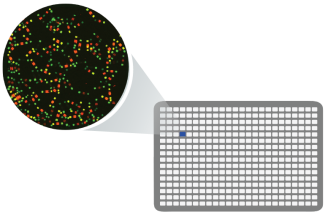


FirePlex®
Reagent mix

\+

Biological
samples

Shake overnight
at RT

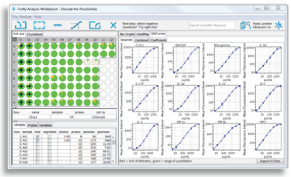


Analytes captured
onto fluorescently
labelled particles

Add imaging dye

Image particles on
high-content imager

Collect Data



Data analysis with
FirePlex® Analysis
Workbench Software

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been validated and quality controlled in functional assays.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens, handled with care, and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink, or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage

- Δ CAUTION! **Immediately upon receipt, please store each kit component at the temperature indicated in the Materials Supplied Section (Section 5).**
- Δ **Kits have a storage time of 6 months from date of receipt. Shelf life for individual kits can be found by referring to the specific product's certificate of analysis (CoA).**

5. Materials Supplied

Item	Quantity	Storage Condition Upon Receipt
Sample Diluent [S#]▲	1 X 25 mL	4°C
Reagent Diluent [R#]▲	1 X 8 mL	4°C
1X PBS	1 X 8 mL	4°C
5X Capture Particle Mix*▲	1 X 700 µL	-20°C
35X Detector Antibody Mix (Ex/Em 488-565/575 nm)*▲	1 X 110 µL	-20°C
Protein Standard Mix [HT#] (Lyophilized)▲	1 or 2 units	-20°C
Imaging Dye Concentrate*	1 X 875 µL	RT
384-well Imaging Plate	1 unit	RT
384-well Imaging Plate Seal	2 units	RT
384-well Imaging Plate Lid	1 unit	RT
*Light-sensitive reagent. Please protect from light, and store in a dark place when not in use.		
▲Please refer to product datasheet or CoA for the component corresponding to your specific panel.		

Δ **Note:** The FirePlex-384 Reagent Diluent, Sample Diluent, and Protein Standard mix are specific to a given catalogue number (i.e., *ab234897*). These components are annotated accordingly with the letters and numbers indicated in the square brackets (i.e., *FirePlex-384 Reagent Diluent [R1]*). The product-specific naming for your given component can be found by referring to the CoA or reagent label.

6. Materials Required, Not Supplied

REQUIRED MATERIALS AND EQUIPMENT	
Equipment	**Recommended models/Suppliers**
Orbital shaker (2-3 mm orbit) that holds microplates set to room temp, 1000 RPM	VWR 12620-926 or VWR 12620-930
Microtubes or microplate for dilution of standards and samples	200 – 1,500 µL volume
384-well compatible multi-channel pipette (working volume 2-125 µL)	—
Vortex	—
Microcentrifuge	—
Computer to run analysis software	Compatible with Windows, Mac OS, and Unix. Recommend 64-bit operating system with 64-bit Java and min. 8GB of memory (preferably 16GB)
High-Content Imager	See Section 18 for list of supported instruments. For all other instruments, please contact FirePlexSupport@abcam.com to assess instrument compatibility
OPTIONAL MATERIALS AND EQUIPMENT	
Equipment	**Recommended models/Suppliers**
Reagent Dispenser	Liquid handling instrument, such as ThermoFisher MultiDrop™ Combi
Additional Nunc 384-well Optical Bottom Plates, non-treated	ThermoFisher #242764
Additional Black Polystyrene Universal Microplate Lid	Corning #3935
Additional Adhesive Film for Microplates	—

7. Limitations

Modifications to the components or procedures may result in loss of performance.

8. Technical Hints

- When generating the protein standard samples, or performing serial dilutions of samples, pipette tips **must** be changed after each dilution step.
- All samples should be mixed thoroughly and gently. Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Protect FirePlex-384 Capture Particle Mix, Detector Antibody Mix, and Imaging Dye from light during incubation steps and storage.
- Avoid multiple freeze/thaw of protein standard and biological samples.

Δ For optimal assay performance, adequate mixing during incubation steps is critical and depends upon both speed and orbital diameter (Refer to Section 6). Users should determine the optimal mixing speed if using a shaker with orbital diameters that differ from those indicated in Section 6.

Δ CAUTION! Prior to running the assay for the first time, users are **required** to follow the instructions provided in the FirePlex-384 Imager Setup Kit (ab229130) to prepare their HCI. The validated imaging protocol obtained can then be used for imaging FirePlex particles.

9. Software Installation

- Analysis of the TIFF files generated from running FirePlex-384 assays requires installation of the FirePlex Analysis Workbench. This software can be downloaded from:

  https://www.abcam.com/kits/multiplex-immunoassays-firefly-analysis-workbench-software

- Please note that there are two versions of the FirePlex Analysis Workbench:
  - a Java based version which automatically downloads the newest version of software if one is available when the software is opened, or
  - a Windows based version which is updated manually by the user.
- For any assistance in selecting the software version best suited for your needs, or using the software, please contact FirePlexSupport@abcam.com

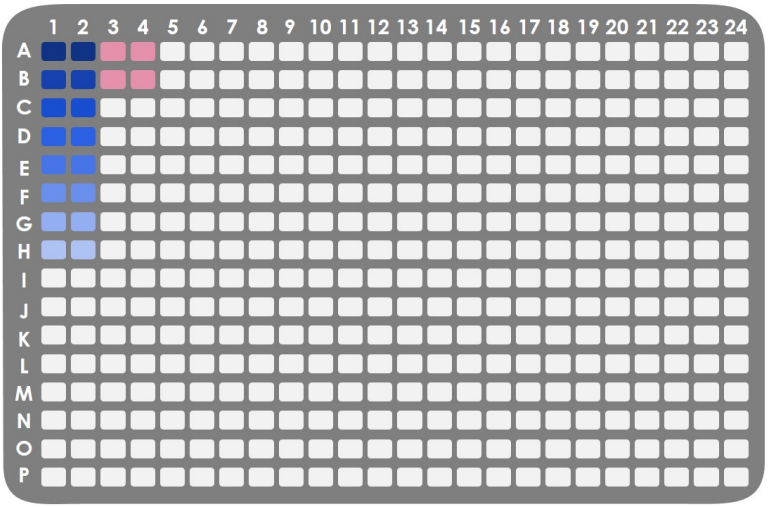
10. High-Content Imager Set Up

Δ CAUTION! Prior to running the assay for the first time, users are **required** to follow the instructions provided in the FirePlex-384 Imager Setup Kit (ab229130) to prepare their HCI. The validated imaging protocol obtained can then be used for imaging FirePlex particles.

11. Recommended Plate Layout

- For each assay performed, we recommend:
  - Designing your plate layout before starting the assay.
  - Assaying a minimum of two replicates (duplicates) per sample.
  - Including four blank control wells (see plate layout below).

The eight-point standard curve should be placed in two adjacent columns as shown in the suggested plate layout below. Placement of the standard curve in other locations on the plate can be easily accomplished, please contact FirePlexSupport@abcam.com for assistance in how to do this.



Standard Curve

Wells	Input	Wells	Input
A1-2	Standard 1	E1-2	Standard 5
B1-2	Standard 2	F1-2	Standard 6
C1-2	Standard 3	G1-2	Standard 7
D1-2	Standard 4	H1-2	Standard 8
A3-4, B3-4	Blank		

12. Standard Preparation

- The following section describes the preparation of an eight-point standard curve for duplicate measurements (**recommended**) on one (1) 384-well plate. Volumes indicated below are the minimum requirements. These volumes can be adjusted if the user-specific assay set-up requires accounting for additional overage volumes.
- Serially diluted standards should be prepared **immediately prior to use**. Take care to change tips when preparing serial dilutions.

Δ CAUTION! **Depending upon the analyte composition of the panel, the kit is supplied with either 1 or 2 vials of protein standard mix. Please pay special attention to instructions in step 12.1 as to how to prepare the protein standard mix.**

12.1 To prepare your protein standard mix:

12.1.1 Reconstitute the vial(s) of Lyophilized Protein Standard Mix [HT#] with the volume of **Sample Diluent [S#]** indicated in the table below.

# of Protein Standard Tubes	Volume required for reconstitution (µL)
1	250
2	125

12.1.2 Incubate at room temperature for 5 minutes and mix thoroughly.

Δ **Note:** If 2 vials of protein standard mix are supplied with the kit, first reconstitute each protein standard mix, and then combine equal volumes of the two tubes into a single mix. The concentration of this protein standard mix now corresponds to the concentration required for Standard #1.

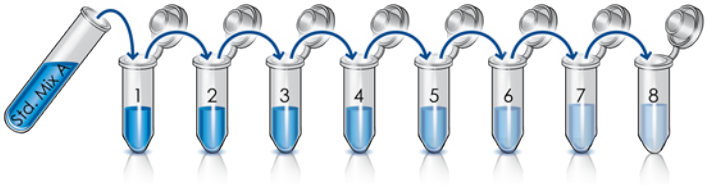
12.1.3 After resuspension the protein standard should be placed on ice.

12.2 Label eight microcentrifuge tubes **Standard #1-8** and add 40 µL of **Sample Diluent [S#]** to tubes #2-8.

Δ **Note**: We recommend using the above format for protein standard preparation to ensure optimal mixing. However, this can be modified to be performed in a multi-well plate if desired.

12.3 Transfer 60 µL of the Standard #1 stock solution prepared in Step 12.1 to the microcentrifuge tube labeled Standard #1. This volume of Standard #1 can be used to prepare one eight-point standard curve measured in duplicate, required for assaying one 384-well plate.

Any remaining Protein Standard Mix should be aliquoted and stored at -80°C.



12.4 Perform 7 serial dilution steps starting with Standard #1 transferring to tubes #2-8.

12.4.1 Transfer 20 µL from Standard #1 into the tube for Standard #2 containing 40 µL od Sample Diluent (3x dilution step total per tube).

12.4.2 Add 20 µL Standard #2 into the Standard #3 tube and vortex thoroughly to mix. Discard the pipet tip.

12.4.3 Repeat the serial dilution steps until you have reached the final tube #8. Vortex thoroughly to mix.

13. Sample Preparation

- For optimal assay performance, samples must always be used either at the recommended dilution or further diluted. Optimal sample dilutions should, however, be determined by the end user.
- Samples generating values higher than the highest standard should be further diluted with **Sample Diluent [S#]**.
- We recommend assaying all samples in duplicate.
- The following protocol can be used for cell culture supernatant (CCS), serum, and plasma collected using citrate, EDTA, or heparin.
- For help with sample optimization or for the use of other sample types not listed in this protocol booklet, please contact FirePlexSupport@abcam.com for assistance
- Store un-diluted biological samples at -80°C. Avoid repeated freeze-thaw cycles.

  Δ **Optional:** To clarify samples, centrifuge samples at 2,000 x g for 15 minutes. Transfer clarified sample to a new tube. Take care to transfer sample from the middle of the tube, avoiding the transfer of cell pellets or the upper lipid layer.

13.1 Dilute biological samples **2-fold into Sample Diluent [S#]** (i.e., 1:1 dilution by adding 15 µL biological sample to 15 µL sample diluent). Vortex thoroughly to mix. 12.5 µL of diluted sample input is required per well.

  Δ **Note**: 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).

Δ **Note: When adjusting the sample concentration for the dilution factor remember to account for the additional 2-fold dilution that occurs in the well. For example, if a sample is diluted 2-fold prior to adding to the assay plate then final in well dilution is 4-fold. Therefore interpolated concentrations need to be multiplied by 4 to determine the actual analyte concentration in the undiluted sample.**

14. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- It is recommended to assay all standards, controls, and samples in duplicate.
- For all pipette steps into 384-well plate we recommend using a multi-channel pipette, liquid handling robot, or dispenser.

Day One

14.1 Prepare 1X Capture Particle Mix

14.1.1 Add 2,800 µL of **Reagent Diluent [R#]** to supplied 5X Capture Particles to make 1X Capture Particles. Vortex thoroughly to mix.

14.2 Prepare 1X Detector Antibody Mix

14.2.1 Centrifuge 35X Detector Antibody Mix tube for 5 seconds at 1,000 x g.

14.2.2 To prepare 3500 µL of 1X Detector Antibody mix: Add 3400 µL of **Reagent Diluent [R#]** to a fresh tube, and then transfer 100 µL of the supplied 35X Detector Antibody Mix to the same tube. Vortex thoroughly to mix.

14.3 Prepare Reaction Mix

14.3.1 Use the table below to determine the volumes of 1X Capture Particle Mix and 1X Detector Antibody Mix needed depending on the number of wells to be run.

# of Wells	1X Capture Particle Mix (µL)	1X Detector Antibody Mix (µL)	Final Reaction Mix (µL)
1	8.125	8.125	16.25
48	390	390	780
96	780	780	1,560
192	1,560	1,560	3,120
288	2,340	2,340	4,680
384	3,120	3,120	6,240

14.3.2 Vortex tube containing 1X Capture Particle Mix thoroughly (~10 sec) and transfer the required volume to a new tube.

Δ CAUTION! Take care to vortex particle mix thoroughly before transfer to prevent particle settling.

14.3.3 Next, vortex tube containing 1X Detector Antibody Mix thoroughly (~10 sec) and transfer the required volume to the tube containing 1X Capture Particle Mix.

14.4 Vortex the Reaction Mix thoroughly and transfer to a trough. Add 12.5 µL of reaction mix to each well of a 384-well plate.

Δ CAUTION! Take care to mix Reaction Mix as you go to prevent particle settling. If using a trough, gently rotate back and forth 3-4 times before every pipette step.

14.5 Add 12.5 µL of Standard or Sample (diluted according to instructions in Sections 12 and 13, respectively) to each well. Add 12.5 µL of **Sample Diluent [S#]** to the four blank wells.

14.6 Cover the plate with the supplied black Imaging Plate Lid and shake at 1,000 rpm overnight (12-18 hours) at room temperature. We recommend sealing plates using one of the supplied 384-well Plate Seals for the overnight incubation.

Δ CAUTION! During overnight incubation, ensure plates are protected from light by using either the supplied black Imaging Plate Lid, or an opaque plate seal.

Day Two

14.7 Prepare Imaging Dye

14.7.1 **Immediately prior to use,** prepare the Imaging Dye working solution. To do this, add 4125 µL of 1X PBS to the supplied tube of Imaging Dye Concentrate. Vortex thoroughly before use.

- Δ **Note:** Any remaining Imaging Dye working solution can be stored at RT in the dark and used again, **for up to 4 weeks.**

14.8 After the overnight incubation, remove the assay plate from the shaker and gently remove the Plate Seal.

- Δ **Note:** If condensation is apparent on the Plate Seal, the assay plate can be centrifuged prior to removal of the plate seal.

14.9 Add 10 µL of Imaging Dye Working Solution to each well, and re-seal the plate using a new Plate Seal.

- Δ **Note:** Ensure that the edges of the plate are clear of any liquid prior to sealing, to ensure a tight and even seal.
- Δ **Note:** If transferring Imaging Dye Working Solution to a trough, use a pipette for sufficient transfer (do not pour).

14.10 Shake plate for 20 minutes at room temperature, at 1,000 rpm.

- Δ **Note:** If a Plate Seal was not used, remove any condensation that has built up on the lid using a Kimwipe prior to replacing lid on the plate.

14.11 Remove plate from orbital shaker and allow particles to settle for ~5 minutes.

14.12 Wipe bottom of imaging plate with a Kimwipe and then image on High-Content Imager.

15. Particle Image Acquisition

15.1 Ensure that the bottom of your imaging plate is free from dust (wipe with Kimwipe if necessary). Load plate into your imager.

Load the imaging protocol that you have previously validated on your machine from having used the FirePlex HT Imager Setup kit (ab229130). This method should have Z-axis, excitation and emission filters, and general exposure times that will generate readable TIFF images that can be successfully converted into *.fci* files for use in the FirePlex Analysis Workbench (FAW)

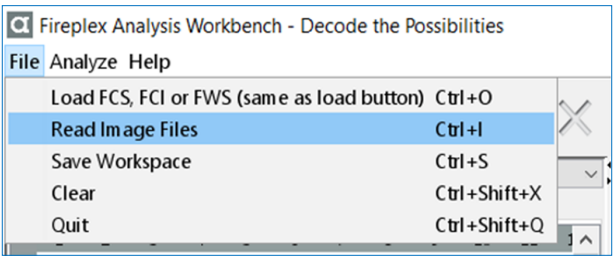
- Δ **Note:** Prior to imaging the whole plate, it is highly recommended to test a single well, (select a well containing the highest positive control) to ensure that the settings still obtain fluorescence within the linear range for each channel as determined when the Imager Setup Kit was run.

16. Data Analysis using FirePlex Analysis Workbench

16.1 Launching Software and Opening Image Files

16.1.1 Open FirePlex Analysis Workbench (FAW).

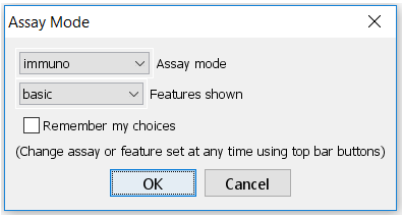
16.1.2 Open the "File" menu and click "Read Image Files



16.1.3 If launching the software for the first time, an "Assay Mode" window will appear. From the "Assay Mode" dropdown menu, select the "immuno" option to activate the immunoassay analysis features.

Δ **Note:** Steps 17.1.3-17.1.6 are only required the first time the software is launched. For future data analyses, users can proceed directly to Step 17.1.7.

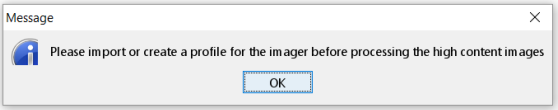
16.1.4 In the "Features shown" dropdown menu, keep the "basic" mode selection.



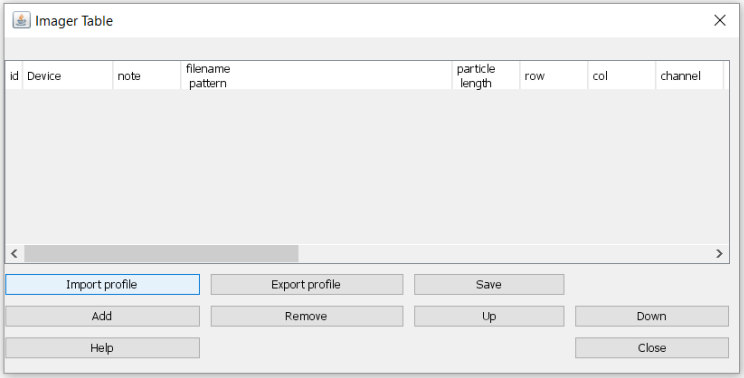
16.1.5 If desired, check "Remember my choices" so that FAW remembers your choice.

16.1.6 Click "OK" when done.

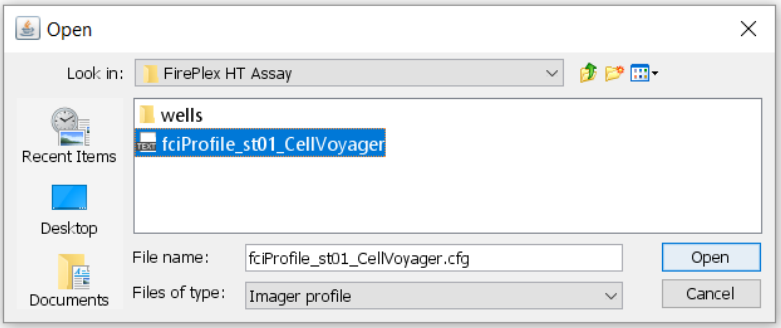
16.1.7 A warning message will appear to instruct you to "Import a High Content Imager Profile". Click OK.



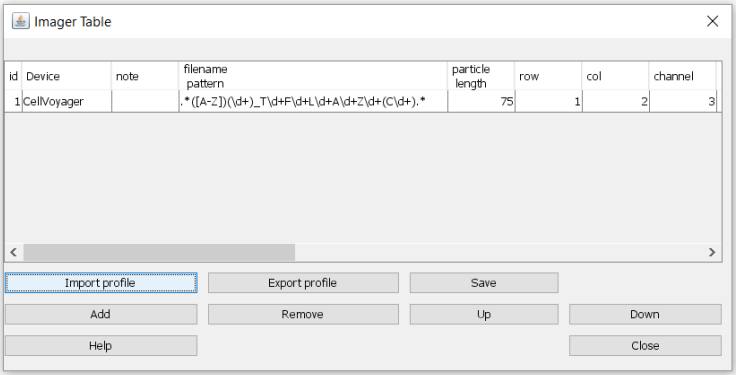
16.1.8 An “Imager Table” window will open.



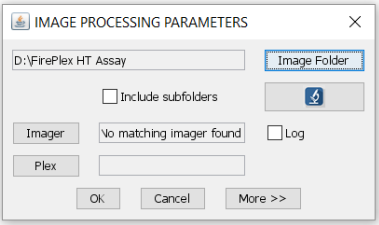
16.1.9 Click the “Import Profile” button and then locate the “fciProfile_XXXX.cfg” configuration file you have been provided. This *.cfg* configuration file enables conversion of the TIFF images into a compressed *.fci* file that is read by the FAW for decoding and quantitation analysis. Highlight the *.cfg* file and click “Open”.



16.1.10 The Imager Table will now be populated with information from the *.cfg* file. Click "Close" once you see your machine's name and information present.

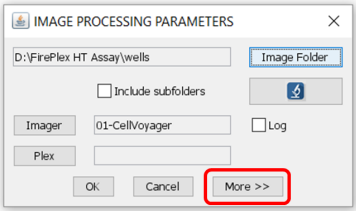


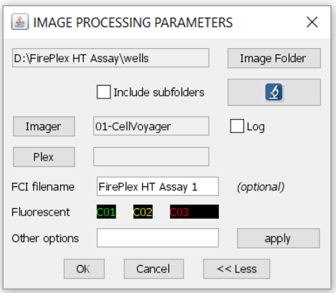
16.1.11 The "Image Processing Parameters" window will open.



16.1.12 Click the "Image Folder" button, locate and select the folder where you have saved your exported TIFF files for the experiment and then click "Open".

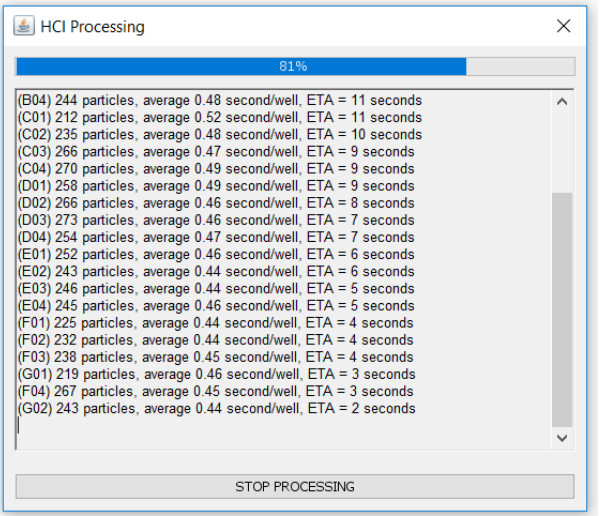
16.1.13 Your Image Processing Parameters window will now populate the Imager window. Click the "More" button to expand the window to allow file naming for your *.fci* file. You can now enter text into the field labeled "FCI filename". Enter a name for your *.fci* file here, otherwise the file will automatically receive a timestamped filename (e.g. "20171114142359.fci).





16.1.14 Following the image analysis a *.fci* file will be generated. This file can be uploaded into FAW for easy access to the analyzed image data. The FCI file will be saved in the folder with the TIFF images with the default date and time filename, e.g. "20171114142359.fci".

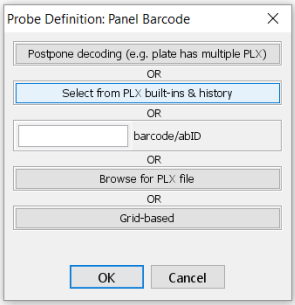
16.1.15 A HCI processing window will then appear which displays the progress of the image analysis as shown below.



16.2 Specifying the Analyte Panel (Plex File)

The analytes used in the multiplex panel and their code positions are specified in a panel file, with suffix ".plx", also known as a PLX file.

16.2.1 When the image analysis is complete, and if a Plex file had not been specified earlier, the "Probe Definition: Panel Barcode" window will appear.



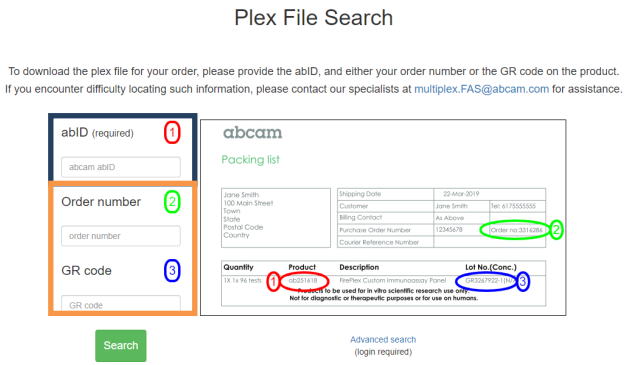
16.2.2 If the panel being run is a standard kit as shown on the Abcam website, then the Plex file will already be preloaded into the software.

16.2.3 If a SelectPlex panel is being run, the Plex file for the HT SelectPlex products can be accessed as follows:

16.2.4 Go to https://www.abcam.com/fireplexht, and scroll to the section titled "Pre-designed and custom panels", and click on the link "access your FirePlex-384 SelectPlex plex file here"

16.2.5 You will be redirected to a page to enter the abID, and either the Order Number or GR code for your Custom Panel in the corresponding text boxes (see screenshot below)

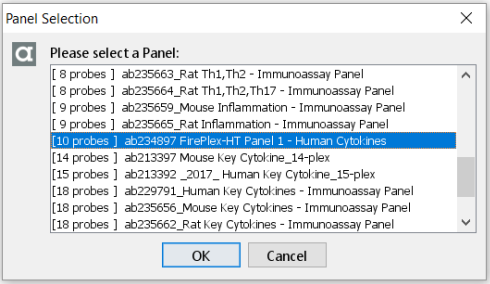
Δ **Note:** Instructions and screenshots on the page will indicate where the abID and Order Number can be found.



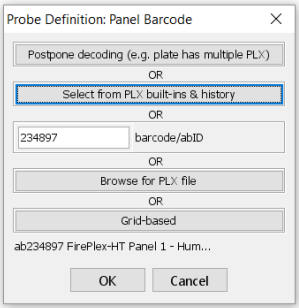
16.2.6 The plex file can be downloaded by clicking on the hyperlink under the "Filename" column.

Δ **Note:** You can also access the Certificate of Analysis (CoA) for your SelectPlex panel by clicking on the hyperlink under the "CoA" column of this table.

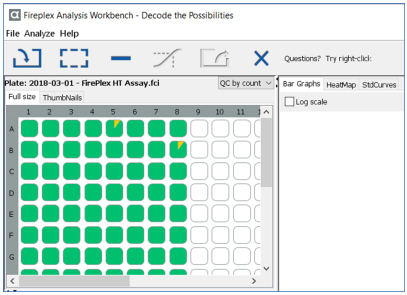
16.2.7 Click the "Select from PLX built-ins" button and locate the PLX file for your HT Assay Kit (For example: ab234897 FirePlex-384 Panel 1 – Human Cytokines). If you are using a Custom FirePlex-384 assay, click the "Browse for PLX file" button and locate where the PLX file is saved and select it for import.



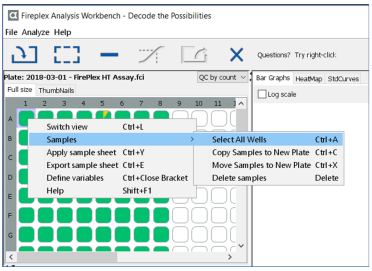
16.2.8 Click “OK” to return to the “Probe Definition” screen, where the PLX file name will be visible beneath the “Browse for PLX file” button. Click OK.

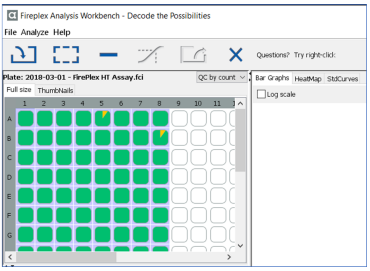


16.2.9 The plate data will load and present in a plate orientation display on the left side of the FAW screen.

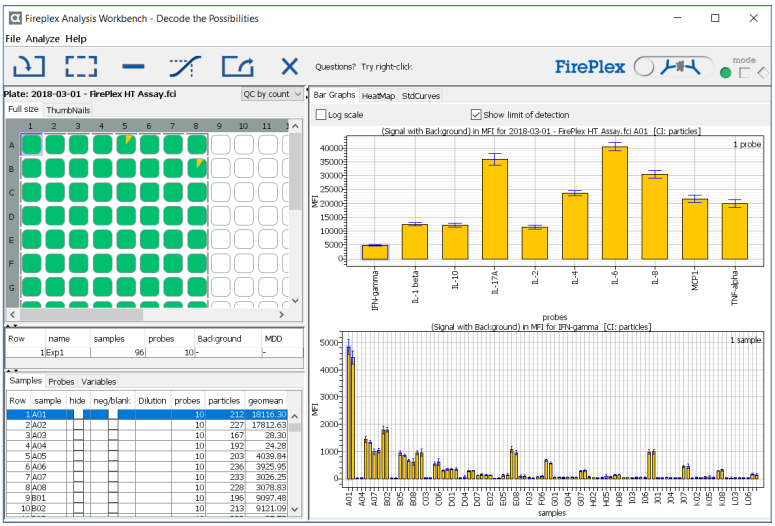


16.2.10 Using the Right-click button of your mouse, open the mouse menu that shows “Sample”, then highlight “Select All Wells”. All of the wells on the plate will now be highlighted with a light blue color. Alternatively, you can use the “Ctrl+A” shortcut keystroke on your keyboard. Locate the “Make Experiment” icon and use the Left-click button of your mouse to activate this option.





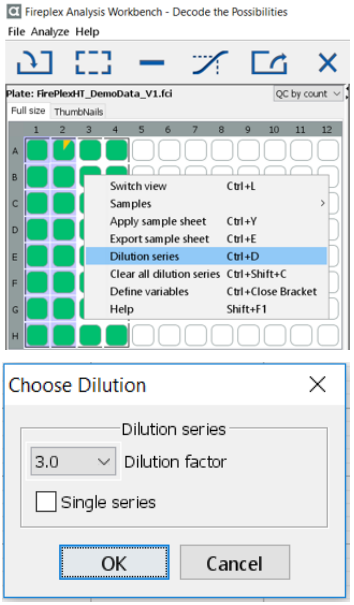
16.2.11 All of the highlighted wells will now display their fluorescence reporter values. Bar graphs representing signal values will appear on the screen on the right side of the FAW.



Δ **Note:** Raw Mean Fluorescence Intensity (MFI) data can be directly exported from your experiment at this point. To do so, skip to Section 17.4 of this booklet. To use the built-in standard curve analysis in the FAW, proceed to Section 17.3 and follow the instructions.

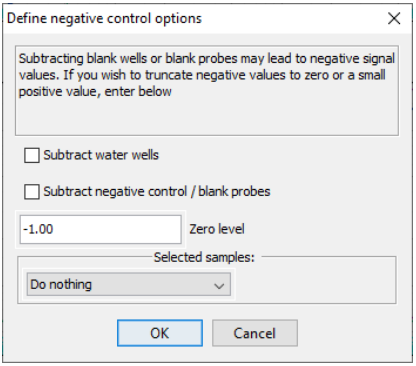
16.3 Assigning Standard Curves and Control Wells

16.3.1 Left-click on the plate to highlight all of the wells that contain the standard curve dilution points (e.g. A01 to H02). Once highlighted, Right-click on the plate with your mouse and select the "Dilution series" option. A "Choose Dilution" window will appear. Make sure 3.0 is chosen in the "Dilution Factor" dropdown menu. Click OK when done.

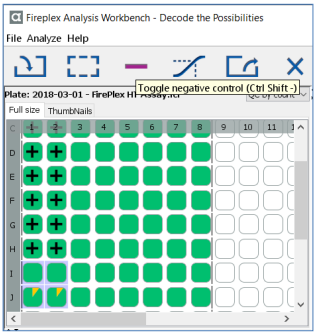


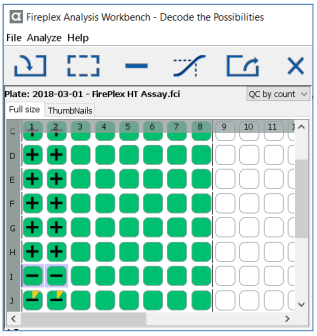
16.3.2 The wells that were highlighted in step 17.3.1 will automatically be labeled as standard curve wells and will be labeled with (+) signs ⊕.

16.3.3 Highlight all your Blank/Negative control wells with the left-click button on your mouse. Click on the "Toggle negative control" icon ▬. A pop-up window appears where you can define specific settings for your negative controls (you can also clear negative controls through this window). Select the desired settings and press OK.



The negative control wells will be labeled with (-) signs ⊖.





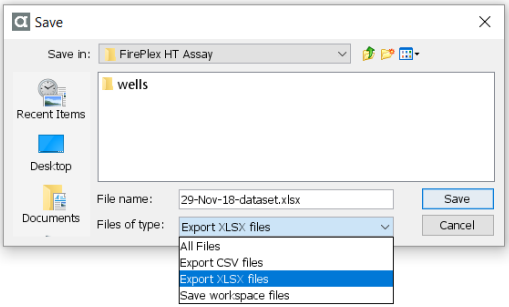
16.3.4 All of the wells will now display their concentration units in "pg/ml" in the bar graph windows.

Δ **Note: When adjusting the sample concentration for the dilution factor remember to account for the additional 1:2 dilution that occurs in the well. For example, if a sample is diluted 2-fold prior to adding to the assay plate then final in well dilution is 4-fold dilution. Therefore interpolated concentrations need to be multiplied by 4 to determine the actual analyte concentration in the undiluted sample.**

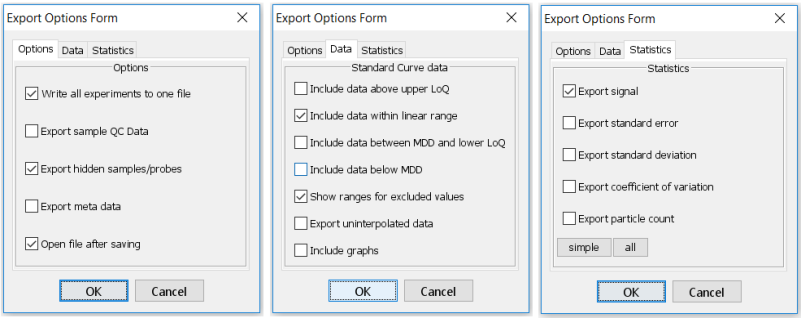
16.4 Data Export

16.4.1 To export the analyzed data, locate the "Save workspace or export all experiments to CSV file(s)" icon from the toolbar, and click the icon. This will open a "Save" window, where you will be able to specify a file name and save location for your experiment's data export file.

16.4.2 The standard export format is comma-separated value (.csv) file that will read in Microsoft Excel, but with no formatting. To export the data report to a formatted Excel document (recommended), locate the "Files of type" dropdown and select the "Export XLSX files" option. The FAW program will remember your .xls or .csv selection preference for future data exports. Click the "Save" button.



16.4.3 An “Export Options Form” window will appear that has 3 tabs (Options, Data, Statistics). It is recommended to keep the 3 ticked options as they are, but you may select as many data export options as needed for your analysis. Click OK when done to generate the Excel file with your experimental data in pg/ml units.



17. Supported High-Content Imagers

The table below contains the recommended specifications for HCI instruments, as well as list of instruments currently validated for use with FirePlex-384 Immunoassays. To evaluate the compatibility of other instruments, please contact our technical support team at FirePlexSupport@abcam.com.

17.1 General Recommended Imager Specifications:

Field of View	*Detection*	*Color Channel*	*Excitation*	*Emission*
Entire Area of single Well on a 384 plate	16-bit CMOS or sCMOS Camera(s)	Green	440-500 nm	500-535 nm
		Yellow	490-565 nm	570-630 nm

17.2 Compatible Imagers for FirePlex-384 Assay:

For the most up-to-date list of validated High-Content Imagers, please go to:

https://www.abcam.com/kits/fireplex-384-immunoassays

Or contact FirePlexSupport@abcam.com

18. Notes

Technical Support

For all FirePlex-related technical support inquiries, please contact FirePlexSupport@abcam.com