FirePlex®-384 TGF-beta (Human, Mouse, Rat, NHP) Immunoassay Panel Protocol

Protocol Booklet Version 2.1 November 2018

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

This assay protocol is applicable to the FirePlex-384 TGF-beta panel. 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

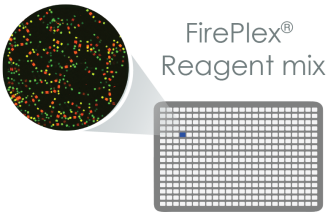
FirePlex-384® multiplex immunoassays utilize patented FirePlex particles for quantitation of up to 10 protein analytes from 6.25 µL of biological sample.

- 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 Human 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 1x384-well plate.

2. Protocol Summary



FirePlex® Reagent mix

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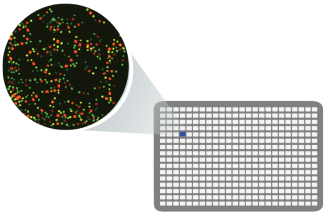
Biological samples

Shake overnight at RT

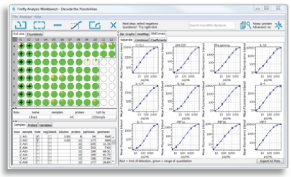


Analytes captured onto fluorescently labelled particles

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

- **CAUTION! Immediately upon receipt, please store each kit component at the temperature indicated in the Materials Supplied Section (Section 5).**
- **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
FirePlex-384 Sample Diluent S1	1 unit (25 ml)	4°C
FirePlex-384 Reagent Diluent R2	1 unit (8 ml)	4°C
FirePlex-384 – 1X PBS	1 unit (8 ml)	4°C
FirePlex-384 5X Capture Particle Mix*▲	1 unit (700 µl)	-20°C
FirePlex-384 35X Detector Antibody Mix (Ex/Em 488-565/665 nm)* ▲	1 unit (110 µl)	-20°C
FirePlex-384 TGF-ß Protein Standard Mix (Lyophilized)▲	1 unit	-20°C
FirePlex-384 – Imaging Dye Concentrate*	1 unit (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.		

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 - 1500 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 Multiplex.FAS@abcam.com to assess instrument compatibility
1N (1 M) HCl	Sigma-Aldrich H9892 or equivalent—
NaOH	Sigma-Aldrich S8045 or equivalent—
HEPES	Sigma-Aldrich 54457 or equivalent—

OPTIONAL MATERIALS AND EQUIPMENT	
Equipment	**Recommended models/Suppliers**
Reagent Dispenser	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

- Before running this assay on a given High-Content Imager (HCI) for the first time, users **must** perform a test run of the particles using the FirePlex-HT Imager Setup Kit (ab229130).
- 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.
- 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.

9. Software Installation Guide

We recommend installing the FirePlex Analysis Workbench software onto your computer before beginning the assay. The software is required to check that your HCI settings are correct and for data analysis.

9.1 First Time Use

9.1.1 Ensure Java is installed on your machine. Under Windows, typing Java to the start menu should bring up a variety of java-related options. On a Mac, System Preferences should show a Java icon. If not present, Java can be downloaded from
https://java.com/en/download/
If you have a 64-bit machine it is advantageous to use the 64-bit version of Java, but the 32-bit version will also work if that's what you have installed already.

9.1.2 To download the workbench, go to
http://www.abcam.com/kits/multiplex-immunoassays-firefly-analysis-workbench-software

9.1.3 Click "Download the software" button.

9.1.4 Clicking the button downloads a short Java web-start script and launches the script.

9.1.5 The script will download FirePlex Analysis Workbench (FAW) to your desktop.

9.1.6 The above steps should be performed on the computer used for data analysis. It is not necessary to download the workbench on the computer directly connected to the High-Content Imager.

9.2 Subsequent Use

9.2.1 Whenever the FirePlex Analysis Workbench is launched, the application will download the newest version if available.

9.2.2 An internet connection is not needed for subsequent use, except for update purposes.

9.3 Troubleshooting

9.3.1 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 web browser and double-click the "firecode.jnlp" file to download and launch the software.

9.3.2 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.

9.3.3 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.

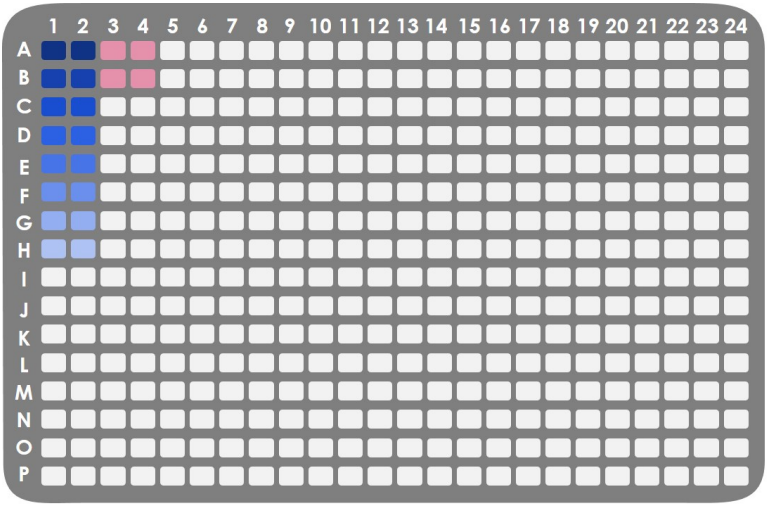
9.3.4 On some systems, Java Web Start may ask for permission to access the Internet to check for a new version of Java. Although not required for the Analysis Workbench unless your Java version is older than 2006, it is recommended to stay up to date for security purposes.

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-HT 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 by altering the default settings within FirePlex Analysis Workbench software. Please refer to the Software section (Section 17) of this protocol booklet for how to accomplish 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.
- Unlike some biological sample types, the protein standard mix does not require acid/base treatment before use.

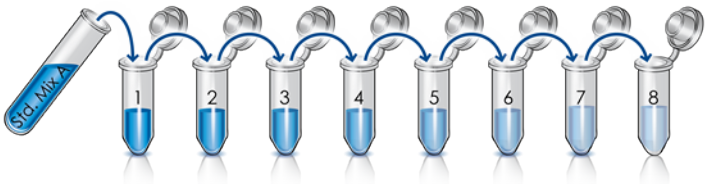
12.1 Reconstitute 1 vial of lyophilized TGF-ß Protein Standard Mix with the volume of **Sample Diluent S1** indicated on the tube. The concentration in this vial now corresponds to the concentration required for Standard #1.

Incubate at room temperature for 5 minutes and mix thoroughly. 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 S1** 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 Transfer 20 µL from Standard #1 into the tube for Standard #2 and vortex thoroughly to mix. Then, add 20 µL from the tube for Standard #2 into the Standard #3 tube, etc., as indicated in the image above, making sure to switch pipette tips after each transfer. Continue until you have reached the final tube, having transferred 20 µL from tube #7 into tube #8. **Mix well between each step.**

13. Sample Preparation

13.1 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. This panel is a multi-dilution panel in serum/plasma where TGF-ß 1 requires at least a 1:100 final in-well dilution and TGF-ß 2 and 3 require at least a 1:4 final in-well dilution to be in the linear range of the assay. In cell culture supernatants, we recommend a 1:4 final in-well dilution for all analytes.

- Samples generating values higher than the highest standard should be further diluted with **Sample Diluent S1**.
- We recommend assaying all samples in duplicate.
- The following protocol can be used for cell culture supernatant (CCS), urine, bronchoalveolar lavage and serum/plasma samples collected using citrate, EDTA, or heparin.
- Animal serum used in the preparation of cell culture media may contain high levels of latent TGF-β. For best results, do not use animal serum for growth of cell cultures when assaying for TGF-β production. If animal serum is used as a supplement in the media, precautions should be taken to prepare the appropriate control and run the control in the assay to determine the baseline concentration of TGF-β.
- Note that serum, plasma, urine, cell culture supernatants and bronchoalveolar lavage (BAL) samples require acid/base treatment before use (instructions see below)!
- Acid/base treatments require preparation of a 1N HCl solution and 1.2 N (1.2 M) NaOH/0.5 M HEPES solution.
- Cell and tissue lysates do not require acid/base treatment because the latency peptide is not yet cleaved from the pro-form of the protein.

– Do not acid activate/neutralize the standards or blanks!
– Store un-diluted biological samples at -80°C. Avoid repeated freeze-thaw cycles.

13.2 To clarify serum and plasma samples, centrifuge samples at 2,000 x g for 15 minutes. To clarify cell and tissue lysates from cell debris, centrifuge samples at 10,000 X g for 5 minutes. Transfer clarified sample to a new tube.

Δ **Note**: Transfer serum/plasma sample from the middle of the tube, taking care to avoid transferring cell pellets or the upper lipid layer.

13.3 **Serum, plasma, urine, culture media/cell culture supernatants and bronchoalveolar lavage (BAL) samples require acid/base treatment before use:**

- To 25 µL of biological sample, add 6.25 µL of 1 N (1 M) HCl and mix well.
- Incubate 10 minutes at room temperature.
- Neutralize the acidified sample by adding 6.25 µL of 1.2 N (1.2 M) NaOH/0.5 M HEPES.
- Mix well and dilute immediately in S1 sample buffer as follows:
- For 25% (1:4) final in-well dilutions, add 12.5 µL of S1 diluent to the sample. This results in a 50% (1:2) dilution before adding the sample to the assay. 12.5 µl of diluted sample input is required per well.
- For 1% (1:100) final in-well dilutions, we recommend (for most accurate pipetting) to take 2 µl of the 1:2 diluted sample from above to add to 48 µl of S1diluent to create a 2% (1:50) dilution before adding the sample to the assay. 12.5 µL of diluted sample input is required per well.

If larger amounts of sample are available, the following table can be used as reference to adjust the acid/base treatment procedure to larger volumes:

Starting Sample Volume [µl]	I M HCl [µl]	1.2 M NaOH/0.5M HEPES [µl]
100	25	25
50	12.5	12.5
25	6.25	6.25

Be sure to dilute samples appropriately further in S1 diluent to reach the desired final in-well dilution.

13.4 Cell and tissue lysates do not require acid/base treatment prior to addition to the assay. Dilute those sample types **1:2 into Sample Diluent S1** (i.e. add 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**: When determining the total volume of diluted sample required, we recommend accounting for a minimum 10% overage.

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 1:2 prior to adding to the assay plate then final in well dilution is 1:4. 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 2800 µL of **Reagent Diluent R2** to supplied 5X Capture Particles to make 1X Capture Particles. Vortex thoroughly to mix.

14.1.2 Store any remaining 1X Capture Particle Mix at 4°C. Protect from light.

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 R2** to a fresh tube, and then transfer 100 µL of the supplied 35X Detector Antibody Mix to the same tube. Vortex thoroughly to mix.

14.2.3 Store any remaining 1X Detector Antibody Mix at 4°C. Protect from light.

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	1560
192	1560	1560	3120
288	2340	2340	4680
384	3120	3120	6240

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. If using a

Multidrop™ Combi reagent dispenser for liquid transfer, refer to Section 15.

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 S1** 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.

Note: Any remaining Imaging Dye working solution can be stored at RT in the dark and used again, **for up to 1 week**.

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. If using a Multidrop™ Combi reagent dispenser for Imaging Dye addition, see Section 15.

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 ~2 minutes.

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

15. Settings for Multidrop™ Combi Reagent Dispenser

- A Multidrop™ Combi can be used for addition of the Reaction Mix and the Imaging Dye working solution into the 384-well plate. This protocol is only recommended if using the volumes required for a full 384-well plate.
- A small plastic tubing cartridge is necessary for use with this product.
- It is recommended to test this protocol first with water to confirm you have the appropriate reagent volumes to meet the dead volume requirements of your specific instrument.

15.1 Prior to each run, prime the tubing with water.

15.2 Choose the default protocol option on the main menu and then enter the settings indicated in the table below.

	Reaction Mix	Imaging Dye Working Solution
Plate type	384 standard (15mm)	384 standard (15mm)
Volume and cassette	12.5 µL small cassette	10 µL small cassette
Column Selection	Full plate	Full plate
Predispense	130 µL	130 µL

15.3 Vortex the Reaction Mix or Imaging Dye working solution thoroughly and then transfer the entire volume to a 50 mL conical tube.

Δ **Note:** You must use the Reaction Mix volume required for a 384 well plate as indicated in the table in step 14.3.1.

15.4 To insert an air gap into the tubing, press the prime button twice without the tubing in a solution.

15.5 If dispensing the Reaction Mix, vortex or mix the 50 mL tube thoroughly immediately prior to dispensing. Then, place the tubing into the 50 mL tube and make sure the tubing sits at the bottom of the tube and press start.

Δ CAUTION! Take care to mix the Reaction Mix immediately prior to dispensing to prevent particle settling.

15.6 After the run is complete, perform a visual confirmation that the solution was added to all the wells of the plate.

15.7 Hold down the empty button to dispense the remaining solution in the tubing back into the source 50 mL tube.

15.8 Clean the tubing by first priming the tubing with water, then with ethanol, and then with water.

15.9 Between uses, store the tubing as indicated in the manufacturer's instructions.

16. Particle Image Acquisition

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

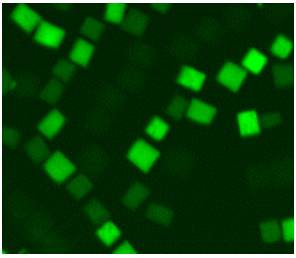
16.2 Load the imaging protocol that you have previously validated on your machine from having used the FirePlex HT Imager Setup kit (ab229130). This method should have Z-axis, excitation and emission filters, and general exposure times that will generate readable TIFF images that can be successfully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

16.3 Before imaging your plate on a given High-Content Imager for the first time, perform an imaging test on one of the Standard 1 control wells (e.g. well A01 that has highest standard curve

reporter signal). This step **does not** need to be repeated for subsequent image acquisitions on the same instrument.

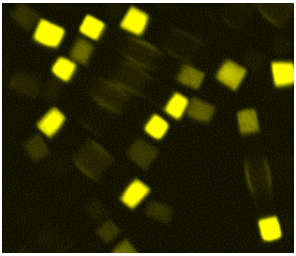
16.3.1 Using the 4x-5x magnification objective and the **Green fluorescence** excitation and filter set (e.g. **Blue Light excitation** [440-500 nm LED] with **Green emission** [515-535 nm]), confirm that the Z-axis offset is set such that the particles are in focus. If needed, adjust the Z-axis offset so that the fluorescent rectangular green sections are sharp.

16.3.2 Test the Green fluorescence exposure and confirm that the 3 distinct levels of Green fluorescence present on every particle are visible. The two ends of the particle will have high and medium Green fluorescence, respectively; the center particle region will have low Green fluorescence (see image below).

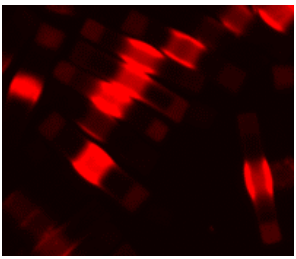


Δ **Note:** Exposures should be adjusted such that the particle regions with high Green fluorescence have pixel values well-below the saturation point for the HCI instrument being used (e.g. if your machine has a 65k gray-scale range, target the highest Green fluorescence to be between 15k-20k gray-scale value). Exposure times should be limited to <1 second for this parameter.

16.3.3 Test the **Yellow fluorescence** detection using an appropriate excitation and emission filter set (e.g. **Blue Light excitation** [440-500 nm LED] with **Yellow emission** [570-630 nm]). Yellow fluorescence will primarily be on the ends of the particles (see image below). Locate the brightest Yellow regions and tune the exposure to yield gray-scale values that are about half the saturation max of your machine (e.g. if your machine has a 65k gray-scale range, target the highest Yellow fluorescence to be between 30k-40k gray-scale value).

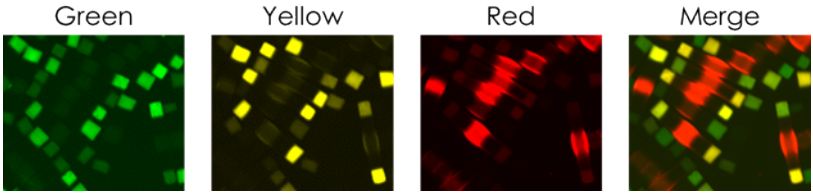


16.3.4 Adjust the **Red fluorescence** detection using an appropriate excitation and emission filter set (e.g. **Green/Yellow Light excitation** [490-565 nm LED] with **Red emission** [650-760 nm]). Red fluorescence will be focused in the middle section of some of the particles (see image below). Locate the brightest Red regions and tune the exposure to yield gray-scale values that are about half the saturation max of your machine (e.g. if your machine has a 65k gray-scale range, target the highest Red fluorescence to be between 30k-40k gray-scale value).



16.3.5 Ensure that Z-axis offset values that you have work for each fluorescent parameter.

16.3.6 Acquire a test image all 3 colors of the Standard 1 well. All 3 fluorescent images should be below pixel saturation and should appear similar to the images below.



16.4 Highlight all the wells of your plate you wish to acquire and initiate a full scanning protocol.

16.5 Export all the TIFF files for conversion into *.fci* files using the FirePlex Analysis Workbench.

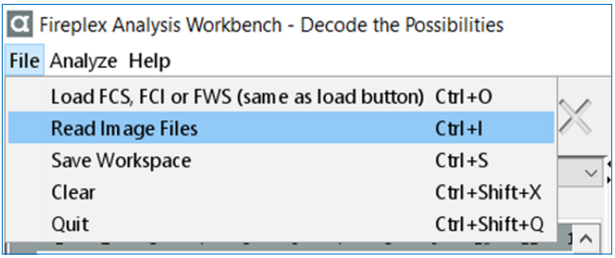
16.6 Store the imaging plate at RT, protected from light. The same plate can be re-imaged up to another 12 hours after imaging dye is added, if needed. Repeat steps 14.10 to 14.11 before re-imaging the plate to redistribute the particles across the plate.

17. Data Analysis using FirePlex Analysis Workbench

17.1 Launching Software and Opening Image Files

17.1.1 Open FirePlex Analysis Workbench (FAW).

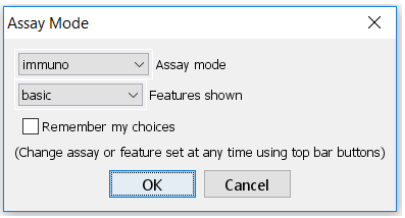
17.1.2 Open the "File" menu and click "Read Image Files



17.1.3 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.

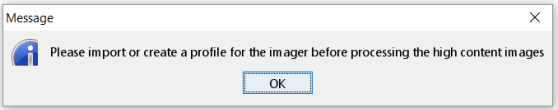
17.1.4 In the "Features shown" dropdown menu, keep the "basic" mode selection.



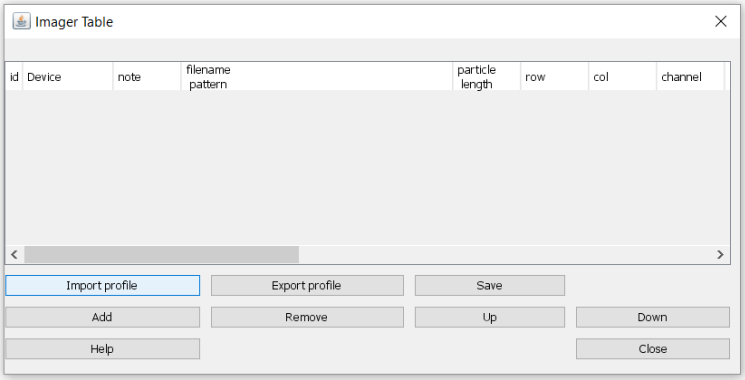
17.1.5 If desired, check "Remember my choices" so that FAW remembers your choice.

17.1.6 Click "OK" when done.

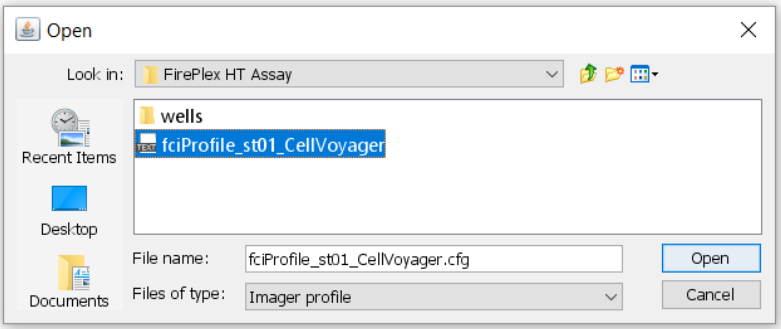
17.1.7 A warning message will appear to instruct you to “Import a High Content Imager Profile”. Click OK.



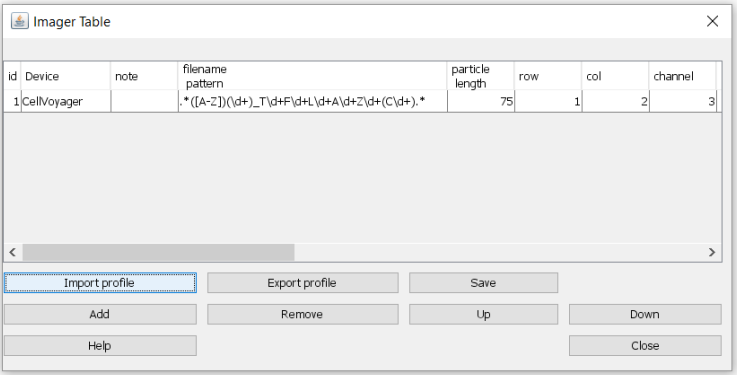
17.1.8 An “Imager Table” window will open.



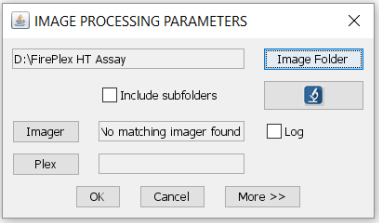
17.1.9 Click the “Import Profile” button and then locate the “fciProfile_XXXX.cfg” configuration file you have been provided. This *.cfg* configuration file enables conversion of the TIFF images into a compressed *.fci* file that is read by the FAW for decoding and quantitation analysis. Highlight the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



17.1.10 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.

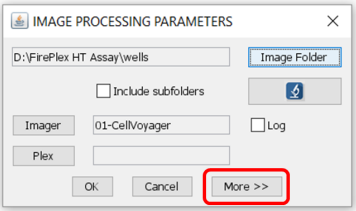


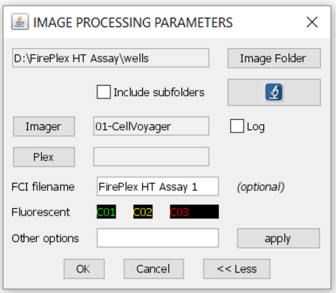
17.1.11 The "Image Processing Parameters" window will open.



17.1.12 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".

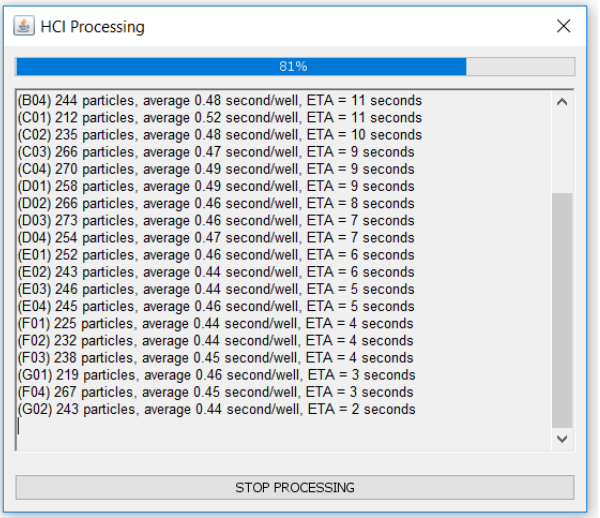
17.1.13 Your Image Processing Parameters window will now populate the Imager window. Click the "More" button to expand the window to allow file naming for your *.fci* file. You can now enter text into the field labeled "FCI filename". Enter a name for your *.fci* file here, otherwise the file will automatically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





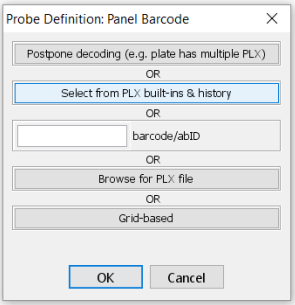
17.1.14 Following the image analysis a *.fci* file will be generated. This file can be uploaded into FAW for easy access to the analyzed image data. The FCI file will be saved in the folder with the TIFF images with the default date and time filename, e.g. "20171114142359.fci".

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

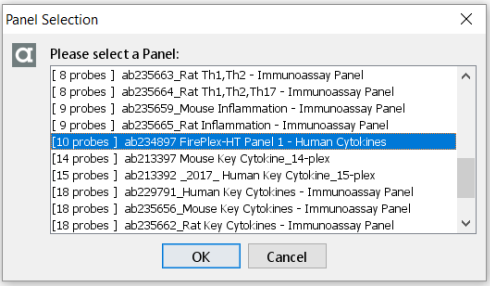


Specifying the Plex File

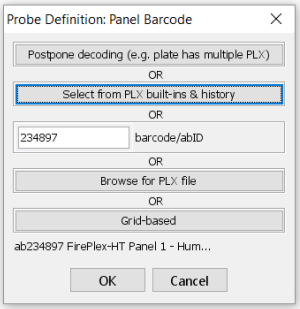
17.1.16 When the image analysis is complete, and if a Plex file had not been specified earlier, the "Probe Definition: Panel Barcode" window will appear.



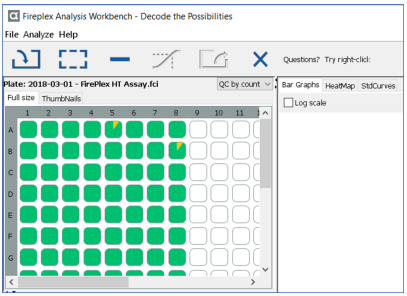
17.1.17 If you are using a Catalogue FirePlex-HT assay, click the "Select from PLX built-ins" button and locate the PLX file for your HT Assay Kit (For example: ab234897 FirePlex-384 Panel 1 – Human Cytokines). If you are using a Custom FirePlex-384 assay, click the "Browse for PLX file" button and locate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



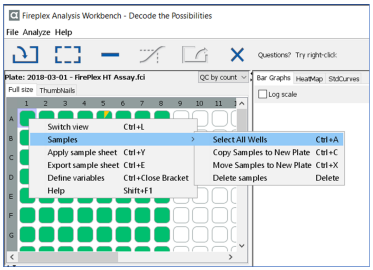
17.1.18 Click "OK" to return to the "Probe Definition" screen, where the PLX file name will be visible beneath the "Browse for PLX file" button. Click OK.

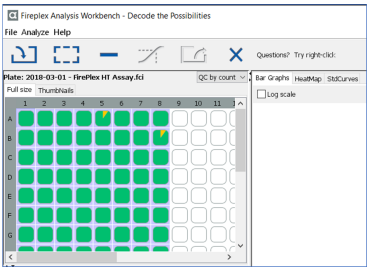


17.1.19 The plate data will load and present in a plate orientation display on the left side of the FAW screen.

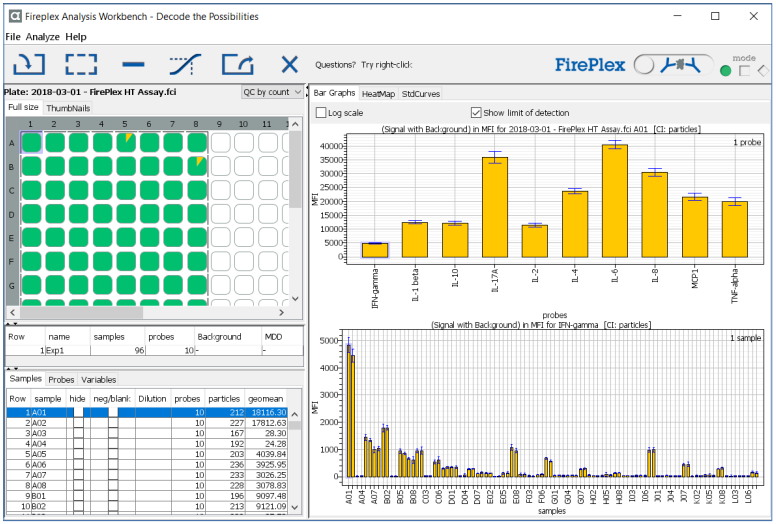


17.1.20 Using the Right-click button of your mouse, open the mouse menu that shows "Sample", then highlight "Select All Wells". All of the wells on the plate will now be highlighted with a light blue color. Alternatively, you can use the "Ctrl+A" shortcut keystroke on your keyboard. Locate the "Make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





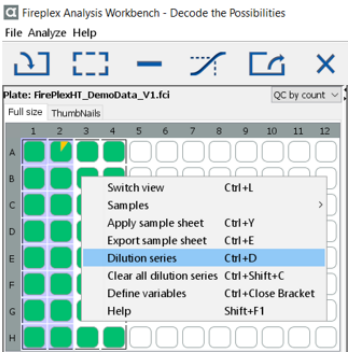
17.1.21 All of the highlighted wells will now display their fluorescence reporter values. Bar graphs representing signal values will appear on the screen on the right side of the FAW.

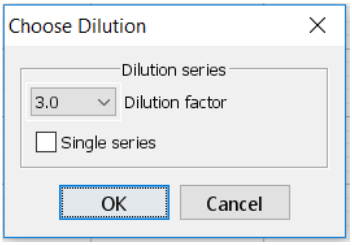


Δ **Note:** Raw Mean Fluorescence Intensity (MFI) data can be directly exported from your experiment at this point. To do so, skip to Section 17.4 of this booklet. To use the built-in standard curve analysis in the FAW, proceed to Section 17.3 and follow the instructions.

17.2 Assigning Standard Curves and Control Wells

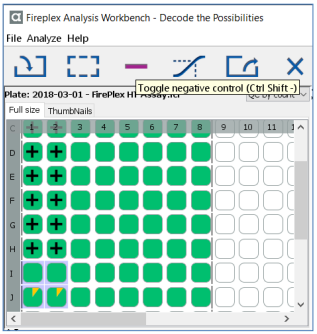
17.2.1 Left-click on the plate to highlight all of the wells that contain the standard curve dilution points (e.g. A01 to H02). Once highlighted, Right-click on the plate with your mouse and select the "Dilution series" option. A "Choose Dilution" window will appear. Make sure 3.0 is chosen in the "Dilution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

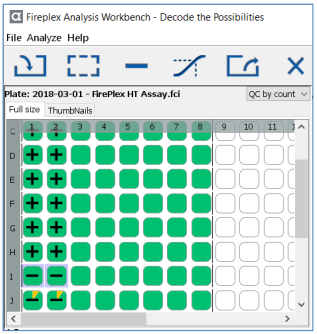




17.2.2 The wells that were highlighted in step 17.3.1 will automatically be labeled as standard curve wells and will be labeled with (+) signs .

17.2.3 Highlight all your Blank/Negative control wells with the left-click button on your mouse. Click on the "Toggle negative control" icon . The negative control wells will be labeled with (-) signs .





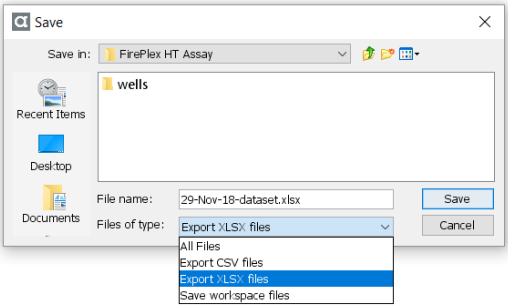
17.2.4 All of the wells will now display their concentration units in "pg/ml" in the bar graph windows.

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 1:2 prior to adding to the assay plate then final in well dilution is 1:4. Therefore interpolated concentrations need to be multiplied by 4 to determine the actual analyte concentration in the undiluted sample.**

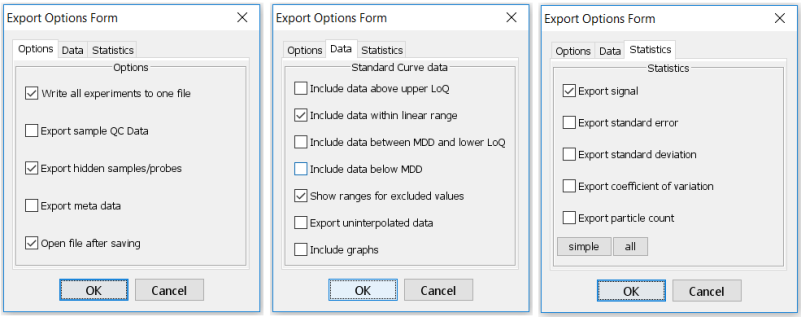
17.3 Data Export

17.3.1 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.

17.3.2 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.



17.3.3 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.



18. 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 Multiplex.FAS@abcam.com

18.1 General Recommended Imager Specifications:

Field of View	*Detection*	*Color Channel*	*Excitation*	*Emission*
Entire Area of single Well on a 384 plate	16-bit sCMOS Camera(s)	Green	440-500 nm	500-535 nm
		Yellow	440-500 nm	570-630 nm
		Red	490-560 nm	650-760 nm

18.2 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/introducing-FirePlex-HT

Imager Class	*Make & Model*	*Light Source*	*Detection*	*Color Channel*	*Excitation*	*Emission*
Microscope	Molecular Devices ImageXpress Micro Confocal	LED	16-bit sCMOS Camera (single)	Green	440-500 nm	514-535 nm
				Yellow	440-500 nm	573-613 nm
				Red	490-560 nm	672-712 nm
Microscope	PerkinElmer Opera Phenix	Laser	16-bit sCMOS Camera (multiple)	Green	488 nm	500-550 nm
				Yellow	488 nm	570-630 nm
				Red	561 nm	650-760 nm
Plate Scanning Cytometer	TTP Labtech Mirrorball	Laser	14-bit PMTs	Green	488 nm	502-537 nm
				Yellow	488 nm	565-605 nm
				Red	488 nm	667-685 nm
Microscope	GE IN Cell Analyzer 6000	Laser	16-bit sCMOS Camera (single)	Green	488 nm	500-525 nm
				Yellow	488 nm	575-620 nm
				Red	561 nm	670-695 nm
Microscope	GE IN Cell Analyzer 2200	LED	16-bit sCMOS Camera (single)	Green	460-490 nm	500-525 nm
				Yellow	460-490 nm	575-620 nm
				Red	530-555 nm	670-695 nm
Microscope	Yokogawa Cell Voyager 7000s	Laser	16-bit sCMOS Camera (multiple)	Green	488 nm	500-550 nm
				Yellow	488 nm	570-630 nm
				Red	561 nm	650-760 nm
Microscope	Yokogawa Cell Voyager 8000	Laser	16-bit sCMOS Camera (multiple)	Green	488 nm	500-550 nm
				Yellow	488 nm	570-630 nm
				Red	561 nm	650-760 nm

19. Notes

Technical Support

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