ab231985
FirePlex® Human
Th1/Th2/Th17 -
Immunoassay Panel
Protocol Booklet

For the quantitative measurement of multiple human targets in serum, plasma, cell culture supernatant and other human biological samples.

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

Our multiplex immunoassays use the FirePlex® particle technology to quantify up to 70 human protein and peptide analytes in the same well, from 12.5 µL sample input.

Assay run-time is 3.5 hours, followed by particle analysis using a validated flow cytometer model and data analysis using our integrated, free-of-charge FirePlex Analysis Workbench software.

FirePlex immunoassays offer:
- Measurement of multiple analytes in the same well, thus conserving time and precious samples.
- Flexibility to select either from our catalog of research focused fixed panels or build custom panels from our large antibody pairs portfolio.
- Antibody pairs that are validated across a broad set of biological sample types, and provide sensitive and reproducible quantitation of analytes in a given sample.

Important - Use of FirePlex Immunoassays with cell extract and tissue homogenate samples requires the purchase of a FirePlex Intracellular Lysis Kit (ab239454).
FirePlex Immunoassay—Quick Guide

Prior to starting
a. Prepare buffers, protein standard dilutions, and capture/detector antibody mixes as instructed (see section 12).
b. Centrifuge samples at 2,000 x g for 15 min to clarify.
c. Dilute samples according to Section 14.
d. Seal empty wells.

Capture
a. Resuspend 1X Capture Particle Solution by inversion and vortex for 5 sec.
b. Add 150 µL 1X Capture Particle Solution to each well.
c. Apply vacuum to filter plate.

Sample incubation
a. Add 175 µL of 1X Wash Buffer to each well and filter. Dry base of plate with a Kimwipe™.
b. Add 50 µL of protein standard or sample. Cover and incubate for 1 hour at RT, or overnight at 4°C, orbital shaking at 750 rpm, then filter.

Detect
a. Wash twice by adding 175 µL of 1X Wash Buffer to each well and filter. Dry base of plate with a Kimwipe™.
b. Add 50 µL of 1X Biotin Detector Antibody. Cover and incubate for 1 hour at RT, orbital shaking at 750 rpm, then filter.

Report
a. Wash twice by adding 175 µL of 1X Wash Buffer to each well and filter. Dry base of plate with a Kimwipe™.
b. Add 50 µL of freshly prepared 1X Reporter Solution. Cover and incubate for 30 min at RT, orbital shaking at 750 rpm, then filter.

Scan
a. Wash twice by adding 175 µL of 1X Wash Buffer to each well and filter. Dry base of plate with a Kimwipe™.
b. Add 175 µL of appropriate** scanning buffer. Scan on flow cytometer.
*Unless otherwise indicated, all incubation steps are performed at room temperature.
**See Section 16.13 for the appropriate buffer for each cytometer.

2. Performance Data

This FirePlex immunoassay panel contains a **Negative Control Particle**, in addition to the protein analytes listed below. For more information, see section 15.1 for an overview and section 18 for advanced data analysis on the Negative Control Particle.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Sensitivity (pg/mL)</th>
<th>Dynamic Range (pg/mL)</th>
<th>Intra-Assay CV (n=36)</th>
<th>Inter-Assay CV (n=3)</th>
<th>Protein Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-gamma</td>
<td>2.95</td>
<td>4.57-10000</td>
<td>2.5%</td>
<td>5.0%</td>
<td>Mix A</td>
</tr>
<tr>
<td>IL-2</td>
<td>1.80</td>
<td>4.57-10000</td>
<td>5.1%</td>
<td>14.4%</td>
<td>Mix A</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.59</td>
<td>1.52-3333</td>
<td>6.6%</td>
<td>13.8%</td>
<td>Mix A</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.12</td>
<td>0.51-1111</td>
<td>4.8%</td>
<td>7.7%</td>
<td>Mix A</td>
</tr>
<tr>
<td>IL-10</td>
<td>2.18</td>
<td>4.57-10000</td>
<td>3.9%</td>
<td>5.7%</td>
<td>Mix A</td>
</tr>
<tr>
<td>IL-17A</td>
<td>0.40</td>
<td>1.52-3333</td>
<td>3.0%</td>
<td>2.1%</td>
<td>Mix A</td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>1.46</td>
<td>4.57-10000</td>
<td>6.5%</td>
<td>6.3%</td>
<td>Mix A</td>
</tr>
<tr>
<td>Negative Control Particle</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- 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 and should be 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 and Stability

Store FirePlex Panel immediately upon receipt. Kit has a storage time of 9 months from date of receipt.
Refer to Section 6 for storage conditions of individual components.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- 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

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage Condition (Before prep)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Human Th1/Th2/Th17 Capture Particles*</td>
<td>1</td>
<td>-20°C</td>
</tr>
<tr>
<td>15X Human Th1/Th2/Th17 Biotin Detectors</td>
<td>1</td>
<td>-20°C</td>
</tr>
<tr>
<td>Human Protein Standard Mix A (lyophilized)</td>
<td>1</td>
<td>-20°C</td>
</tr>
<tr>
<td>10X Wash Buffer</td>
<td>25 mL</td>
<td>+4°C</td>
</tr>
<tr>
<td>2X Human and NHP Assay Diluent</td>
<td>15 mL</td>
<td>+4°C</td>
</tr>
<tr>
<td>5X Reporter Solution*</td>
<td>4 mL</td>
<td>+4°C</td>
</tr>
<tr>
<td>Filter Plate (1 x 96 wells)</td>
<td>1</td>
<td>R/T</td>
</tr>
<tr>
<td>Plate Seals</td>
<td>3</td>
<td>R/T</td>
</tr>
<tr>
<td>Tungsten cleaning wire</td>
<td>1</td>
<td>R/T</td>
</tr>
</tbody>
</table>

*Reagent is light sensitive. Store in a dark place and protect from light at all times.
7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- FirePlex Run Buffer for data acquisition on a given flow cytometer

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage Condition (Before prep)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run Buffer I (ab245836)</td>
<td>1x96 tests</td>
<td>+4°C</td>
</tr>
<tr>
<td>Run Buffer II (ab234450)</td>
<td>1x96 tests</td>
<td>+4°C</td>
</tr>
<tr>
<td>Run Buffer III (ab245837)</td>
<td>1x96 tests</td>
<td>+4°C</td>
</tr>
<tr>
<td>PBS (user supplied)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

⚠️ **Note:** To determine which Run Buffer to use for your specific flow cytometer, **users must first** use the Cytometer Setup Kit V2 – ab245835.

- Validated flow cytometer. Please visit our website to see a current list of validated flow cytometers: [www.abcam.com/FirePlexCytometry](http://www.abcam.com/FirePlexCytometry)

⚠️ **Note:** FirePlex particles are designed to be read using a blue (488 nm) laser with green, yellow, and red detectors and can only be read on validated flow cytometer models.

- Plex file for software data analysis
- Vacuum manifold for 96 well plate (ab204067 recommended)
- Test tubes for dilution of standards or samples
- Deionized water
- Multi-channel pipette (recommended)
- Vortex mixer
- Microcentrifuge
- Plate shaker

⚠️ **Note:** Mixing rates depend upon the orbital radius of your plate shaker. Information about the recommended rate for your plate shaker can be found in the Technical Hints section.
FirePlex Immunoassay Intracellular Lysis Kit, for use with cell extracts and tissue homogenates.

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage Condition (Before prep)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Wash Buffer</td>
<td>25 mL</td>
<td>+4°C</td>
</tr>
<tr>
<td>5X Intracellular Extraction Buffer</td>
<td>10 mL</td>
<td>+4°C</td>
</tr>
<tr>
<td>50X Intracellular Extraction Enhancer</td>
<td>1 mL</td>
<td>+4°C</td>
</tr>
<tr>
<td>2X Intracellular Assay Diluent</td>
<td>15 mL</td>
<td>+4°C</td>
</tr>
</tbody>
</table>

8. Technical Hints

- Before running this assay on a given flow cytometer for the first time, we strongly recommend performing a test run with your flow cytometer to confirm that the settings are accurate, and it is functional. See Section 10 for flow cytometer set up/verification instructions.
- When generating the protein standard samples, or performing serial dilutions of samples, pipette tips must be changed after each dilution step.
- After each wash step, dry the base of the filter plate by pressing down on a thick cushion of paper towels to ensure the base is completely dry.
- When applying vacuum to samples in the filter plate, press down firmly on all four corners of the filter plate and turn off the vacuum as soon as the liquid is cleared from each well to prevent over-drying. Vacuum manifold pressure should be set at ~5 psi.
- 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.
- Ensure unused wells are properly sealed with provided plate seals during all incubation steps.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
- Protect FirePlex particles and 5X Reporter Solution from light at all times.
- Avoid multiple freeze/thaw of protein standard and biological samples.
- Do not combine and mix component lots from multiple kits.
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:

\[
\text{Orbital shaker speed (in RPM)} = \sqrt{\frac{1687500}{\text{orbital diameter (in mm) of your shaker}}}
\]

This kit is sold based on the number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.
9. Software Installation Guide

We recommend installing the FirePlex Analysis Workbench software onto your computer before proceeding with further instructions to run the assay. The software is required to check that your flow cytometer settings are correct (Section 10) and for data analysis after the assay is complete (Section 18).

9.1 First Time Use

Go to www.abcam.com/FireflyImmunoAnalysis
Download our free-of-charge FirePlex Analysis Workbench software.

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

9.1.2 The Java program will automatically be copied to your desktop.

9.1.3 Please be sure to install this software on your computer for data analysis. It is not necessary to install on the computer directly connected to the flow cytometer.

9.2 Subsequent Use

9.2.1 Whenever the analysis workbench is updated, the application will download the new version, otherwise it will use the version it has already downloaded in order to save time.

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
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. Flow Cytometer Set Up/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. Also, please ensure that you use the flow cytometer settings file provided and have optimized and verified this protocol to work on your own cytometer.

△ Note: Flow cytometer setup is critical, as FirePlex particles behave differently from beads and cells used in conventional flow cytometry or other bead-based multiplex assays. In addition, the Red channel (assay reporter) signal on the Setup Kit particles (ab245835) and your FirePlex immunoassay will not match each other. The actual assay run will have more red fluorescence than the Cytometer Setup Kit, so the Red channel PMT voltage will likely need to be reduced to ensure the Red channel signal is within the linear range of the PMT.

These instructions and settings files are found at:
www.abcam.com/FirePlexCytometry
11. Plate Preparation

- For each assay performed, we recommend:
  - Designing your plate layout before starting the assay.
  - Each sample should be assayed with a minimum of two replicates.
- A minimum of four wells must be used as the blank control.
- For first time experiments, two wells are required for optimizing flow cytometer target channel gain settings (see schematic below) after the assay procedure is complete. These two wells are separate from Flow Cytometer Set Up/Verification (Section 10).
- The 96 well plate included with this kit is supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Recommended plate layout is below:

![Plate Layout Diagram]

A 1-2 - Standard #1
B 1-2 - Standard #2
C 1-2 - Standard #3
D 1-2 - Standard #4
E 1-2 - Standard #5
F 1-2 - Standard #6
G 1-2 - Standard #7
H 1-2 - Standard #8

A 3-4 - Standard #9 (Blank)
B 3-4 - Standard #9 (Target channel gain optimization)
12. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for assaying 96 wells.
- Prepare only as much reagent as is needed on the day of the experiment. Diluted reagents should not be stored for later use. The instructions below for preparation of 1X Wash Buffer, 1X Human Assay Diluent, 1X Reporter Solution and 1X Intracellular Lysis Assay Diluent provide sufficient reagent to run one full 96 well plate.

12.1 1X Wash Buffer
Prepare 1X Wash Buffer by diluting 10X Wash Buffer with deionized water. To prepare 200 mL 1X Wash Buffer, combine 20 mL 10X Wash Buffer with 180 mL deionized water. Mix thoroughly and gently.

12.2 1X Human Assay Diluent (for use with biological fluids such as serum, plasma, cell culture supernatant)
Prepare 1X Human Assay Diluent by diluting the 2X Human and NHP Assay Diluent with 1X Wash Buffer. To prepare 20 mL 1X Human Assay Diluent, combine 10 mL 2X Human and NHP Assay Diluent with 10 mL 1X Wash Buffer. Mix thoroughly and gently.

12.3 1X Reporter Solution
Immediately prior to use: Prepare sufficient volume of 1X Reporter Solution (50 µL per well) by diluting 5X Reporter Solution in 1X Wash Buffer. Prepare only enough 1X Reporter Solution as required. To prepare 5 mL 1X Reporter Solution combine 1 mL 5X Reporter Solution with 4 mL 1X Wash Buffer. Mix thoroughly and gently.

12.4 Run Buffer
Supplied ready to use.
12.5 1X Intracellular Lysis Assay Diluent (for use with cell extracts and tissue homogenates only)
Prepare 1X Intracellular Lysis Assay Diluent by diluting 5X Intracellular Extraction Buffer and 2X Intracellular Assay Diluent with 1X Wash Buffer. To make 20 mL 1X Intracellular Lysis Assay Diluent combine 6 mL 1X Wash Buffer, 10 mL 2X Intracellular Assay Diluent, 4 mL 5X Intracellular Extraction Buffer. Mix thoroughly and gently. If required protease inhibitors can be added.

12.6 1X Intracellular Extraction Buffer (for preparation of cell extracts and tissue homogenates only in section 14.8-14.10)
Prepare 1X Intracellular Extraction Buffer by diluting 5X Intracellular Extraction Buffer and 50X Intracellular Extraction Enhancer Solution with 1X Wash Buffer. To make 20 mL 1X Intracellular Extraction Buffer combine 15.6 mL 1X Wash Buffer, 4 mL 5X Intracellular Extraction Buffer and 0.4 mL 50X Intracellular Extraction Enhancer. Mix thoroughly and gently. If required protease inhibitors can be added.

12.7 1X Capture Particle Solution
12.7.1 Vortex the vial of 10X Capture Particle solution for 10 seconds to thoroughly resuspend the particles.
12.7.2 Dilute the vial of 10X Capture Particle solution to 1X in a new tube by adding the corresponding volume of 1X Wash Buffer, using the table below.

<table>
<thead>
<tr>
<th>Number of assay wells</th>
<th>10X Capture Particle solution</th>
<th>1X Wash Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>96</td>
<td>1.63 mL</td>
<td>14.67 mL</td>
</tr>
<tr>
<td>72</td>
<td>1.23 mL</td>
<td>11.07 mL</td>
</tr>
<tr>
<td>48</td>
<td>0.81 mL</td>
<td>7.29 mL</td>
</tr>
<tr>
<td>24</td>
<td>0.42 mL</td>
<td>3.78 mL</td>
</tr>
</tbody>
</table>

Note: Particles should be protected from light during handling. We recommend a minimum of 4 extra wells to account for pipetting dead volume and to use in the set-up of your flow cytometer. The table above incorporates the extra wells already into the
calculations for you.
**1X Biotin Detector Antibody Solution**

**12.7.3** Centrifuge the vial of 15X Biotin Detector solution for 1 minute at 1,000 x g.

**12.7.4** Dilute the vial of 15X Biotin Detector solution to 1X in a new tube by adding 1X Human Assay Diluent, using the table below.

<table>
<thead>
<tr>
<th>Number of assay wells</th>
<th>15X Biotin Detector solution</th>
<th>1X Human Assay Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>96</td>
<td>0.363 mL</td>
<td>5.087 mL</td>
</tr>
<tr>
<td>72</td>
<td>0.273 mL</td>
<td>3.827 mL</td>
</tr>
<tr>
<td>48</td>
<td>0.180 mL</td>
<td>2.520 mL</td>
</tr>
<tr>
<td>24</td>
<td>0.093 mL</td>
<td>1.307 mL</td>
</tr>
</tbody>
</table>

⚠️ **Note:** We recommend a minimum of 4 extra wells to account for pipetting dead volume and to use in the set-up of your flow cytometer. The table above incorporates the extra wells already into the calculations for you.
13. Standard Preparation

General sample information:
- The following section describes the preparation of a standard curve for duplicate measurements (recommended).
- Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of positive controls for every use.
- Each vial of Protein Standard contains a mixture of proteins. To ensure all of the proteins in your multiplex panel are included, please refer to the Protein Standard Mix datasheet for a complete protein listing.

13.1 Centrifuge the Human Protein Standard Mix A vial for 1 minute at 1,000 x g to pellet the lyophilized contents.

13.1.1 For serum, plasma, cell culture supernatants and other biological fluid measurements, reconstitute the Human Protein Standard Mix A vial by adding 200 µL of 1X Human Assay Diluent.

13.1.2 For cell extract and tissue homogenate measurements, reconstitute the Human Protein Standard Mix A vial by adding 200 µL of 1X Intracellular Lysis Assay Diluent.

13.2 Incubate at room temperature for 5 minutes and mix thoroughly and gently. Each vial contains a 5X Protein Standard Stock Solution. After resuspension, the protein standard should be placed on ice. Any remaining 5X Standard Stock Solution should be aliquoted and stored at -80ºC.

13.3 Label nine tubes, Standards #1 – 9.

13.3.1 For serum, plasma, cell culture supernatants and other biological fluid measurements, add 240 µL of 1X Human Assay Diluent to the tube labeled Standard #1. Add 200 µL of 1X Human Assay Diluent to tubes labeled Standards #2 – 8. Add 250 µL of 1X Human Assay Diluent to Standard #9.

13.3.2 For cell extract and tissue homogenate measurements, add 240 µL of 1X Intracellular Lysis Assay Diluent to the tube labeled Standard #1. Add 200 µL of 1X Intracellular Lysis Assay Diluent to tubes labeled Standards #2 – 8. Add 250 µL of 1X Intracellular Lysis Assay Diluent to Standard #9.
13.4 Add 60 µL of each 5X Protein Standard Stock Solution listed in the table below to the tube labelled Standard #1. Mix by pipetting up and down.

<table>
<thead>
<tr>
<th>Protein Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Protein Standard Mix A</td>
</tr>
</tbody>
</table>

13.5 Transfer 100 µL of Standard #1 to Standard #2 which corresponds to preparing the 3-fold dilution series until Standard #8. Standard #9 contains no protein and is the Blank control.

Δ **Note:** Pipette tips need to be changed after each dilution step to avoid contamination between standards.
14. Sample Preparation

- Recommended sample dilutions for each sample type can be found below. Optimal sample dilutions should however be determined by the end user.

- For optimal assay performance, samples must always be used either at the recommended dilution or further diluted.

- To prevent clogging of the filter plate it is important that samples are clarified via centrifugation as stated below.

- Samples generating values higher than the highest standard should be further diluted in the 1X Assay Diluent.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Starting Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Culture Supernatant</td>
<td>1:4</td>
</tr>
<tr>
<td>Serum</td>
<td>1:4</td>
</tr>
<tr>
<td>Plasma – Citrate</td>
<td>1:4</td>
</tr>
<tr>
<td>Plasma – EDTA</td>
<td>1:4</td>
</tr>
<tr>
<td>Plasma – Heparin</td>
<td>1:4</td>
</tr>
<tr>
<td>Urine</td>
<td>1:4</td>
</tr>
<tr>
<td>Saliva</td>
<td>1:4</td>
</tr>
<tr>
<td>Cerebrospinal fluid</td>
<td>1:4</td>
</tr>
<tr>
<td>Synovial fluid</td>
<td>1:4</td>
</tr>
<tr>
<td>Milk (defatted)</td>
<td>1:4</td>
</tr>
<tr>
<td>Bronchial lavage</td>
<td>1:4</td>
</tr>
<tr>
<td>Cell Extract</td>
<td>50 – 500 µg/mL</td>
</tr>
<tr>
<td>Tissue Homogenate</td>
<td>50 – 500 µg/mL</td>
</tr>
</tbody>
</table>
14.1 **Cell Culture Supernatants**
Centrifuge cell culture media at 2,000 x g for 15 minutes to remove debris. Collect supernatants and assay. Or dilute samples into 1X Human Assay Diluent and assay. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

14.2 **Serum**
Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2,000 x g for 15 minutes and collect serum. Dilute samples into 1X Human Assay Diluent and assay. Store un-diluted serum at -20ºC or below. Avoid repeated freeze-thaw cycles.

14.3 **Plasma**
Collect plasma using citrate, EDTA or heparin. Centrifuge samples at 2,000 x g for 15 minutes. Dilute samples into 1X Human Assay Diluent and assay. Store un-diluted plasma samples at -20ºC or below for up to 3 months. Avoid repeated freeze-thaw cycles.

14.4 **Urine**
Centrifuge urine at 400 x g for 5 minutes to remove debris. Collect supernatants, dilute in 1X Human Assay Diluent and assay. Store un-diluted samples at -20ºC or below. Avoid repeated freeze-thaw cycles.

14.5 **Saliva**
Centrifuge saliva at 2,000 x g for 15 minutes to remove debris. Collect supernatants, dilute samples into 1X Human Assay Diluent and assay. Store un-diluted samples at -20ºC or below. Avoid repeated freeze-thaw cycles.

14.6 **Milk**
De-fat milk samples as follows. Centrifuge milk samples at 500 x g for 15 minutes at 4°C and collect the aqueous fraction using syringe attached to needle. Centrifuge the aqueous fraction at 3,000 x g for 15 minutes at 4°C and collect the final aqueous fraction (de-fatted milk) using syringe attached to needle. Dilute the de-fatted milk samples in 1X Human Assay Diluent and assay. Store un-diluted de-fatted milk at -20ºC or below. Avoid repeated freeze-thaw cycles.

14.7 **Cerebrospinal fluid, Synovial fluid, Bronchial lavage**
Centrifuge sample at 2,000 x g for 15 minutes to remove debris. Collect supernatants, dilute samples into 1X Human Assay Diluent and assay. Store un-diluted samples at -20ºC or below. Avoid repeated freeze-thaw cycles.
14.8 Preparation of extracts from cell pellets:

14.8.1 Collect non-adherent cells by centrifugation or scrape to collect adherent cells from the culture flask. Typical centrifugation conditions for cells are 500 x g for 5 minutes at 4°C.

14.8.2 Rinse cells twice with PBS.

14.8.3 Solubilize pellet at 2x10^7 cell/mL in chilled 1X Intracellular Extraction Buffer.

14.8.4 Incubate on ice for 20 minutes.

14.8.5 Centrifuge at 18,000 x g for 20 minutes at 4°C.

14.8.6 Transfer the supernatants into clean tubes and discard the pellets.

14.8.7 The sample total protein concentration in the extract should be quantified using a BCA assay or alternative protein assay. Aliquot and store extracts at -80°C until ready for use.

14.8.8 Dilute samples to desired concentration in 1X Intracellular Lysis Assay Diluent and assay samples immediately.

14.9 Preparation of extracts from adherent cells by direct lysis (alternative protocol):

14.9.1 Remove growth media and rinse adherent cells 2 times in PBS.

14.9.2 Solubilize the cells by addition of chilled 1X Intracellular Extraction Buffer directly to the plate. Use 0.75 mL - 1.5 mL 1X Intracellular Extraction Buffer per confluent 15 cm diameter plate.

14.9.3 Scrape the cells into a microfuge tube and incubate the lysate on ice for 15 minutes.

14.9.4 Centrifuge at 18,000 x g for 20 minutes at 4°C.

14.9.5 Transfer the supernatants into clean tubes and discard the pellets.

14.9.6 The sample total protein concentration in the extract should be quantified using a BCA assay or alternative protein assay. Aliquot and store extracts at -80°C until ready for use.

14.9.7 Dilute samples to desired concentration in 1X Intracellular Lysis Assay Diluent and assay samples immediately.

14.10 Preparation of extracts from tissue homogenates:

14.10.1 Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (dounce homogenizer is recommended).

14.10.2 Homogenize 100 to 200 mg of wet tissue in 0.5 mL – 1 mL of chilled 1X Intracellular Extraction Buffer. For lower amounts of tissue adjust volumes accordingly.
14.10.3 Incubate on ice for 20 minutes.
14.10.4 Centrifuge at 18,000 x g for 20 minutes at 4°C.
14.10.5 Transfer the supernatants into clean tubes and discard the pellets.
14.10.1 The sample total protein concentration in the extract should be quantified using a BCA assay or alternative protein assay. Aliquot and store extracts at -80°C until ready for use.
14.10.2 Dilute samples to desired concentration in 1X Intracellular Lysis Assay Diluent and assay samples immediately.

15. Experimental Design

15.1 Controls within each well
Negative Control Particles are conjugated to a monoclonal Rabbit IgG and are included in each panel to ensure assay specificity. These particles can be used for background normalization in crude biological samples with wide variations in target protein expression levels or for validation of unique biological samples. These particles function similarly to isotype controls and are used to eliminate capture-independent signal. To perform data analysis using a Negative Control Particle, refer to Section 18.

15.2 Negative control wells
It is recommended that the user run four negative control wells, i.e. replacing the sample input with assay diluent, every time an assay is performed. See section 11 for a recommended plate layout for your experiment. Significant signal in negative control wells can indicate problems executing the assay.

15.3 Replicates
It is recommended to run samples in duplicate. 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).
16. 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.

16.1 Cover unused and previously used wells with the provided plate seal during all incubation and vacuum filtration steps.

16.2 Invert the 1X Capture Particle Solution end-over-end for 2 minutes and vortex for 10 seconds to fully resuspend the particles. Add 150 µL of 1X Capture Particle Solution to each well. **Mixing is vital to ensure that each well receives an equal number of particles. To prevent the particles from falling out of suspension, the 1X Capture Particle Solution should be remixed every 4 wells. If using a multi-channel pipette, add 1X Capture Particle Solution to a reservoir and mix thoroughly by pipetting. The 1X Capture Particle Solution should be mixed after each addition to the plate wells. If needed, use a single channel pipette to transfer the last few wells as total volume is limiting.**

16.3 Remove the buffer by applying vacuum to the filter plate. Then add 175 µL of 1X Wash Buffer to each well and remove the buffer by applying vacuum to the filter plate. After removing the buffer, dry the base of the plate by pressing down on tissue paper to ensure the bottom of each filter well is dry and prevent wicking.

16.4 Add 50 µL of standard or sample (diluted according to instructions in section 13 and 14) to each well.

16.5 Cover with plate lid and incubate for 1 hour at room temperature with shaking at 750 rpm in the dark.

△ Note: If measuring low abundance targets, it is recommended to incubate overnight at 4°C with shaking at 750 rpm in the dark.

16.6 Remove the plate lid and apply vacuum to the filter plate to remove the buffer. Wash each well twice by adding 175 µL of 1X Wash Buffer and then remove the buffer by applying vacuum to the filter plate. After the final removal of buffer, dry the base of the plate by pressing down on tissue paper to ensure the bottom of each filter well is dry and prevent wicking.

16.7 Add 50 µL 1X Biotin Detector antibody mix to each well. Cover with plate lid and incubate for 1 hour at room temperature with shaking at 750 rpm in the dark.
16.8 Remove the plate lid and apply vacuum to the filter plate to remove the buffer. Wash each well twice by adding 175 µL of 1X Wash Buffer and then remove the buffer by applying vacuum to the filter plate. After the final removal of buffer, dry the base of the plate by pressing down on tissue paper to ensure the bottom of each filter well is dry and prevent wicking.

16.9 Add 50 µL of freshly prepared 1X Reporter Solution to each well.

16.10 Cover with plate lid and incubate for 30 minutes at room temperature with shaking at 750 rpm in the dark.

16.11 Remove the plate lid and apply vacuum to the filter plate to remove the buffer. Wash each well twice by adding 175 µL of 1X Wash Buffer and then remove the buffer by applying vacuum to the filter plate. After the final removal of buffer, dry the base of the plate by pressing down on tissue paper to ensure the bottom of each filter well is dry and prevent wicking.

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

⚠️ Note: The appropriate Run Buffer for your cytometer (i.e. Run Buffer I, II, III, or PBS) should be determined prior to starting the assay by using the FirePlex Cytometer Setup Kit V2 – ab245835 (see Section 10).

16.13 To acquire data, proceed to Section 17 or cover the plate and store overnight (up to 18 hours) at 4°C.

⚠️ Note: Overnight storage is not recommended for targets that are expected to generate values <20 pg/mL, as prolonged storage of the sample at 4°C may lead to reduced sensitivity of the assay.
17. Flow Cytometer Acquisition

17.1 Flow Cytometer with plate handler

17.1.1 Prior to acquiring your multiplex immunoassay kit, use the Cytometer Setup Kit (ab211043) and complete flow cytometer set up according to the instructions and confirm that your cytometer accurately resolves the FirePlex particles and can decode the Setup particles in the FirePlex Analysis Workbench.

17.1.2 Download and use the preconfigured flow cytometer settings file for your validated flow cytometer model from www.abcam.com/FirePlexCytometry

17.1.3 On the day of your actual multiplex immunoassay acquisition, two blank wells (i.e. wells B3 and B4) should be used to optimize your flow cytometer target channel gain settings. Complete flow cytometer target channel gain optimization according to the instructions for your validated flow cytometer model.

⚠️ Note: This step is not necessary for users of BD Bioscience’s Accuri™ C6 or C6 Plus.

17.2 Flow Cytometer with single tube loader

17.2.1 Mix each well thoroughly by pipetting up and down 5 times to ensure maximum recovery of particles.

17.2.2 Immediately transfer each well to a 1.5mL Eppendorf tube or 5mL FACS tube. Label each tube with the well name.

17.2.3 Download and use the preconfigured flow cytometer settings file for your validated flow cytometer model from www.abcam.com/FirePlexCytometry

17.2.4 For first time experiments, two blank wells should be used to optimize your flow cytometer target channel gain settings. Complete flow cytometer target channel gain optimization according to the instructions for your validated flow cytometer model.

⚠️ Note: This step is not necessary for users of BD Bioscience’s Accuri™ C6 or C6 Plus.
18. Data Analysis

For detailed instructions of how to use the FirePlex Analysis Workbench software, please review the User Guide which can be found at: Help Menu > Help Front Page. Specific help on features can be obtained by right-clicking a GUI element, for instance a button, a chart or a table and selecting “Help”.

18.1 Instructions for uploading and selecting your data.

18.1.1 Open FCS files using the “Load FCS file” button. Select either a single FCS file for your entire plate or all of the FCS files for each well at the same time by selecting them in the folder and pressing open.
18.1.2 When prompted, select the analytes in your multiplex panel by loading your Plex (.PLX) file. If using a Premixed Focus Panel Kit, then enter the product abid into the ‘Panel Barcode’ box. The Plex file defines the correspondence between cytometer code spots and protein identity. It is critical to make sure that you choose the correct Plex. A Plex file containing fewer analytes or more analytes than your panel will return incorrect results.

18.1.3 Highlight wells of interest in the Full-size plate view tab (standards and unknowns) and join those together by selecting the ‘Make Experiment’ button.

18.1.4 The Analysis Workbench software will automatically decode and assign MFI levels of each analyte per well at this step.
18.1.5 Data generated from step 18.1.4 can either be exported as raw data in a .csv file using the ‘Export’ button to analyze the data using an alternative software.

Or users can proceed to generate a standard curve using the Analysis Workbench software.

18.2 Generating a standard curve

18.2.1 The standard wells can be defined if the standard plate layout of section 11 is used. Right click any occupied well of the plate and select “Dilution series”.

18.2.2 In the new menu window, select “2 columns, 8 rows” and leave A01 as the starting well. The software will take A01 & A02 as the highest concentration, B01 & B02 as STD2, etc.

Note: If the standard wells are not a regular array, see the software guide for alternative methods of defining dilutions.
18.2.3 A ‘plus’ sign will appear inside every well to indicate the well has been marked as the protein standard.

18.2.4 Highlight all blank wells in your plate layout and assign them as Negative controls by selecting the ‘Negative’ button.

18.2.5 Standard curves and unknown analyte values will automatically be generated at this stage. Standard curves are generated using the 4PL equation and are reported in pg/mL. Standard curves can be viewed in the ‘StdCurves’ tab.

18.2.6 If your multiplex panel contains a negative control particle and you would like to perform negative control particle normalization, select the ‘Standard Curve’ button:

18.2.7 From the new menu, select ‘Subtract blank wells and/or blank probes’ and hit ‘Ok’.

18.2.8 Negative control particle normalized standard curves and unknown analyte values will automatically be generated at this stage.

18.2.9 Export standard curve and analyte data as .csv file using the ‘Export’ button and analyze. There is no need to change the default selection in the Export Options popup unless preferred.

Analyte interpolated values should be corrected for the sample dilution factor after export from the FirePlex Analysis Workbench software.
## 19. Troubleshooting

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<th>Reason</th>
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<td>Poor standard curve</td>
<td>Inaccurate pipetting</td>
<td>Prewet pipette tips and be sure to change tips after each dilution step</td>
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<tr>
<td></td>
<td>Improper standard dilution</td>
<td>Prior to opening, briefly spin each stock standard tube and dissolve the powder thoroughly by gentle mixing</td>
</tr>
<tr>
<td>Low Signal or Sensitivity</td>
<td>Improper storage of the FirePlex protein standards</td>
<td>Store your reconstituted standards at -80°C, all other assay components at +4°C.</td>
</tr>
<tr>
<td></td>
<td>Inadequate reagent volumes or improper dilution</td>
<td>Ensure sufficient incubation times; Check pipettes and ensure correct calibration</td>
</tr>
<tr>
<td>Large CV</td>
<td>Plate is insufficiently washed</td>
<td>Review manual for proper wash technique. Check vacuum manifold seal for air leaks</td>
</tr>
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<td></td>
<td>Contaminated wash buffer</td>
<td>Prepare fresh wash buffer</td>
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<tr>
<td>Low Particle or Event Count</td>
<td>Particle settling or aggregation</td>
<td>Invert and vortex 30X stock and 1X Capture Particle Solution thoroughly before assay use</td>
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<td></td>
<td>Vacuum too weak</td>
<td>Adjust the vacuum pressure to be ~5 psi</td>
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<td>Incorrect flow cytometer settings</td>
<td>Check for correct Flow Cytometer settings.</td>
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<tr>
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<td>Blot the filter plate on dry tissue paper by holding down firmly for 5 seconds</td>
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<tr>
<td>Clogged Filter Plate</td>
<td>High lipid content in biological fluid samples</td>
<td>Centrifuge the samples at 10,000 x g for 10 minutes at 4°C. Re-collect the soluble fraction of the sample.</td>
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<tr>
<td>Uneven buffer removal from wells</td>
<td>Variable particle counts per well</td>
<td>Repeat assay set up. Ensure thorough mixing of 1X Capture Particle Solution before addition to plate</td>
</tr>
<tr>
<td>Precipitate in Diluent</td>
<td>Precipitation and/or coagulation of components within the Assay Diluent.</td>
<td>Precipitate can be dissolved by gently warming the Assay Diluent to 37°C.</td>
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Technical Support

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