Version 5 Last updated 11 November 2021

# ab243554 FirePlex®-96 Discovery Cytokines (Mouse) Immunoassay Panel Protocol Booklet

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

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

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# 1. Overview

Abcam multiplex immunoassays use the FirePlex® particle technology to quantify up to 70 mouse 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).

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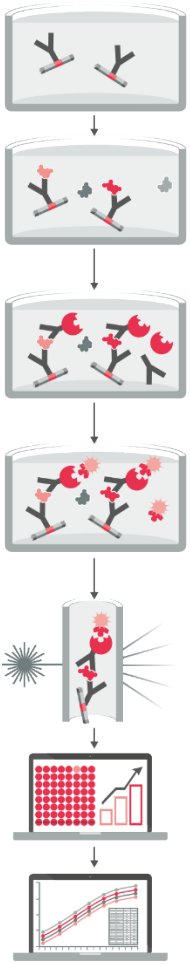
# FirePlex Immunoassay– Quick Guide

| | | |
|---|---|---|
| **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 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 3X 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.**

## 2. Performance Data

This FirePlex immunoassay panel contains a **Negative Control Particle**, in addition to the protein analytes listed below.

| Analyte | Sensitivity (pg/mL) | Dynamic Range (pg/mL) | Intra-Assay CV (n=36) | Inter-Assay CV (n=3) | Protein Standard |
|---|---|---|---|---|---|
| BCA1 | 2.52 | 4.57 – 3,333 | 6% | 16% | Mix 2 |
| CCL27 | 0.45 | 1.52 – 3,333 | 5% | 7% | Mix 2 |
| CD137 | 0.94 | 4.57 – 10,000 | 6% | 15% | Mix 2 |
| CD23 | 28.97 | 123 – 270,000 | 6% | 14% | Mix 2 |
| CD31 | 2.50 | 13.71 – 30,000 | 4% | 6% | Mix 2 |
| CD40 | 0.98 | 4.57 – 10,000 | 7% | 10% | Mix 2 |
| CXCL1 | 0.11 | 0.51 – 1,111 | 8% | 8% | Mix 1 |
| CXCL11 | 2.43 | 13.71 – 30,000 | 6% | 17% | Mix 2 |
| CXCL16 | 0.44 | 1.52 – 3,333 | 7% | 18% | Mix 2 |
| DLL1 | 1.77 | 4.57 – 10,000 | 9% | 20% | Mix 2 |
| Eotaxin | 0.30 | 1.52 – 3,333 | 4% | 6% | Mix 2 |
| Eotaxin-2 | 1.18 | 4.57 – 10,000 | 9% | 19% | Mix 1 |
| FAS Ligand | 1.40 | 4.57 – 10,000 | 5% | 3% | Mix 2 |
| Fractalkine | 2.57 | 13.71 – 30,000 | 7% | 14% | Mix 2 |
| Galectin 3 | 1.36 | 4.57 – 10,000 | 5% | 18% | Mix 2 |
| G-CSF | 0.12 | 0.51 – 1,111 | 7% | 13% | Mix 2 |
| GM-CSF | 0.23 | 1.52 – 3,333 | 8% | 17% | Mix 1 |
| GRO gamma | 0.11 | 0.51 – 1,111 | 7% | 13% | Mix 2 |
| HGF | 1.56 | 4.57 – 10,000 | 9% | 13% | Mix 2 |
| ICAM2 | 1.11 | 4.57 – 10,000 | 4% | 20% | Mix 2 |
| IFN-A2 | 0.08 | 0.51 – 1,111 | 8% | 19% | Mix 1 |
| IFN-gamma | 0.11 | 0.51 – 1,111 | 7% | 20% | Mix 1 |
| IL-1 beta | 0.26 | 1.52 – 3,333 | 8% | 20% | Mix 1 |
| IL-10 | 1.01 | 4.57 – 10,000 | 8% | 17% | Mix 1 |
| Analyte | Sensitivity (pg/mL) | Dynamic Range | Intra-Assay | Inter-Assay | Protein Standard |

| | | (pg/mL) | CV (n=36) | CV (n=3) | |
|---|---|---|---|---|---|
| IL-11 | 1.58 | 4.57 – 10,000 | 5% | 12% | Mix 2 |
| IL-12/23 p40 | 0.08 | 0.51 – 1,111 | 7% | 9% | Mix 1 |
| IL-12p70 | 0.32 | 1.5 – 3,333 | 6% | 8% | Mix 1 |
| IL-13 | 0.26 | 1.5 – 3,333 | 4% | 43% | Mix 1 |
| IL-16 | 0.08 | 0.51 – 1,111 | 4% | 8% | Mix 1 |
| IL-17A | 0.14 | 0.51 – 1,111 | 8% | 27% | Mix 1 |
| IL-17F | 0.13 | 0.51 – 1,111 | 6% | 20% | Mix 2 |
| IL-18 | 14.31 | 41.2 – 90,000 | 5% | 10% | Mix 1 |
| IL-19 | 1.16 | 4.57 – 10,000 | 5% | 17% | Mix 1 |
| IL-1a | 0.28 | 1.52 – 3,333 | 9% | 7% | Mix 1 |
| IL-2 | 4.00 | 13.7 – 30,000 | 8% | 7% | Mix 1 |
| IL-20 | 0.10 | 0.51 – 1,111 | 10% | 15% | Mix 1 |
| IL-21 | 0.92 | 4.57 – 10,000 | 5% | 19% | Mix 1 |
| IL-22 | 0.08 | 0.51 – 1,111 | 6% | 9% | Mix 1 |
| IL-27a | 7.06 | 41.2 – 90,000 | 8% | 19% | Mix 1 |
| IL-28 | 0.09 | 0.51 – 1,111 | 8% | 14% | Mix 1 |
| IL-3 | 0.27 | 1.52 – 3,333 | 7% | 12% | Mix 1 |
| IL-31 | 0.75 | 1.52 – 3,333 | 5% | 8% | Mix 1 |
| IL-33 | 0.36 | 1.52 – 3,333 | 5% | 11% | Mix 1 |
| IL-4 | 0.29 | 1.52 – 3,333 | 7% | 13% | Mix 1 |
| IL-5 | 0.10 | 0.51 – 1,111 | 10% | 20% | Mix 1 |
| IL-6 | 6.29 | 13.7 – 10,000 | 6% | 19% | Mix 1 |
| IL-6 R alpha | 7.28 | 41.2 – 90,000 | 9% | 5% | Mix 1 |
| IL-7 | 0.36 | 1.52 – 3,333 | 10% | 10% | Mix 1 |
| IL-9 | 0.07 | 0.51 – 1,111 | 13% | 22% | Mix 1 |
| IP-10 | 0.23 | 1.52 – 3,333 | 6% | 8% | Mix 1 |
| KIM-1 | 0.11 | 0.51 – 1,111 | 5% | 3% | Mix 2 |
| Leptin | 4.15 | 13.7 – 30,000 | 7% | 7% | Mix 2 |
| MCP1 | 0.08 | 0.51 – 1,111 | 9% | 8% | Mix 1 |
| MCP3 | 0.10 | 0.51 – 1,111 | 9% | 11% | Mix 2 |
| M-CSF | 1.40 | 4.57 – 10,000 | 6% | 7% | Mix 2 |
| MER | 5.34 | 13.7 – 30,000 | 8% | 7% | Mix 2 |
| MIF | 6.17 | 41.2 – 90,000 | 5% | 26% | Mix 2 |
| MIG | 0.35 | 1.52 – 3,333 | 6% | 14% | Mix 1 |
| MIP1a | 0.29 | 1.52 – 3,333 | 9% | 18% | Mix 1 |
| MIP1b | 0.07 | 0.51 – 1,111 | 9% | 8% | Mix 1 |
| Analyte | Sensitivity (pg/mL) | Dynamic Range | Intra-Assay | Inter-Assay | Protein Standard |

| | | (pg/mL) | CV (n=36) | CV (n=3) | |
|---|---|---|---|---|---|
| MMP8 | 9.95 | 41.2 – 90,000 | 6% | 18% | Mix 2 |
| Osteopontin | 8.41 | 41.2 – 90,000 | 6% | 11% | Mix 2 |
| PD1 | 7.81 | 41.2 – 90,000 | 4% | 6% | Mix 2 |
| RAGE | 7.90 | 13.7 – 30,000 | 6% | 11% | Mix 2 |
| SCF | 0.34 | 1.52 – 3,333 | 6% | 10% | Mix 2 |
| sTNF RI | 0.43 | 1.52 – 3,333 | 10% | 17% | Mix 1 |
| TACI | 0.25 | 1.52 – 3,333 | 10% | 10% | Mix 2 |
| TNF-alpha | 1.15 | 4.57 – 10,000 | 8% | 17% | Mix 1 |
| VEGFA | 0.08 | 0.51 – 1,111 | 7% | 10% | Mix 2 |
| Negative Control Particle | N/A | N/A | N/A | N/A | N/A |

## 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 shelf life of 6 months from date of receipt at customer site.**

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

| Item | Quantity | Storage Condition (Before prep) |
|---|---|---|
| 10X Mouse Discovery Cytokines Capture Particles* | 1 x 1,800 uL | -20°C |
| 5X Mouse Discovery Cytokines Biotin Detectors | 1 x 400 uL | -20°C |
| Mouse Discovery Cytokines Protein Standard Mix 1 and Mix 2 (lyophilized) | 2 Each (Lyophilized) | -20°C |
| 10X Wash Buffer | 1 x 25 mL | +4°C |
| 2X Rodent Assay Diluent | 1 x 15 mL | +4°C |
| 5X Reporter Solution* | 1 x 4 mL | +4°C |
| Filter Plate (1 x 96 wells) | 1 | R/T |
| Plate Seals | 3 units | R/T |
| *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

| Item | Quantity | Storage Condition (Before prep) |
|---|---|---|
| Run Buffer I (ab245836) | 1x96 tests | +4°C |
| Run Buffer II (ab234450) | 1x96 tests | +4°C |
| Run Buffer III (ab245837) | 1x96 tests | +4°C |
| PBS (user supplied) | - | - |

  - **Note:** To determine which Run Buffer to use for your specific flow cytometer, **users must first** use the Cytometer Setup Kit V2 – ab245835.
  - **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
- Paper towels
- **FirePlex Immunoassay Intracellular Lysis Kit**, for use with cell extracts and tissue homogenates (ab239454)
- 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.

# 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.

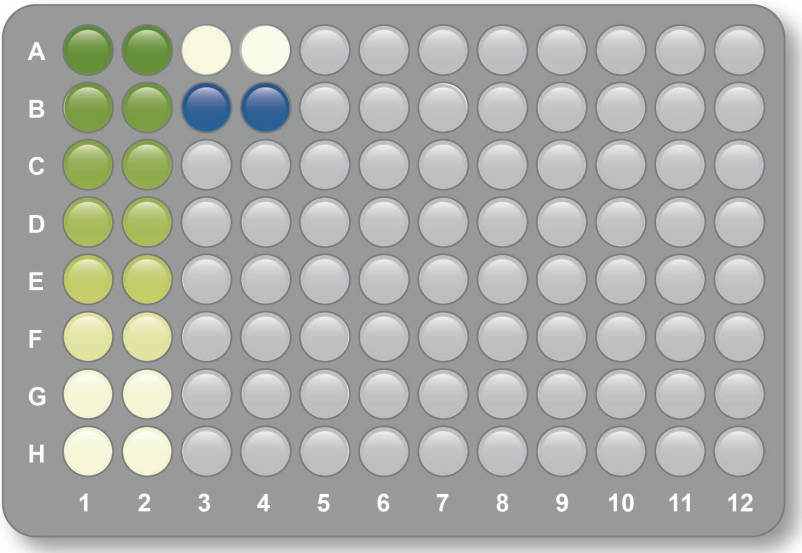
- For optimal assay performance, adequate mixing during incubation steps is critical and depends upon both speed and orbital diameter. The mixing speed of 750 RPM recommended in this manual is for a shaking incubator with an orbital diameter of 3 mm. Customers should determine the orbital diameter of their shaking incubator prior to use. For shakers with a different orbital diameter, adjust the rpm according to the formula:

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

- 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. 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:




**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)**

# 10. 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 Mouse Assay Diluent, 1X Reporter Solution and 1X Intracellular Lysis Assay Diluent provide sufficient reagent to run one full 96 well plate.

## 10.1 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.

## 10.2 1X Mouse Assay Diluent (for use with biological fluids such as serum, plasma, cell culture supernatant)

Prepare 1X Mouse Assay Diluent by diluting the 2X Rodent Assay Diluent with 1X Wash Buffer. To prepare 20 mL 1X Mouse Assay Diluent, combine 10 mL 2X Rodent Assay Diluent with 10 mL 1X Wash Buffer. Mix thoroughly and gently.

## 10.3 3X Reporter Solution

*Immediately prior to use:* Prepare sufficient volume of 3X Reporter Solution (50 µL per well) by diluting 5X Reporter Solution in 1X Wash Buffer. Prepare only enough 3X Reporter Solution as required.

To prepare 5 mL 3X Reporter Solution combine 3 mL 5X Reporter Solution with 2 mL 1X Wash Buffer. Mix thoroughly and gently.

## 10.4 Run Buffer

Supplied ready to use.

## 10.5 1X Capture Particle Solution

**10.5.1** Vortex the vial of 10X Capture Particle solution for 10 seconds to thoroughly resuspend the particles.

**10.5.2** 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.

| Number of assay wells | 10X Capture Particle solution | 1X Wash Buffer |
|---|---|---|
| 96 | 1.63 mL | 14.67 mL |
| 72 | 1.23 mL | 11.07 mL |
| 48 | 0.81 mL | 7.29 mL |
| 24 | 0.42 mL | 3.78 mL |

Δ **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. Listed values include these.

## 10.6 1X Biotin Detector Antibody Solution

**10.6.1** Centrifuge the vial of 5X Biotin Detector solution for 1 minute at 1,000 x g.

**10.6.2** Dilute the vial of 5X Biotin Detector solution to 1X in a new tube by adding 1X Mouse Assay Diluent, using the table below.

| Number of assay wells | 5X Biotin Detector solution | 1X Mouse Assay Diluent |
|---|---|---|
| 96 | 1.090 mL | 4.360 mL |
| 72 | 0.820 mL | 3.280 mL |
| 48 | 0.540 mL | 2.160 mL |
| 24 | 0.280 mL | 1.120 mL |

Δ **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. Listed values include these.

# 11. 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.

**11.1** Centrifuge each protein standard vial for 1 minute at 1,000 x g to pellet the lyophilized contents.

**11.1.1** For serum, plasma, cell culture supernatants and other biological fluid measurements, reconstitute the Mouse Protein Standard Mix A vial by adding 200 µL of 1X Mouse Assay Diluent. If there are more than one vial of protein standard mix, the combined total volume should make 200 µL. If there is vial A and B, add 100 µL 1X Mouse Assay Diluent to each vial and then combine to make the 5X stock solution.

**11.1.2** For cell extract and tissue homogenate measurements, reconstitute the Mouse Protein Standard Mix A vial by adding 200 µL of 1X Intracellular Lysis Assay Diluent.If there are more than one vial of protein standard mix, the combined total volume should make 200 µL. If there is vial A and B, add 100 µL 1X Intracellular Lysis Assay Diluent to each vial and then combine to make the 5X stock solution.

**11.2** 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 -80°C.

**11.3** Label nine tubes, Standards #1 – 9.

**11.3.1** For serum, plasma, cell culture supernatants and other biological fluid measurements, add 180 µL of 1X Mouse Assay Diluent to the tube labeled Standard #1. Add 200 µL of 1X Mouse Assay Diluent to tubes labeled Standards #2 – 8. Add 250 µL of 1X Mouse Assay Diluent to Standard #9.

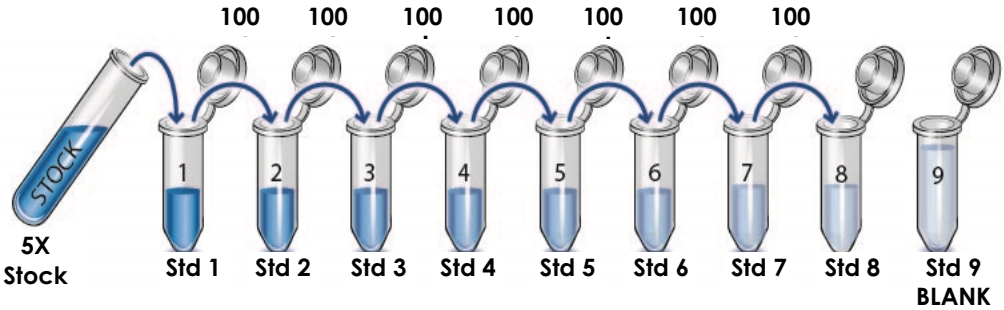
11.3.2 For cell extract and tissue homogenate measurements, add 180 µL of 1X Intracellular Lysis Assay Diluent to the tube labeled Standard #1. Add 200 µL of 1X Intracellular Lysis Assay Diluent to tubes labeled Standards #2 – 8. Add 250 µL of 1X Intracellular Lysis Assay Diluent to Standard #9.

11.4 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.

| Protein Standard(s) |
|---|
| Mouse Discovery Cytokines Protein Standard Mix 1 |
| Mouse Discovery Cytokines Protein Standard Mix 2 |

11.5 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.



# 12. 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.

| Recommended Sample Dilutions | |
|---|---|
| **Sample Type** | **Starting Dilution** |
| Cell Culture Supernatant | 1:4 |
| Serum | 1:4 |
| Plasma – Citrate | 1:4 |
| Plasma – EDTA | 1:4 |
| Plasma – Heparin | 1:4 |
| Cell Extract | 50 – 500 µg/mL |
| Tissue Homogenate | 50 – 500 µg/mL |

## 12.1 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 Mouse Assay Diluent and assay. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

## 12.2 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 Mouse Assay Diluent and assay. Store un-diluted serum at -20°C or below. Avoid repeated freeze-thaw cycles.

## 12.3 Plasma

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

## 12.4 Preparation of extracts from cell pellets:

**12.4.1** 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.

**12.4.2** Rinse cells twice with PBS.

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

**12.4.4** Incubate on ice for 20 minutes.

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

**12.4.6** Transfer the supernatants into clean tubes and discard the pellets.

**12.4.7** 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.

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

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

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

**12.5.2** 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.

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

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

**12.5.5** Transfer the supernatants into clean tubes and discard the pellets.

**12.5.6** 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.

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

## 12.6 Preparation of extracts from tissue homogenates:

**12.6.1** 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).

**12.6.2** 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.

**12.6.3** Incubate on ice for 20 minutes.

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

**12.6.5** Transfer the supernatants into clean tubes and discard the pellets.

**12.6.1** 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.

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

## 13. 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.

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

13.2 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.**

13.3 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.

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

13.5 Cover with plate lid and incubate overnight at +4°C with shaking at 750 rpm in the dark.

13.6 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.

13.7 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.

13.8 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.

13.9 Add 50 µL of **freshly prepared** 3X Reporter Solution to each well.

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

13.11 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.

13.12 Add 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.

13.13 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.

# 14. Flow Cytometer Acquisition

## 14.1 Flow Cytometer with plate handler

**14.1.1** 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.

**14.1.2** 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.

# 15. 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”.

# 16. Troubleshooting

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

# Technical Support

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