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# ProcartaPlex®

## Multiplex Immunoassay

Using Magnetic Beads
For Cell Culture Supernatant Samples
Applicable only for FLEXMAP3D® users

Instructions for
Mouse Assays (384 tests)


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When describing a procedure for publication using this product, please refer to it as the ProcartaPlex® Multiplex Immunoassay from eBioscience.

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### Trademarks

## Contents

## Intended Use

This user manual is for a ProcartaPlex® Immunoassay Kit from the eBioscience division of Affymetrix to perform quantitative, multiplexed protein measurements from serum, plasma, and cell culture supernatant samples using magnetic beads technology from Luminex®. Other biological samples might be suitable for use in the assay.

**NOTE: For the most current version of user documentation, go to our website at www.ebioscience.com**

## How it Works

ProcartaPlex Immunoassays incorporate magnetic microsphere technology licensed from the Luminex Corporation to enable the simultaneous detection and quantitation of multiple protein targets in diverse matrices. The platform allows the simultaneous detection from a single sample of up to 100 protein targets on the Luminex 200/100 and FLEXMAP 3D® platforms and 50 protein targets on the MAGPIX® platform.

## Materials Provided and Storage Conditions

ProcartaPlex Immunoassay Kits contain the components listed below. Refer to the Certificate of Analysis for quantities and details of components supplied. Expiration date is stated on the kit when stored between 2-8°C. Do not use past kit expiration date.

| Components Supplied | Pre-mixed Panels | Simplex Kit | Basic Kit | Custom Panels |
|---|---|---|---|---|
| Antigen Standards, premixed | √ | √ | | √ |
| Detection Antibody, premixed (50X) [1] | √ | √ | | |
| Detection Antibody, premixed (1X)[1] | | | | √ |
| Antibody Magnetic Beads, premixed[1] | √ | √ | | √ |
| Streptavidin-PE (SA-PE) (1X)[1] | √ | | √ | √ |
| Wash Buffer Concentrate (10X)[1] | √ | | √ | √ |
| Detection Antibody Diluent[1] | √ | | √ | |
| Reading Buffer[1] | √ | | √ | √ |
| PCR 8-Tube Strip | √ | | √ | √ |
| Filter Plate | | | | |
| Black Microplate Lid | √ | | √ | √ |
| Plate Seals | √ | | √ | √ |

[1] Contains sodium azide. See WARNING.

[2] 384-Well Filter Plate (Cat. No. EPX38488-000) is not included into this kit and can be ordered separately.

**WARNING: All chemicals should be considered potentially hazardous. We recommend that this product and its components be handled by those trained in laboratory techniques and be used according to the principles of good laboratory practice. This kit contains small quantities of sodium azide. Sodium azide is highly toxic and reactive in the pure form. At this product's concentration, though not classified as hazardous, build up of sodium azide may react with lead and copper plumbing to form highly reactive explosive metal azide. Dispose of the product in accordance with all State and local regulations.**

**NOTE: ProcartaPlex® Supplemental Products can be also purchased as stand alone items. For more information go to our website at www.ebioscience.com/product-line/procartaplex/complementaryproducts.htm contact the technical service under tech@ebioscience.com**

## Precautions and Technical Hints

- Thoroughly read this user manual and Certificate of Analysis that is included with the assay kit. The product insert may contain specific instructions for proper use of your kit.
- For FLEXMAP 3D instrument initiate the startup protocol to warm up the lasers for at least 30 minutes. Ensure that the Luminex machine is calibrated according to the manufacturer's instructions.
- When working with samples and standards, change the pipette tips after every transfer and avoid creating bubbles when pipetting.
- During the incubation steps, cover the 384-Well Filter Plate with the Black Microplate Lid provided in the kit to minimize exposure of the beads to light.
- Be careful not to invert the 384-Well Filter Plate during the assay or allow contents from one well to mix with another well.
- Use a multi-channel pipette and reagent reservoirs whenever possible to achieve optimal assay precision.
- Store the reconstituted standards (including standard diluent sets) on ice before adding to the 384-Well Filter Plate.

## Required Equipment and Materials Not Supplied

- FLEXMAP 3D.
- Glass-distilled or deionized water.
- Adjustable single and multi channel pipettes with disposable tips.
- Multichannel pipette reservoir.
- Beakers, flasks, cylinders necessary for preparation of reagents.
- Vacuum Filtration Manifold.

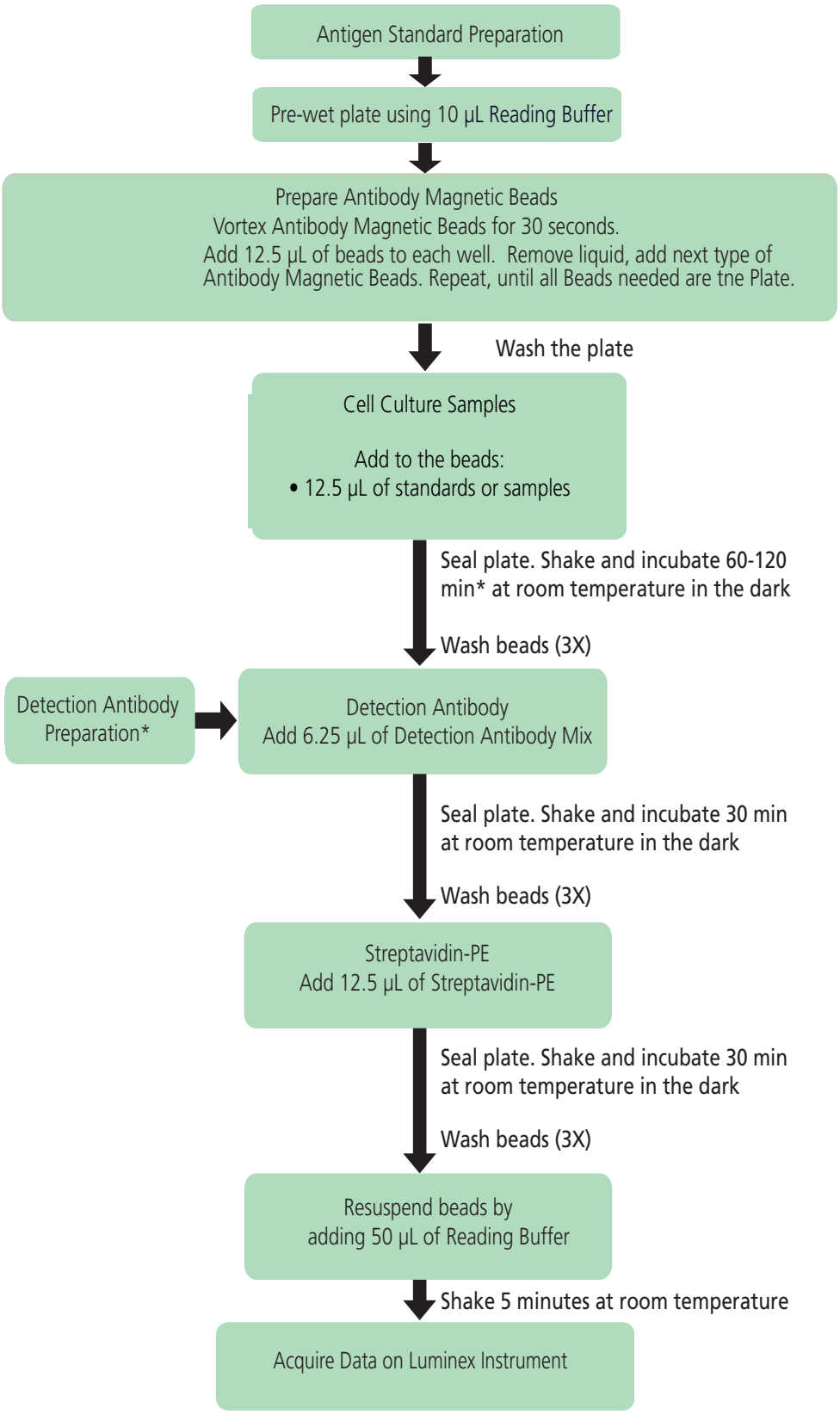
## Sample Preparation

- For frozen samples, thaw samples on ice and mix well by vortexing followed by centrifugation to remove particulates. Avoid multiple freeze/thaw cycles.
- If there is a high lipid content in the sample, centrifuge at 10,000 x g for 10 min at 2-8 °C and transfer contents to a new tube.
- For preparation instruction of lysates from cultured cells refer to **www.ebioscience.com/resources/best-protocols/multiplexing.htm**

### Diluting Samples with High Concentration of Target Analytes

You may need to further dilute your samples if the analyte concentration is above the assay upper limit of quantitation (ULOQ). For cell culture supernatant samples, we recommend using the medium that was used to culture the cells.

## Assay Protocol Overview




*For assays that require higher sensitivity, 120 min or overnight incubation is recommended.

## Preparation of Reagents

### Antigen Standard

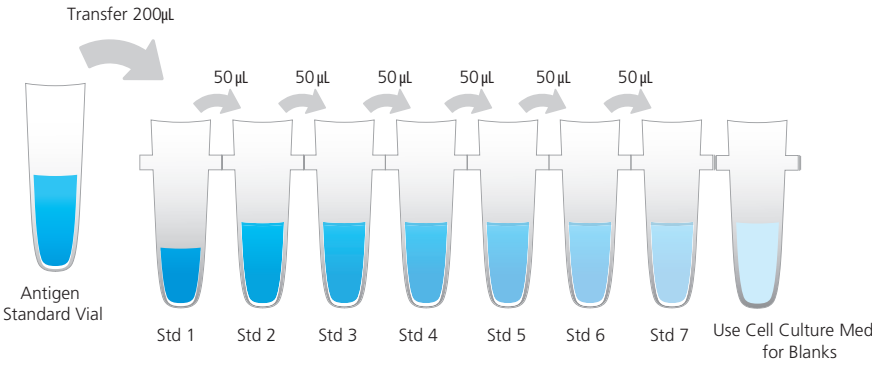
Carefully review the Certificate of Analysis for kit specific Antigen Standard preparation instructions. The majority of kits is supplied with lyophilized multi-standards containing a mix of multiple standard proteins. Some kits contain multiple sets of standards, each with a unique lot number, that require pooling prior to use. Each kit is shipped with two identical vials of each premixed antigen standard set from the same lot to permit the user to run the assay twice if running a partial plate. When preparing Antigen Standards, the final volume after reconstitution and pooling should be 250 µL. When combining multiple kits, ensure that the Antigen Standards of your analytes of interest are only present in one of the used standard vials.

Alternative preparation instructions for combination of pre-mixed panels with simplex kits or different simplex kits with more then 5 antigen standard sets are available upon request or can be downloaded under **www.ebioscience.com/resources/best-protocols/multiplexing.htm**

In Custom Panels with more then 5 antigen standard sets alternative preparation instruction will be included as an extra data sheet.

| Step | Action |
|---|---|
| **Step 1. Reconstitution and pooling of Standards** | **A.** Centrifuge each different antigen standard set vial(s) at 2000 x *g* for 10 sec.<br>**B.** Add 50 µL of cell culture media that was used to culture the cells.<br>**C.** Gently vortex the vial(s) for 30 seconds and centrifuge at 2000 x *g* for 10 seconds to collect contents at the bottom of the vial(s).<br>**D.** Incubate on ice for 10 min to ensure complete reconstitution.<br>**E.** Pool entire contents of each vial into one of the vials and add sample type specific buffer to quantity sufficient (q.s.) to 250 µL. (See table below for example).<br>**F.** Gently vortex the vial for 30 seconds and centrifuge at 2000 x *g* for 10 seconds to collect contents at the bottom of the vial. |

| # of Standard Sets | Reconstitution Volume per vial | Pooled Volume | Buffer to q.s. | Total Volume |
|---|---|---|---|---|
| 1 | 50 µL | 50 µL | 200 µL | 250 µL |
| 2 | 50 µL | 100 µL | 150 µL | 250 µL |
| 3 | 50 µL | 150 µL | 100 µL | 250 µL |
| 4 | 50 µL | 200 µL | 50 µL | 250 µL |
| 5 | 50 µL | 250 µL | 0 µL | 250 µL |

| Step | Action |
|---|---|
| **Step 2. Prepare 4-Fold Serial Dilution** | **A.** Refer to Certificate of Analysis for the value of each premixed standard with assigning S1 values for each analyte for the current lot.<br>**B.** Prepare a 4-fold serial dilution of the reconstituted standard(s) using the PCR 8-tube strip provided. Label tubes Std1, Std2, Std3, Std4, Std5, Std6 and Std7.<br>**C.** Add 200 µL of the reconstituted antigen standards into the first tube of the strip tube and label as Standard 1 (Std1).<br>**D.** Add 150 µL of cell culture media into Std tubes 2-7.<br>**E.** Transfer 50 µL of the reconstituted antigen standards from Tube 1 into Tube 2.<br>**F.** Mix by pipetting up and down for a total of 10 times.<br>**G.** Change the pipette tip and transfer 50 µL of the mixed standards from Tube 2 into Tube 3.<br>**H.** Mix by pipette up and down 10 times.<br>**I.** Repeat steps E-H for Std tubes 4-7.<br>**J.** Add 200 µL of cell culture media into tube 8 which serves as a blank. Keep on ice until ready to use.<br>  <br> |

## Assay Protocol

The Antibody Magnetic Beads can be used with a Vacuum Filtration Manifold and the 384-Well Filter Plate. The Vacuum Manifold can be properly calibrated by placing a standard 384-Well Filter Plate on top of the manifold and then turning on the vacuum. Press down on all 4 corners of the standard 384-Well Filter Plate to form a tight seal and adjust the pressure so that it takes 4-6 seconds to evacuate 50 µL of Wash Buffer from the wells. If the vacuum is too high, beads can get trapped or pulled through the filter. Turn off vacuum as soon as the solution filters through the wells and remove the plate from the manifold.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 1. Prepare 1X Wash Buffer</td><td>Bring the Wash Buffer Concentrate (10X) to room temperature and vortex for 15 seconds. Mix 20 mL of the Wash Buffer Concentrate (10X) with 180 mL dd$H_2O$. Wash Buffer (1X) can be stored at 2-8 °C for up to 6 months.</td></tr>
<tr><td>Step 2. Define the plate map</td><td>A. Mark the standard, sample and blank wells using the plate map at the end of this manual.<br>B. Add 10 µL Reading Buffer (1X) to the 384-Well Filter Plate to pre-wet the wells. Aspirate using the Vacuum Manifold. Blot the bottom of the plate after filtration.</td></tr>
<tr><td>Step 3a. Add the Antibody Magnetic Beads (only 1 bead set)</td><td>A. Vortex the Antibody Magnetic Beads for 30 sec.<br>B. Add 12.5 µL of the Antibody Magnetic Beads to each well. Use a multi-channel pipette for this step as well as the steps below.</td></tr>
<tr><td>Step 3b. Add the Antibody Magnetic Beads (2 or more beads sets)</td><td>IMPORTANT: ProcartaPlex pre-mixed panel and simplex kits can be mixed together for enhanced flexibility. Ensure that the bead regions from your ProcartaPlex panels and simplex kits do not overlap. Some analytes use the same bead region and cannot be combined together in one multiplex assay. Please check the compatibility of our analytes using our online panel configurator or contact our technical service at tech@ebioscience.com.<br>Both simplex and pre-mixed kits are supplied with Antibody Magnetic Beads at working concentration that require 12.5 µL beads per well or 5 mLs per 384-wells. For running a partial plate adjust the volume accordingly.<br>A. Vortex each of the Antibody Magnetic Bead sets for 30 sec and add 5 mL of each bead set for 384-wells to an appropriate sized tube. After all of the bead sets are added, vortex the tube for another 30 sec.<br>B. Add the bead mix to a disposable reservoir and add the appropriate volume of Antibody Magnetic Beads to each well of the 384-Well Filter Plate using the table below:
<table>
<tr><th># of different Magnetic Bead sets to be mixed</th><th>Amount added to each well in µL</th></tr>
<tr><td>2</td><td>25 µL</td></tr>
<tr><td>3</td><td>37.5 µL</td></tr>
<tr><td>4</td><td>50 µL</td></tr>
<tr><td>5</td><td>62.5 µL</td></tr>
<tr><td>6</td><td>75 µL</td></tr>
</table>
If more than 6 bead sets are to be mixed proceed to Step 4 and repeat step 3b until all Antibody Magnetic Beads have been added and washed.</td></tr>
</table>

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 4. Wash Antibody Magnetic Beads</td><td>A. Carefully remove the Plate Seal to avoid splashing the plate contents.<br>B. Remove the liquid in the wells by aspiration using Vacuum Filtration Manifold. Blot the bottom of the plate after filtration.<br>C. Add 50 µL of Wash Buffer (1X) into each well.<br>D. Remove the liquid in the wells by aspiration using Vacuum Filtration Manifold. Blot the bottom of the plate after filtration.</td></tr>
<tr><td>Step 5. Add sample type-specific buffer, samples, standards and blanks and incubate</td><td>A. For cell culture supernatant samples add 12.5 µL standards or samples into dedicated wells.<br>B. For wells designated as blanks add 12.5 µL of cell culture medium.<br>C. Seal the plate with the provided Plate Seal. Cover the plate with the Black Microplate Lid and shake at 500 rpm for 60 to 120 min at room temperature (RT).<br>D. Alternatively, the 384-Well Plate can be incubated overnight. Shake the 384-Well Plate for 30 min at RT at 500 rpm, then transfer the plate to 4°C and store on a level surface. After overnight incubation, shake the plate for an additional 30 min at RT at 500 rpm.</td></tr>
<tr><td>Step 6. Wash the 384-Well Plate</td><td>Wash plate for a total of three times using "Step 4. Wash Antibody Magnetic Beads".</td></tr>
<tr><td>Step 7. Prepare 1X Detection Antibody Mixture</td><td>A. Prepare fresh prior to use detection antibodies. If intending overnight incubation do not prepare detection antibodies at this point.<br>B. Detection antibodies for Custom Panels are provided at a 1X concentration and do not require dilution.<br>C. For simplex and pre-mixed panels, detection antibody is provided at 50X concentration. Add 60 µL of each detection antibody concentrate to the mixing bottle and bring volume of the mixing bottle to a total of 3 mL using detection antibody diluent.Tables below are an example for 384 and 192-wells.<br>Example for using 384-wells<br><table><tr><th># of Vials of Detection Antibody</th><th>Total Volume of Mixed Detection Antibody</th><th>Volume of Diluent to Add</th></tr><tr><td>1</td><td>60 µL</td><td>2940 µL</td></tr><tr><td>2</td><td>120 µL</td><td>2880 µL</td></tr><tr><td>3</td><td>180 µL</td><td>2820 µL</td></tr><tr><td>4</td><td>240 µL</td><td>2760 µL</td></tr></table>Example for using 192-wells<br><table><tr><th># of Vials of Detection Antibody</th><th>Total Volume of Mixed Detection Antibody</th><th>Volume of Diluent to Add</th></tr><tr><td>1</td><td>30 µL</td><td>1470 µL</td></tr><tr><td>2</td><td>60 µL</td><td>1440 µL</td></tr><tr><td>3</td><td>90 µL</td><td>1410 µL</td></tr><tr><td>4</td><td>120 µL</td><td>1380 µL</td></tr></table></td></tr>
</table>

| Step | Action |
|---|---|
| **Step 8. Add Detection Antibody Mixture and incubate** | **A.** Add 6.25 µL of Detection Antibody Mixture (1X) to each well.<br>**B.** Seal the plate with a new Plate Seal, cover plate with Black Microplate Lid and incubate 30 min on a plate shaker at RT at 500 rpm. |
| **Step 9. Wash the 384-Well Plate** | Wash plate for a total of three times using "Step 4. Wash Antibody Magnetic Beads". |
| **Step 10. Add SAPE and incubate** | **A.** Add 12.5 µL of SAPE solution to each well.<br>**B.** Seal the plate with a new Plate Seal, cover plate with Black Microplate Lid and incubate 30 min on a plate shaker at RT at 500 rpm. |
| **Step 11. Wash the 384-Well Plate** | Wash plate for a total of three times using "Step 4. Wash Antibody Magnetic Beads". |
| **Step 12. Prepare the 384-Well Plate for Analysis on a Luminex Instrument** | **A.** Add 50 µL of Reading Buffer into each well.<br>**B.** Seal the plate with a new Plate Seal, cover plate with Black Microplate Lid and incubate 5 min on a plate shaker at RT at 500 rpm.<br>**C.** Remove Plate Seal and run the plate on a Luminex Instrument. |

## Setup of the Luminex Instruments

| Sample Size | DD Gate | Timeout | Bead Event/Bead Region |
|---|---|---|---|
| 50 µL | 5,000 - 25,000 | 60 sec | 50-100 |

Verify the probe height for each plate before reading. Failure to adjust the probe height can cause damage to the instrument. The Luminex system allows for calibration of Low and High RP1 target values. We recommend RP1 Low target value settings for ProcartaPlex Immunoassays.

Please refer to the Certificate of Analysis provided with the kit for bead region and analyte associations when entering the information into the Luminex Acquisition Software.

**NOTE: If there is a malfunction of the Luminex Instrument or software during the run, the 384-Well Plate can be re-read. Remove the 384-Well Plate from the instrument and remove the liquid in the wells by aspiration using the Vacuum Filtration Manifold. Blot the bottom of the plate after filtration. Resuspend the beads in 50 µL of Reading Buffer, seal the 384-Well Plate with a new Plate Seal and shake at 500 rpm for 5 min at room temperature. The assayed samples may take longer to read since there will be less beads in the wells.**

## Analyzing Results

The concentration of the samples can be calculated by plotting the expected concentration of the standards against the MFI generated by each standard. A 4PL or 5PL algorithm is recommended for the best curve fit. Analyze the assayed samples according to the operation manual for the Luminex Instrument (e.g MAGPIX®, Luminex® 100/200™, FLEXMAP 3D®). We offer a free and robust analysis software package for data analysis. ProcartaPlex Analyst 1.0 can be download at: **www.ebioscience.com/resources/procartaplex-analyst-1.0-software.htm**

# Troubleshooting

| Observation | Probable Cause | Recommend Solution |
|---|---|---|
| Low Flow Rate | Samples/beads are stuck in flow cell | Remove the 384-Well Plate and perform a wash and rinse cycle. |
| High CVs | Samples and antigen standards not stored on ice | Prepare the samples and standards on ice before setting up the assay. |
| | Contamination from re-using the Plate Seal | Use a new Plate Seal for each incubation step. |
| | Incomplete washing | After adding the standards and samples, it is very important that any excess standards are removed during the wash step. |
| | Contamination from contents from adjacent wells | Avoid splashing the Wash Buffer during wash steps into adjacent wells. |
| | Poor pipetting techniques | Use a multichannel pipettor and careful pipette techniques. Avoid touching pipette tips to sides of the wells when adding Wash Buffer. |
| Limited dynamic range for BioPlex software users | Instrument calibrated at high PMT settings | Calibrate the instrument using the CAL2 Low RP1 target value. |
| Low bead count | Volume of bead solution is too low | Add 120 µL Reading Buffer into each well and shake at 500 rpm for 5 min at room temperature to resuspend beads prior to reading on the Luminex Instrument. |
| | High bead aggregation | Vortex the bead suspension well before using in the assay and ensure that the beads are properly mixed during the incubation steps. |
| | Dyes contained in the beads are photo-bleached from overexposure to light | Store bead solution and the 384-Well Plate in the dark. |
| | Samples causing the instrument to clog | Remove the 384-Well Filter Plate and perform a wash and rinse to the instrument. Rerun the assay with further dilution of samples. |
| | Probe height is incorrect | Refer to the Luminex Manual for proper adjustment of the needle height. |
| | Instrument needle is partially clogged | Replace or clean needle according to the manufacturer's recommendations. |
| | Beads stuck to the bottom of the plate | Confirm that the plate shaker is set to 500 rpm and shaking for at least 5 min before reading. |
| | Air bubble in the sample loop | Refer to the Luminex manual for proper removal of the air bubble. |
| Low signal or sensitivity | Standards not reconstituted and diluted correctly | Prepare fresh antigen standards following the instructions in the Preparing Antigen Standards section. |
| Poor recovery | Did not use appropriate cell culture media to prepare the standards | Use the same cell culture media that is used to culture the cells. |
| | Samples and antigen standards were not stored on ice | Prepare the samples and standards on ice before setting up the assay. |

# Recommended and Blank Plate Layout

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
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| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
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