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ProcartaPlex®

Multiplex Immunoassay

For Convenience and Mix&Match Panels

Using Magnetic Beads
For Serum, Plasma, and Cell Culture Supernatant Samples

Instructions for Human, NHP and Canine Assays

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Citing ProcartaPlex® Immunoassay in Publications

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

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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. Store kit at 2-8°C. Expiration date is stated on the kit.Do not use after expiration date.

Components Supplied
Antigen Standards, premixed
Detection Antibody, premixed (1X)[1]
Magnetic Beads, premixed (1X)[1]
Streptavidin-PE (SA-PE) (1X)[1]
Wash Buffer Concentrate (10X)[1]
Competitive Conjugate Solution (50X)[1,2]
Universal Assay Buffer (1X)[1]
Universal Assay Buffer Concentrate (10X)[1] (optional)
Reading Buffer[1]
PCR 8-Tube Strip
96-Well Flat Bottom Plate
Black Microplate Lid
Plate Seals

[1]Contains sodium azide. See WARNING.
[2]Will be included in Competitive Assays only. Refer to the table on the next page.

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.

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 Luminex 100/200 and FLEXMAP 3D instruments 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. MAGPIX instrument doesn't require additional warm up.
- 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 96-Well Flat Bottom Plate with the Black Microplate Lid provided in the kit to minimize exposure of the beads to light.
- Be careful not to invert the 96-Well Flat Bottom 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 on ice before adding to the 96-Well Flat Bottom Plate

Required Equipment and Materials Not Supplied

- MAGPIX, Luminex® 100/200, FLEXMAP 3D, or Luminex-based Instrument.
- 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.
- Hand-Held Magnetic Plate Washer, Vortex mixer and Microtiter plate shaker.

Competitive Assays

Competitive Assays are based on competitive ELISA technique and require addition of Competitive Conjugate Solution to samples, standards and blanks. Competitive Assays are listed below:

Species	Analytes
Human	Cortisol

Preparation of Competitive Conjugate Solution (1X) is described in "Preparation of Reagents".

Sample Preparation

- For frozen samples, thaw samples on ice and mix well by vortexing followed by centrifugation at 10,000 x g for 5-10 min to remove particulates. Avoid multiple freeze/thaw cycles.
- If samples are high in lipid content, centrifuge at 10,000 x g for 10 min and transfer contents to a new tube.

For preparation instruction of tissue homogenates and lysates from cultured cells refer to **www.ebioscience.com/resources/best-protocols/multiplexing.htm**

Plasma Sample Preparation

	Action
A.	Collect samples in sodium citrate or EDTA tubes. When using heparin as an anticoagulant, no more than 10 IU of heparin per mL of blood collected should be used since an excess of heparin may give falsely high values of some of the analytes.
B.	Centrifuge samples at 1,000 x *g* at 4 °C for 10 min within 30 min of collection.
C.	Collect the plasma fraction. Use immediately or aliquot and store at -80 °C.

Serum Sample Preparation

We recommend to spin down serum samples at 1,000 x *g* for 10 min at 20-25 °C before running the assay.

Action
A. Allow blood to clot for 20-30 min at 20-25 °C.
B. Centrifuge at 1,000 x *g* for 10 min at 20-25 °C.
C. Collect the serum fraction. (Alternatively, use any standard serum separator tube following the manufacturer's instructions.)
D. Use immediately or aliquot and store at -80 °C.

Dilution of Samples

You may need to further dilute your samples if the analyte concentration exceeds the assay upper limit of quantitation (ULOQ). When preparing dilution of serum and plasma samples use Universal Assay Buffer (1X). For dilution of cell culture supernatant samples, use cell culture medium that was used to culture the cells. Recommended dilution factors for analytes with high normal serum or plasma concentration are listed in the table below.

NOTE: For analytes that show high concentration in serum and plasma additional Universal Assay Buffer (10X) will be included in the kit.

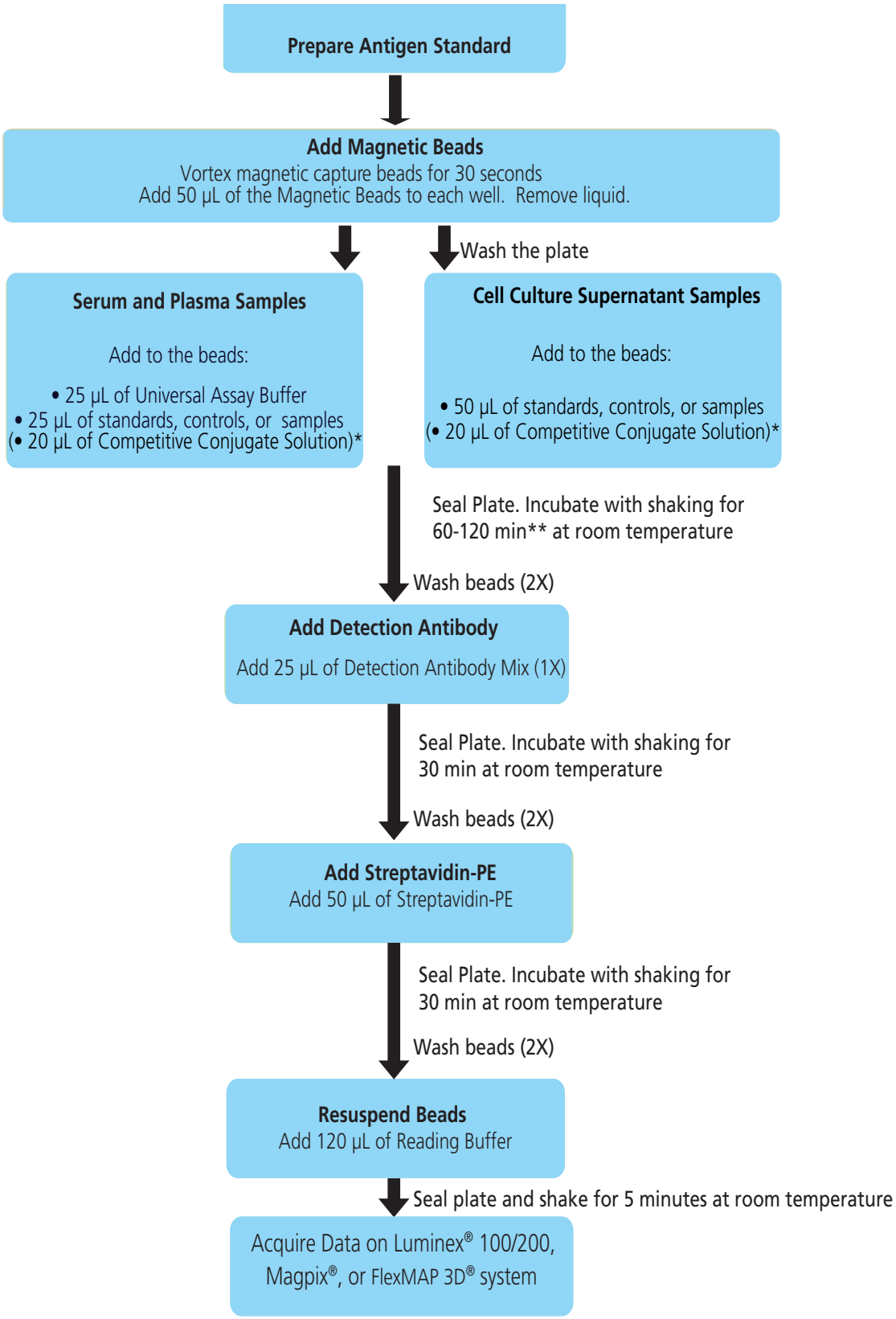
Species	Analytes	Recommended Sample Dilution Factor
Human, NHP	Adiponectin	200
Human	CRP	500
Human	CD44var (var6)	20
Human	Fibrinogen	200000 [1]
Human	Endoglin	20
Human, NHP	ICAM-1	200
Human	MMP-2	50
Human	MMP-3	50
Human	MMP-9	50
Human	NGAL	200
Human	Osteopontin	20
Human	RBP4	200
Human, NHP	RANTES	50
Human	SAA	200
Human	SAP	4000
Human	SCGF-beta	25
Human	L-Selectin	200
Human	TIMP-1	20
Human, NHP	VCAM-1	200
Human	ZAG	200

[1]Dilution required only for plasma samples.

TGF beta (Acidification of Samples)

TGF beta 1 has to be acid treated for proper detection of the bioactive form. Therefore this analyte should be tested as simplex assay. An additional datasheet with the preparation instruction will be included in each kit or can be found at **www.ebioscience.com/resources/best-protocols/multiplexing.htm**

Assay Protocol Overview



*For Competitive Assays, add Competitive Conjugate Solution.
**For assays that require higher sensitivity, 120 min or overnight incubation is recommended.

Preparation of Reagents

Preparing 1X Wash Buffer

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 ddH_2O. Wash Buffer (1X) can be stored at 2-8 °C for up to 6 months.

NOTE: Wash Buffer Concentrate volume might not be sufficent if using automated plate washer. For bulk orders use Cat. No. EPX-66666-001.

Preparing 1X Universal Assay Buffer

For analytes with high serum or plasma concentration 10X Universal Asssay Buffer will be provided. Prepare a 1X working concentration of Universal Assay Buffer by mixing 10 mL of the 10X Universal Assay Buffer with 90 mL ddH_2O.

Preparing 1X Competitive Conjugate Solution for Competitive Assays only

Competitive Conjugate Solution (50X) will only be included in Competitive Assays. Prepare the Competitive Conjugate Solution (1X) by mixing 50 µL of Competitive Conjugate Solution (50X) with 2450 µL Universal Asssay Buffer (1X).

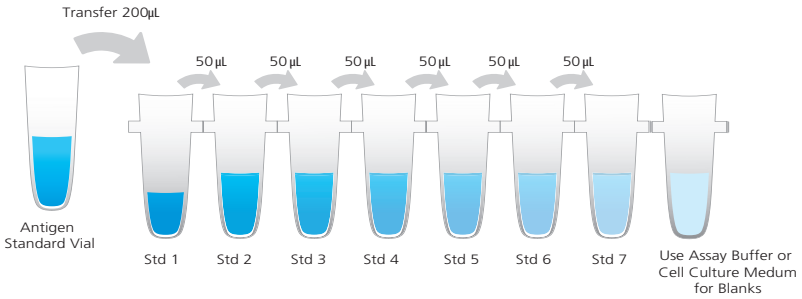
Preparing Antigen Standard

Carefully read 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.

Instructions for combining more than 5 antigen standard sets can be downloaded at: **www.ebioscience.com/resources/best-protocols/multiplexing.htm** (see point: Preparation of Antigen Standard (alternative instructions)).

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. **B.** Add 50 µL of sample type specific buffer into each standard vial. If you want to measure serum or plasma samples , use Universal Assay Buffer (1X) to reconstitute the standard, if you want to analyse cell culture supernatant samples, use the cell culture medium that was used to culture the cells to dissolve the standard. **C.** Gently vortex the vial(s) for 10 seconds and centrifuge at 2000 x *g* for 10 seconds to collect contents at the bottom of the vial(s). **D.** Incubate on ice for 10 min to ensure complete reconstitution. **E.** Pool entire contents of each vial into one of the vials and fill up with sample type specific buffer to a total volume of 250 µL. **F.** Gently vortex the vial for 10 seconds and centrifuge at 2000 x *g* for 10 seconds to collect contents on the bottom of the vial.

# of Standard Sets	Reconstitution Volume per vial	Pooled Volume	Buffer to add	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.** 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. **B.** Add 200 µL of the reconstituted antigen standards into the first tube of the strip tube and label as Standard 1 (Std1). **C.** Add 150 µL of sample type specific standard buffer into Std tubes 2-7. Use Universal Assay Buffer for serum or plasma samples and cell culture media for culture supernatant samples. **D.** Transfer 50 µL of the reconstituted antigen standards from Tube 1 into Tube 2. **E.** Mix by pipetting up and down for a total of 10 times. **F.** Transfer 50 µL of the mixed standards from Tube 2 into Tube 3. **G.** Mix by pipetting up and down for a total of 10 times. **H.** Repeat steps D-G for Std tubes 4-7. **I.** Add 200 µL of Universal Assay Buffer or cell culture medum into tube 8 which serves as a blank. Keep on ice until ready to use. 

Assay Protocol

Step	Action
Step 1. Define the plate map	Mark the standard, sample and blank wells using the plate map at the end of this manual.
Step 2. Addition of Magnetic Beads to the plate	**A.** Vortex the Magnetic Bead vial for 30 sec. **B.** Add 50 µL of the Magnetic Bead solution to each well of the plate. Use a multichannel pipette for this step as well as for the steps below.
Step 3. Wash Magnetic Beads	**A.** Securely insert the 96-Well Flat Bottom Plate into the Hand-Held Magnetic Plate Washer and ensure that the plate is held in place by the tabs. Wait 2 min to allow the beads to accumulate on the bottom of each well. **B.** Remove the liquid in the wells by quickly inverting the Hand-Held Magnetic Plate Washer and 96-Well Flat Bottom Plate assembly over a sink or waste container. Do not remove the 96-Well Flat Bottom Plate from the Hand-Held Magnetic Plate Washer. Blot the inverted assembly onto several layers of paper towels or absorbent surface to remove any residual solution. **C.** Add 150 µL of Wash Buffer (1X) into each well and wait 30 seconds to allow the beads to accumulate on the bottom of each well. **D.** Remove the Wash Buffer in the wells by quickly inverting the Hand-Held Magnetic Plate Washer and 96-Well Flat Bottom Plate assembly over a sink or waste container. Do not remove the 96-Well Flat Bottom Plate from the Hand-Held Magnetic Plate Washer. Blot the inverted assembly onto several layers of paper towels or absorbent surface to remove any residual solution. **E.** Remove the 96-Well Flat Bottom Plate from the Hand Held Magnetic Plate Washer and proceed to the next step.
Step 4. Add sample type-specific buffer, samples, standards and blanks and incubate	**A.** For serum and plasma: Add 25 µL of Universal Assay Buffer (1X) to each well followed by 25 µL of prepared standards or samples into dedicated wells. For cell culture supernatants: Add 50 µL prepared standards or samples into dedicated wells. **B.** For wells designated as blanks, add an additional 25 µL of Universal Assay Buffer for serum or plasma samples. For cell culture supernatant samples, add 50 µL of cell culture medium. **C.** *If not working with a Competitive Assay skip this step and PROCEED with D.* *For Competitive Assays only: Dilute Competitive Conjugate Solution (50X)* as described in Preparation of Reagents. *Add 20 µL of the diluted Competitive Conjugate Solution (1X) to each well and proceed with step D.* **D.** 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). **E.** Alternatively, the 96 well plate can be incubated overnight. Shake the 96 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.
Step 5. Wash the 96-Well Plate	Wash the plate twice following "Step 3. Wash Magnetic Beads".

Step	Action
Step 6. Add Detection Antibody Mixture and incubate	**A.** Add 25 µL of Detection Antibody Mixture (1X) to each well. **B.** Seal the plate with a new Plate Seal, cover the plate with the Black Microplate Lid and incubate 30 min on a plate shaker at RT at 500 rpm.
Step 7. Wash the 96-Well Plate	Wash the plate twice following "Step 3. Wash Magnetic Beads".
Step 8. Add SAPE and incubate	**A.** Add 50 µL of SAPE solution to each well. **B.** Seal the plate with a new Plate Seal, cover plate with the Black Microplate Lid and incubate 30 min on a plate shaker at RT at 500 rpm.
Step 9. Wash the 96-Well Plate	Wash the plate twice following "Step 3. Wash Magnetic Beads".
Step 10. Prepare the 96-Well Plate for Analysis on a Luminex Instrument	**A.** Add 120 µL of Reading Buffer into each well. **B.** Seal the plate with a new Plate Seal, cover the plate with the Black Microplate Lid and incubate 5 min on a plate shaker at RT at 500 rpm. **C.** Remove Plate Seal and run the plate on a Luminex Instrument.

Setup of the Luminex Instruments

Instrument	Sample Size	DD Gate	Timeout	Bead Event/Bead Region
Luminex 100/200 FLEXMAP 3D	50 µL	5,000 - 25,000	60 sec	50-100
MAGPIX	50 µL	N/A	N/A	50-100

Prior to running the assay, ensure that the probe height has been calibrated with 96-Well Flat Bottom Plate supplied with the kit. Failure to adjust the probe height can cause damage to the instrument or low bead count. The Luminex system allows for calibration of Low and High RP1 target values. We recommend RP1 Low target value settings for ProcartaPlex Immunoassays. When entering the information into the Luminex Acquisition Software please refer to the Certificate of Analysis provided with the kit for bead region and S1 values for each analyte of the current lot.

NOTE: If there is a malfunction of the Luminex Instrument or software during the run, the 96-Well Flat Bottom Plate can be re-read. Remove the 96-Well Flat Bottom Plate from the instrument, insert the 96-Well Flat Bottom Plate into the Hand-Held Magnetic Plate Washer, wait 2 min, then remove the buffer in the wells by quickly inverting the 96-Well Flat Bottom Plate over a sink or waste container. Blot the assembly onto several layers of paper towels to remove any residual solution. Resuspend the beads in 120 µL of Reading Buffer, remove from the Hand-Held Magnetic Plate Washer, seal the 96-Well Flat Bottom Plate with a new Plate Seal and Lid 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 well.

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 downloaded 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 96-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 96-well plate in the dark.
	Samples causing the instrument to clog	Remove the 96 Well Flat Bottom 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

Standards		Samples									
Standard 1	Standard 1	1	1	9	9	17	17	25	25	33	33
Standard 2	Standard 2	2	2	10	10	18	18	26	26	34	34
Standard 3	Standard 3	3	3	11	11	19	19	27	27	35	35
Standard 4	Standard 4	4	4	12	12	20	20	28	28	36	36
Standard 5	Standard 5	5	5	13	13	21	21	29	29	37	37
Standard 6	Standard 6	6	6	14	14	22	22	30	30	38	38
Standard 7	Standard 7	7	7	15	15	23	23	31	31	39	39
Blank	Blank	8	8	16	16	24	24	32	32	40	40

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												