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Platinum ProcartaPlex®

Multiplex Immunoassay

Using Magnetic Beads
For Serum and Plasma (EDTA, Citrat) Samples

Instructions for Platinum
Human Simplex 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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Trademarks

Contents

Intended Use

This user manual is for a Platinum ProcartaPlex® Multiplex Immunoassays from the eBioscience division of Affymetrix to perform quantitative, multiplexed protein measurements from serum and plasma 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

Overcome Matrix Effects

The components in complex biological matrices such as serum and plasma may cause so called matrix effects which can impact the readout of many cytokines (low spike recovery and dilution linearity). eBioscience division of Affymetrix developed matrix type specific sample diluents which assure high performance specifications comparable to those of traditional ELISA assys. The newly developed surrogate matrices for dilution of serum or plasma samples included in Platinum ProcartaPlex kits give spike- and dilution-recovery results in the range of 70-130%.

How it Works

Platinum 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 80 protein targets on the Luminex® 200/100™ and FLEXMAP 3D® platforms and 50 protein targets on the MAGPIX® platform.

IMPORTANT: Platinum ProcartaPlex Simplex kits can only be combined with one another.

Materials Provided and Storage Conditions

Platinum ProcartaPlex Immunoassay Simplex and Basic 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	Simplex kit	Basic kit
Antigen Standards, premixed		√
Detection Antibody, premixed (50X) [1]	√	
Antibody Magnetic Beads, premixed (50X) [1]	√	
Controls High		√
Controls Low		√
Streptavidin-PE (SA-PE) (1X)[1]		√
Wash Buffer Concentrate (10X)[1]		√
Serum Assay Diluent (1X)[1]		(√)
Plasma Assay Diluent (1X)[1]		(√)
Detection Antibody Diluent (1X)[1]		√
Reading Buffer[1]		√
PCR 8-Tube Strip		√
96-Well Flat Bottom Plate		√
Black Microplate Lid		√
Plate Seals		√

[1] Contains sodium azide. See WARNING.

(√) Depending on the sample type used Assay Diluent for Serum or Plasma will be provided

WARNING: All chemicals should be considered potentially hazardous. We recommend that this product and its components be handled by those trained in laboratory techniques. 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.
- Platinum ProcartaPlex Simplex Kits can not be combined with Platinum ProcartaPlex Panels or kits from different ProcartaPlex format.
- For Luminex® 100/200™, 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 (including standard diluent sets) on ice before adding to the 96-Well Flat Bottom Plate

Required Equipment and Materials Not Supplied

- Luminex-based instrument e.g MAGPIX®, Luminex® 100/200™, 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.
- Hand-Held Magnetic Plate Washer, Vortex mixer and Microtiter plate shaker.

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.

Plasma Sample Preparation

Action
A. Collect samples in sodium citrate or EDTA tubes.
B. Centrifuge samples at 1,000 x *g* at 4 °C for 10 min within 30 min of blood collection.
C. Collect the plasma fraction.
D. Use immediately or aliquot and store at -80 °C.

NOTE: Only EDTA and Citrat Plasma Samples have been tested and validated with this kit.

Serum Sample Preparation

We recommend spinning down serum samples at 1000 x *g* for 10 min at 20-25 °C before running the assay.

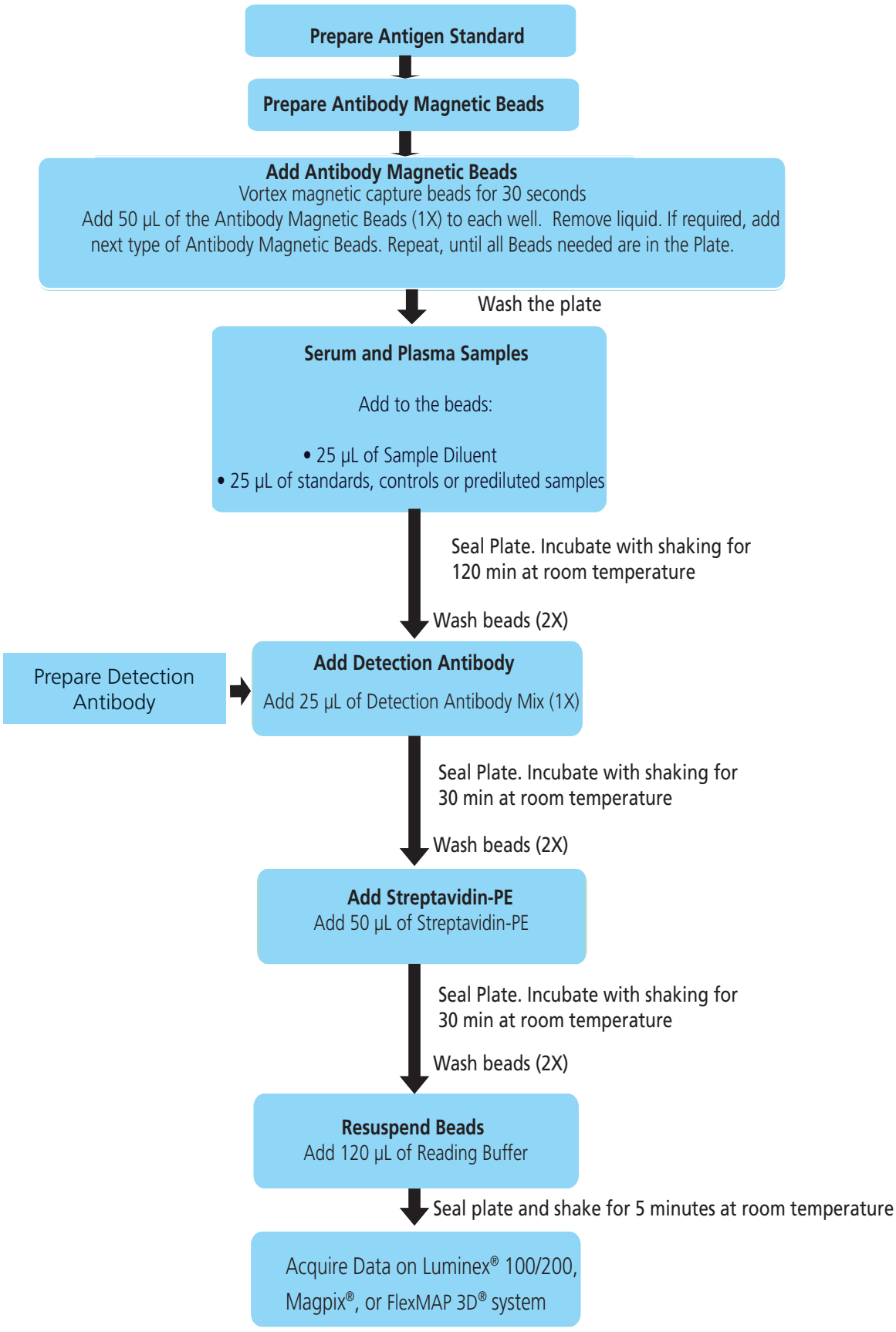
Action
A. Allow blood to clot for 20-30 min at 20-25 °C.
B. Centrifuge at 1,000 x *g* for 10 min at 20-25 °C.
C. Collect the serum fraction. (Alternatively, use any standard serum separator tube following the manufacturer's instructions.)
D. Use immediately or aliquot and store at -80 °C.

Diluting Samples

Dilute the samples 4-fold in appropriate assay diluent (1X) according to following scheme:

1:4 Dilution	Sample Volume	Diluent Volume
Dilution	20 µL	60 µL

Assay Protocol Overview

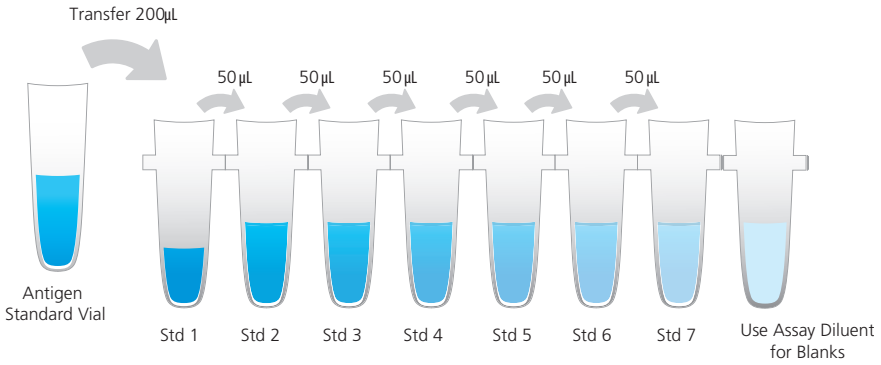


Preparation of Reagents

Antigen Standard and Controls

Platinum ProcartaPlex Immunoassay Kits are supplied with lyophilized multi-standard and controls containing a mix of multiple proteins. Each kit is shipped with two identical vials of each premixed antigen standard and high/low control set from the same lot to permit the user to run the assay twice if running a partial plate.

NOTE: After usage remaining standards and controls cannot be stored and have to be discarded.

Step	Action
Step 1. Reconstitution of Standards and Controls	**A.** Centrifuge the antigen standard set vial and controls at 2000 x *g* for 10 sec. **B.** Add 250 µL of appropriate assay diluent into the standard set vial and controls. Gently vortex the vials for 30 seconds and centrifuge at 2000 x *g* for 10 seconds to collect contents at the bottom of the vial. **C.** Incubate on ice for 10 min to ensure complete reconstitution. **NOTE: After reconstitution controls are ready to be used for the Assay Protocol (Step 5).**
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. **B.** Prepare a 4-fold serial dilution of the reconstituted standard using the PCR 8-tube strip provided. Label tubes Std1, Std2, Std3, Std4, Std5, Std6 and Std7. **C.** Add 200 µL of the reconstituted antigen standards into the first tube of the strip tube and label as Standard 1 (Std1). **D.** Add 150 µL of appropriate assay diluent into Std tubes 2-7. **E.** Transfer 50 µL of the reconstituted antigen standards from Tube 1 into Tube 2. **F.** Mix by pipetting up and down for a total of 10 times. **G.** Change the pipette tip and transfer 50 µL of the mixed standards from Tube 2 into Tube 3. **H.** Mix by pipette up and down 10 times. **I.** Repeat steps E-H for Std tubes 4-7. **J.** Add 200 µL of appropriate assay diluent into tube 8 which serves as a blank. Keep on ice until ready to use. 

Expected Range for High/Low Controls

Control High: S2-S3 (Approximately)

Control Low: S5-S6 (Approximately)

NOTE: All control ranges were evaluated in appropriate assay diluent and 2 hours incubation at RT.

Antibody Magnetic Beads (1X)

Antibody Magnetic Beads in Platinum Simplex Kits are provided as 50X concentrate. If you want to combine Simplex Kits 120 µL of each 50X concentrated Antibody Magnetic Beads must be added to the mixing bottle and volume brought to 6 mL. Table below are an example for 96 and 48-wells.

Example for 96-wells

# of Vials of Antibody Magnetic Beads	Total Volume of Mixed Antibody Magnetic Beads	Volume of Wash Buffer (1X) to Add
1	120 µL	5880 µL
2	240 µL	5760 µL
3	360 µL	5640 µL
4	480 µL	5520 µL
5	600 µL	5400 µL
6	720 µL	5280 µL

Example for 48-wells

# of Vials of Antibody Magnetic Beads	Total Volume of Mixed Antibody Magnetic Beads	Volume of Wash Buffer (1x) to Add
1	60 µL	2940 µL
2	120 µL	2880 µL
3	180 µL	2820 µL
4	240 µL	2760 µL
5	300 µL	2700 µL
6	360 µL	2640 µL

Assay Protocol

Step	Action
Step 1. Prepare 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.
Step 2. Define the plate map	Mark the standard, sample, control and blank wells using the plate map at the end of this manual.
Step 3. Add the Antibody Magnetic Beads	**A.** Vortex the Antibody Magnetic Beads (1X) for 30 sec. **B.** Add 50 µL of the Antibody Magnetic Beads (1X) to each well. Use a multi-channel pipette for this step as well as the steps below.
Step 4. Wash Antibody 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 and wait 2 min to allow the Antibody Magnetic 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 plate and then blotting onto an absorbent towel 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 5. Add appropriate assay diluent, samples, standards, controls and blanks and incubate	**A.** Add 25 µL of appropriate assay diluent to each well for serum or plasma followed by 25 µL of prepared standards, controls or prediluted samples into dedicated wells. **B.** For wells designated as blanks, add an additional 25 µL of appropriate assay diluent for serum or plasma samples. **C.** Seal the plate with the provided Plate Seal. Cover the plate with the Black Microplate Lid and shake at 500 rpm for 120 min at room temperature (RT).
Step 6. Wash the 96-Well Plate	Wash the plate twice using "Step 4. Wash Antibody Magnetic Beads".

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Step 7. Prepare 1X Detection Antibody Mixture</td><td>A. For simplex kits detection antibody is provided at 50X concentration. If you want to combine simplex kits add 60 µL of each different detection antibody concentrate to the mixing bottle and bring volume to a total of 3 mL.Tables below are an example for 48 and 96-wells.

Example for using 96-wells

<table>
<tr><th># of Vials of Detection Antibody</th><th>Total Volume of Mixed Detection Antibody</th><th>Volume of Detection Antibody 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 48-wells

<table>
<tr><th># of Vials of Detection Antibody</th><th>Total Volume of Mixed Detection Antibody</th><th>Volume of Detection Antibody 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>
<tr><td>Step 8. Add Detection Antibody Mixture and incubate</td><td>A. Add 25 µL of Detection Antibody to each well.
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.</td></tr>
<tr><td>Step 9. Wash the 96-Well Plate</td><td>Wash plate twice using "Step 4. Wash Antibody Magnetic Beads".</td></tr>
<tr><td>Step 10. Add SAPE and incubate</td><td>A. Add 50 µL of SAPE solution to each well.
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.</td></tr>
<tr><td>Step 11. Wash the 96-Well Plate</td><td>Wash plate twice using "Step 4. Wash Antibody Magnetic Beads".</td></tr>
<tr><td>Step 12. Prepare the 96-Well Plate for Analysis on a Luminex Instrument</td><td>A. Add 120 µL of Reading Buffer into each well.
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.
C. Remove Plate Seal and run the plate on a Luminex Instrument.</td></tr>
</table>

Performance Characteristics

Spike and Dilution Recovery

Similar to our Platinum ELISA, Platinum ProcartaPlex Immunoassays have to pass more than 30 qualification criteria to guarantiee reproducible high performance of the kits. One release criteria of Platinum ProcartaPlex kits is that both spike and dilution recovery have to be 100%+/-30% in serum, citrat and EDTA plasma.

	Spike Recovery			Dilution Recovery		
	Serum	Plasma		Serum	Plasma	
		Citrat	EDTA		Citrat	EDTA
BDNF	82%	85%	86%	95%	106%	102%
Eotaxin	105%	94%	88%	82%	90%	78%
GM-CSF	90%	94%	89%	110%	84%	84%
GRO alpha	108%	107%	111%	90%	87%	84%
HGF	92%	113%	113%	97%	100%	95%
IFN alpha	99%	88%	113%	103%	91%	92%
IFN gamma	77%	100%	102%	92%	94%	91%
IL-10	79%	91%	91%	104%	90%	89%
IL-12p70	102%	94%	90%	96%	99%	99%
IL-13	98%	97%	94%	90%	93%	90%
IL-15	103%	96%	84%	101%	80%	84%
IL-16	84%	95%	91%	90%	87%	79%
IL-17A	81%	79%	78%	103%	88%	87%
IL-1 alpha	108%	97%	92%	113%	100%	94%
IL-1 beta	102%	93%	93%	95%	100%	95%
IL-1RA	111%	103%	90%	108%	97%	100%
IL-2	102%	82%	87%	84%	92%	92%
IL-20	94%	95%	99%	95%	93%	88%
IL-21	98%	83%	85%	97%	81%	83%
IL-4	97%	99%	96%	90%	96%	90%
IL-5	106%	85%	85%	93%	104%	91%
IL-6	84%	87%	86%	95%	94%	97%
IL-7	110%	103%	102%	92%	97%	93%
IL-8	85%	95%	92%	94%	90%	89%
IL-9	92%	95%	86%	106%	87%	89%
IP-10	99%	97%	86%	87%	95%	96%
LIF	98%	110%	100%	95%	99%	94%
MCP-2	118%	97%	95%	99%	94%	97%
MIP-1 alpha	86%	86%	76%	95%	96%	86%
MIP-1 beta	87%	95%	89%	91%	95%	82%
OPG	85%	102%	105%	92%	105%	94%
PDGF-BB	88%	96%	87%	101%	103%	101%
PECAM-1	89%	121%	117%	88%	90%	82%
P-Selectin	83%	105%	96%	89%	97%	99%
RANTES	99%	84%	85%	90%	99%	93%
SCF	103%	94%	94%	98%	92%	91%
TNF-RII	84%	79%	93%	94%	97%	102%
TNF alpha	99%	111%	112%	104%	75%	81%
tPA	77%	103%	106%	91%	101%	99%
TSLP	94%	102%	102%	99%	88%	92%
VEGF-A	87%	93%	86%	94%	93%	92%
VEGF-D	88%	95%	83%	105%	90%	86%

Overall mean spike and dilution recovery. Mean recoveries for each matrix were calculated across the complete portfolio of ProcartaPlex®. Platinum assays target specification of 100% +/- 30% are fulfilled, also regarding min/max values. The spike and dilution recovery was evaluated in a minimum of 5 individual donor samples per matrix. Spikes with recombinant proteins were performed using 3 different known concentrations (high, medium and low), covering the whole range of the assay. For dilution recovery (spiked) samples were prediluted 1:4 and then diluted in 4-fold serial dilution from 1:16 to 1:256. Recovery values may vary within different sample collectives. Therefore, the values provided above have to be seen as example values.

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

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