Rev. 11/14

Super-X Plex™

Cytokine Assay Protocol

Non-Magnetic Beads

Flow Cytometry Assay

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1- ASSAY Principles / Introduction

ANTIGENIX AMERICA Super-X Plex™ multiplex assay utilizes multiple bead populations differentiated by size and levels of fluorescence intensity. This enables the resolution of distinct bead populations on the flow cytometer data output. With this technology, the user can measure or **Multi-Plex up to 24 analytes** in a single reaction well!

The Super-X Plex Cytokine Assays utilize any flow cytometer with a **488nm laser** and measures analyte concentrations based on the measured R-Phycoerythrin emission. Analyte concentrations of the sample are determined by comparison to known concentrations of a standard curve.

Assay Protocol Overview: (Total Assay Time **Approx. 2 hours)**

1. **Add** Antibody-Coated beads to wells

   Remove Buffer

2. **Add** Standards and Samples to wells

   Incubate 1 hour **@** RT with shaking

   Wash 3X

3. **Add** Biotin Detection Antibody to wells

   Incubate 30 Minutes **@** RT with shaking

   Wash 3X

4. **Add** Streptavidin-PE conjugate

   Incubate 20 Minutes **@** RT with shaking

   Wash 2X - and add Reading Buffer

5. **Read on Flow Cytometer**

2. **Required Equipment Not Supplied:**

- Plate Shaker : Barnstead/Lab-Line Titer Plate Shaker (Thermo Scientific, Waltham, MA) or equivalent. The shaker should have 3 mm orbit with ability to maintain 600-800 rpm.
- Filter Plate Washer - Use **ANTIGENIX Filter Plate Washer** (**cat# FPW100)**) with vacuum source
- A flow cytometer equipped with a 488nm laser and capable of detecting FS, SS, PE and PECy5 (and/or PECy7).

ASSAY PROTOCOL

1 Preparing Reading Buffer and Wash Buffer

1.1 Reading Buffer (1X)

Bring the 10x Reading Buffer to room temperature and vortex for 15 seconds. Mix 5 mL of the 10x Reading Buffer with 45 mL ddH_20. (or 1.5 mL of 10X reading buffer with 13.5 mL distilled water) .The 1x Reading Buffer can be stored at 2-8 °C for up to 3 months.

1.2 Wash Buffer (1X)

Bring the 10x Wash Buffer to room temperature and vortex for 15 seconds. Mix 15 mL (or 5 mL) of the 10x Wash Buffer with 135 mL (or 45 mL) ddH_20. The 1x wash Buffer can be stored at room temperature for up to 3 months.

2 Preparing Antibody-Coated Bead Suspension

2.1 ANTIGENIX Single Plex Kits:

The capture beads provided with each kit is a 45x concentrated stock (1 µL per test). Dilution of the stock beads to working suspension with 1x Reading Buffer as needed.

2A Determine the number of analytes in the panel (e.g. a 7-plex panel)

2B Determine the number of wells in the experiment. We recommend adding an additional at least 2 wells to account for pipetting recovery. For example, if a total of 48 wells is needed in the experiment, prepare enough for 50 wells.

2C Determine the total volume of working bead suspension needed for the experiment. Each tube/well needs 45 µL of the working bead suspension. The total volume is calculated by multiplying the number of wells (as above) by 45 µL. For example, 50 wells × 45 µL = 2,250 µL total working bead suspension.

2D Determine the volume needed for each 45x Analyte-Specific Ab Conjugated Beads (i.e. 1.0 µL needed for each well). Therefore, for example, a total of 50 wells needs 50 µL of each 45x capture bead stock.

2E Determine the volume of 1x Reading Buffer needed to prepare the working bead suspension. Calculate the 1x Reading Buffer volume by subtracting the volume for each capture bead stock:

____ µL from Step 2C – ___ µL from Step 2D x ___ number of Plex (Step 2A) = ____ µL of 1x Reading Buffer

For example, a 7-plex panel for 50 wells: 2,250 - 50 x 7 = 1900 µL of 1x Reading Buffer

2F Add the appropriate volume (determined in Step 2E) of 1x Reading Buffer to a test tube labeled with "Working Bead Suspension".

2G Centrifuge each capture bead vial at 2,000 x g for 10 sec.

2H Vortex each capture bead vial for 15 second.

2I Add the appropriate volume (determined in Step 2D -above.) of each capture bead stock into the "Working Bead Suspension" tube.

2J Mix by gentle vortexing. If not use immediately, store the working bead suspension at 2-8°C with light protection. It can be stored at 2-8 °C for up to 1 week.

ANTIGENIX MULTIPLEX PANELS:

NOTE- Antibody-coated beads for the MultiPlex Panels are provided as Pre-Mixed (1X) and are ready to use. No preparation is necessary.

3.- Preparing Detection (dAb- Biotin) Antibody Working Solution

ANTIGENIX Single Plex Kits:

The Detection (dAb) Antibody (Biotin) provided with each kit is a 25x concentrate

(1 µL per test). **Dilute** the stock **biotinylated dAb** to working dilution with

Human/Primate or Mouse/Rat **Diluent** as follows:

3A. Determine the number of analytes in the panel (e.g. a 7-plex panel)

3B Determine the number of wells in the experiment. We recommend adding an additional 2 wells to account for pipetting recovery. For example, if a total of 48 wells is needed in the experiment, prepare enough for 50 wells.

3C Determine the total volume of working dAb solution needed for the experiment. Each tube/well needs 25 µL of the working detection antibody solution. The total volume is calculated by multiplying the number of wells (calculated in Step 3B.) by 25 µL. For example, 50 wells × 25 µL = 1,250 µL total working detection antibody solution.

3D Determine the volume needed for each 25x Biotinylated Detection Antibody stock (i.e. 1.0 µL needed for each well). Therefore, for example, a total of 50 wells needs 50 µL of each 25x detection antibody stock.

3E Determine the volume of 3x Human/Primate or Mouse/Rat dAb Diluent needed to prepare the working dAb solution.

____ µL from Step 3C ÷ 3 = ____ µL of 3x NR(non-rodent) or Mouse/Rat dAb Diluent

For example, a total of for 50 wells: 1,250 ÷ 3 = 417 µL of 3x Human/primate or Mouse/Rat dAb Diluent

3F Calculate the ddH_2O volume by subtracting the volume for each capture bead stock and 3x 3x Human/Primate or Mouse/Rat dAb Diluent:

____ µL from Step 3C – ___ µL from Step 3D x ___ number of Plex (Step 3A) - ____ µL from Step 3E = ____ µL of ddH_2O

For example, a 7-plex panel for 50 wells: 1,250 - 25 x 7 - 417 = 658 µL of ddH_2O

3G Add the appropriate volume (determined in Step 3F) of ddH_2O to a test tube labeled with "Working dAb Solution ".

3H Add the appropriate volume (determined in Step 3E) of 3x Human/Primate or Mouse/Rat dAb Diluent

3I Add the appropriate volume (determined in Step 3D) of each dAb stock into the " dAb Solution Tube" .

3J Mix by gentle vortexing. The working dAb solution can be stored at 2-8 °C for up to 24 hrs.

3.2 ANTIGENIX Multiplex Panels:

Transfer the entire content of 2x Human/Primate or Mouse/Rat dAb diluent to the 2x Premixed Biotin-dAb vial. Mix by gentle vortexing. The working solution can be stored at 2-8 °C for up to 24 hrs.

4. - Antigen Standard Prep:

Reconstitution of the lyophilized standards

If there is only one standard vial in the kit

4A. Centrifuge the antigen standard vial at 2000 x g for 10 sec.

4B Add 250 µL of CCS (cell culture supernatant), SPB (serum/plasma/bodily fluid) or TL (Tissue/cell lysate) standard diluent into the vial.

Note: Reconstitute volume may be different for **"custom assay or Special"** panels. Refer to the "Standard Info Sheet" enclosed in the kit for details.

4C Vortex gently for 15 sec.

4D Incubate on ice for 5-10 min.

4E Vortex gently for 15 sec before Serial Dilution Preparation (See next section)

If Multiple standard vials are provided in the kit

4AA Centrifuge the antigen standard vials at 2000 x g for 10 sec.

4BB Add 250 µL of CCS (cell culture supernatant), SPB (serum/plasma/bodily fluid) or TL (Tissue/cell lysate) standard diluent into the first vial.

Note: Reconstitute volume may be different for custom assay panels. Refer to the "Standard Info Sheet" enclosed in the kit for details.

4CC Vortex gently for 15 sec.

4DD Incubate on ice for 5 min

4EE Vortex gently for 15 sec

4FF Transfer the entire content to the next vial

4GG Vortex gently for 15 sec.

4HH Incubate on ice for 5 min

4II Vortex gently for 15 sec

4JJ If more than 2 standard vials in the kit, repeat Steps 4FF to 44II for the rest of the vials.

Serial Dilution preparation (Standard Curve)

Prepare 3x serial dilutions (160 µL in total, enough for duplicated wells) according to Table 1. Mix each addition by pipetting up and down **6-8 times**. Change pipette tips after each addition to avoid contamination from one concentration to the other. Keep the standards on ice until use.

Table 1: Preparation of antigen Standard Curve

Standard	Amount from Previous Standard (µL)	Standard Diluent (µL)
Standard 1 (Undiluted)		Prepared in Section 4
Standard 2 (1/3)	80	160
Standard 3 (1/9)	80	160
Standard 4 (1/27)	80	160
Standard 5 (1/81)	80	160
Standard 6 (1/243)	80	160
Standard 7 (1/729)	80	160
Blank	0	160

Note: If very **low levels** (less than 10 pg/mL) of cytokines are expected in the samples, we recommend **add one** or **two more standard points**, Standards: 8 (1/2187); 9 (1/6561).

Note: Serial dilution factor may be different for custom assay panels. Refer to the "Standard Info Sheet" enclosed in the kit for details.

5. - Running the Super-X Plex™ assay

5.1 Prepare the plate template. Mark the standard, sample and blank wells. Standards and samples should be run in duplicates or triplicates. If the whole plate will not be used, seal the unused well with a plate seal.

Important: Place the filter plate on top of the inverted filter plate lid during the entire assay process to prevent touching the plate bottom on any surface.

5.2 Vortex working bead suspension for 15 second.

5.3 Add 45 µL of capture bead working suspension to each well.

Note: Save the remaining capture bead working suspension and store at 2-8°C with light protection. It can be used for setting up the flow cytometer.

5.4 Remove solution by using the ANTIGENIX Filter Plate Washer (cat# FPW100).

5.5 Gently tap the plate bottom onto several layers of paper towels to remove residual buffer (after the above removal with plate washer).

5.6 Add 30 µL of CCS (Cell Culture Supernatant) Assay Buffer, Serum/Plasma or Tissue Lysate (TL) Assay Buffer to each sample well.

Note: Cell culture supernatant samples can be run without diluting in Assay Buffer if very low levels (less than 20 pg/mL) of cytokines are expected. If it is the case, skip this step and add 45 µL of cell culture supernatant samples to each sample well in Step 5.7.

5.7 Add 15 µL of samples to each sample well.

5.8 Add 45 µL of standards to each standard well.

5.9 Cover the plate with a plate seal.

5.10 Incubate on plate shaker (set at 700 rpm) for 60 min @ room temperature. Protect from light.

5.11 Remove the plate seal.

5.12 Wash the wells three times with 100 µL wash buffer using plate washer.

5.13 Gently tap the plate bottom onto several layers of paper towels to remove residual buffer after the last wash.

5.14 Add 25 µL of biotinylated antibody working solution to each well.

5.15 Cover the plate with a plate seal.

5.16 Incubate on the shaker (set at 700 rpm) for 30 min at room temperature. Protect from light.

5.17 Remove the plate seal.

5.18 Wash the wells three times with 100 µL wash buffer using plate washer.

5.19 Gently tap the plate bottom onto several layers of paper towels to remove residual buffer after the last wash.

5.20 Add 25 µL of streptavidin-PE working solution to each well.

5.21 Cover the plate with a plate seal.

5.22 Incubate on the shaker (set at 700 rpm) for 20 min at room temperature. Protect from light.

5.23 Remove the plate seal and remove solution with ANTIGENIX filter plate washer.

5.24 **Wash wells TWICE** with 100uL (1X) Wash Buffer

NOTE: If your flow cytometer does **not use sheath fluid** - **WASH** the wells **three times** with 100 µL wash buffer (1X).

5.25 Gently tap the plate bottom onto several layers of paper towels to remove residual buffer after the last wash.

5.26 Add 150 µL of Reading Buffer to each well to resuspend the beads.

5.27 Cover the plate with a plate seal.

5.28 Place the plate on the microtiter shaker and shake for 30 seconds at 700 rpm.

5.29 Remove the plate seal.

5.30 Read on a flow cytometer.

Note: If the assayed plate is not read immediately, it can be stored at 2-8°C for up to 16 hr. The plate should be sealed with a plate seal and protected from light.

6. - Setting Up Flow cytometers

Fluorescence channels

The maximum emission of the **bead** classification **dye** is at **700 nm.** It can be detected on "PE-Cy5" channels of most of the flow cytometers with blue (488 nm) excitation. It can also be detected on PE-Cy7 channels with blue (488 nm) excitation or APC channel with red (635 or 640nm) excitation if such a flu2orescence channel is available.

The **detection dye** of the Super-X Plex assays is **PE** (R-Phycoerythrin) and can be detected on the PE channel with blue (488 nm) excitation.

6.1 Preparing instrument setup beads

6A. Blank beads: Aliquot 75 µL bead suspension from one of Blank wells from Step **5.29** into a sample tube or a well of a 96-well plate depending on the sample loading mechanism of a flow cytomter. Add 100 to 300 µL of Reading Buffer to the tube/well. Remaining capture bead working suspension from Step **5.6** can also be used for this purpose.

6B Standard 1 beads: Aliquot 75 µL bead suspension from one of the Standard 1 wells from Step **5.29** into a sample tube or a well of a 96-well plate depending on the sample loading mechanism of a flow cytomter. Add 100 to 300 µL of Reading Buffer to the tube/well.

Note: Add 75 µL of Reading Buffer to both Blank and Standard 1 wells. Acquisition for both wells will be slower (less bead concentrations) during the sample acquisition step.

When running a panel the first time, we recommend running one extra well of Standard 1 for instrument setup purpose.

6.2 Setting up a display layout/template

1. Perform start up and verification of fluidic stability and optical alignment by following cytometer manufacturer's recommendations.

2. Open a new protocol.

3. Create the following plots and histograms:

   3A) A dot plot with FS (X-axis) and SS (Y-axis) in linear display mode.

   3B) 2 histograms of "PE-Cy5" in Log display mode.

   3C) 2 dot plots with PE (X-axis) and "PE-Cy5" (Y-axis) in Log display mode.

   3D) If PE-Cy7 or APC channel is available, create 2 histograms of "PE-Cy5" in Log display mode and 2 dot plots with PE (X-axis) and PE-Cy7 or APC (Y-axis) in Log display mode.

4. Set all compensation to zero.

5. Save the protocol.

6.3 Running the setup beads

6.3 Run the Blank beads prepared in Step **6A** .

1) Adjust FS and SS gains so that the bead populations are on scale (Figure 1).

2) Create Gate 1 for the smaller (4 micron size, S4) beads and Gate 2 for the larger (5 micron size, S5) beads (Figure 1).

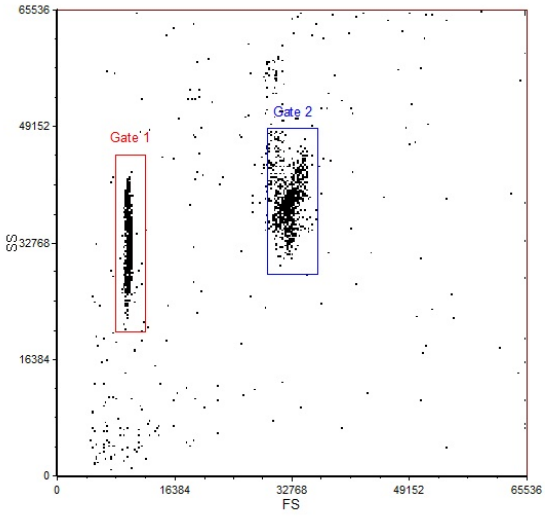

Figure 1

3) Apply Gate 1 to one of the "PE-Cy5" histograms and one of the PE/"PE-Cy5" dot plots.
4) Apply Gate 2 to the other "PE-Cy5" histogram and the other PE/"PE-Cy5" dot plot
5) Adjust "PE-Cy5" PMT voltage so that all bead populations are clearly separated on the histograms and dot plots (Figure 2).

In this example, S4 has 4 bead populations, S4P3, S4P7, S4P9 and S4P11. S4P3 (Size 4 micron, Peak #3) is the dimmest and S4P11 (Size 4 micron, Peak #11) is the brightest.

6) Adjust PE PMT voltage so that the dimmest bead population is positioned within the first decade on the PE-axis of the plot (Figure 2).

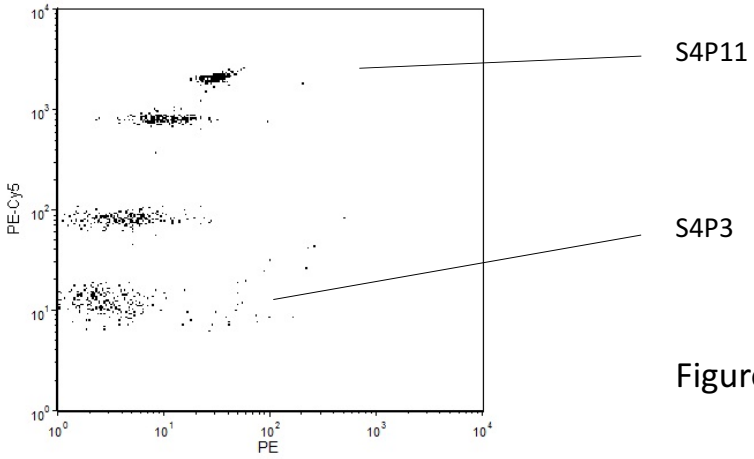

Figure 2

7) Save the protocol.

6.3.1 Run the Standard 1 beads prepared in Step **6B**. (section 6)

1) Verify all the bead populations on the PE-axis are on scale (Figure 3).
2) Adjust PE PMT voltage if needed. If adjustment is needed, make sure rerun the Standard 8 and make sure the dimmest bead population is still visible on the PE/"PE-Cy5" dot plots.

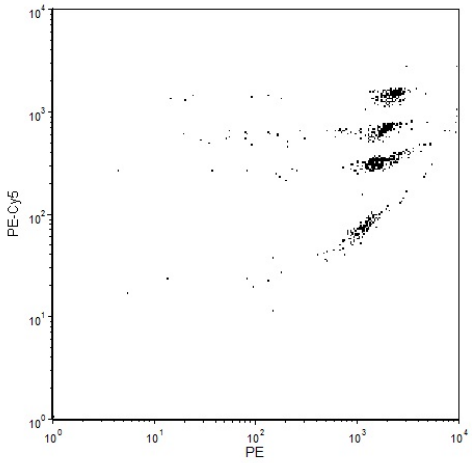

Figure 3

3) Apply proper "PE-Cy5" - %PE color compensation so that the bead populations are in a horizontal position (see Figure 4 as an example of proper color compensation setting).

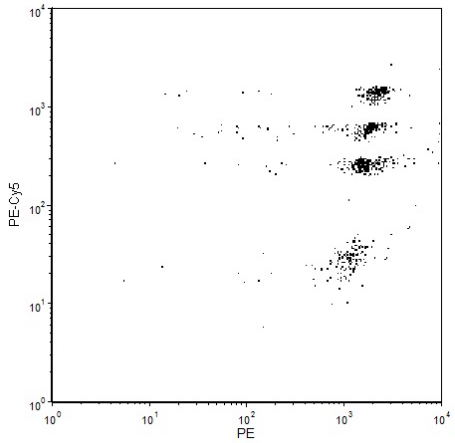

Figure 4

Over compensation should be avoided (**see Figure 5** example of over compensation).

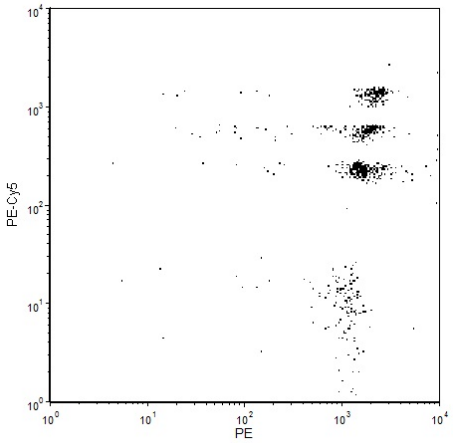

Figure 5

4) Save the protocol .

Note: If PE-Cy7 or APC fluorescence channel is available, carry out Steps 6.3 and 6.3.1 for the PE-Cy7 channel for the proper PMT voltage and color compensation (usually zero) settings.

7. Analyzing samples

If the flow cytometer does not equipped with a 96-well plate loader, transfer the samples from the wells to sample tubes. Acquire 100 events each bead population of the larger beads (Gate 2). For example, if there are 3 bead populations in Gate 2 (larger beads), acquire 3 x 100 = 300 events per sample. We have found that as few as 50 events for a bead population is sufficient.

Save the FCS files and analyze the data using FCAP Array 3.0.

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8. Helpful Hints

1. Set up the ANTIGENIX Filter Plate Washer (cat# FPW100) according to the instruction in the Product Insert. Adjust the vacuum pressure so that 100 µL of 1x Wash Fuffer in the wells can be clear in 3-5 second.

2. When working with samples and standards, change the pipette tips after every transfer and avoid creating bubbles when pipetting.

3. We recommend, whenever possible, using a multi-channel pipette for reagent additions to achieve optimal assay precision.

4. When apply plate seal to the filter plate, do not use a rubber roller. Use finger to gently press over the plate seal to seal the plate.

5. Sample handling:

a. Cell Culture Supernatant: Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -80 . Avoid repeated freeze-thaw cycles.

b. Serum: If using serum separator tubes, allow samples to clot for 30 minutes at room temperature. Centrifuge for 10 minutes at 1000×g. Collect the serum fraction and assay promptly or aliquot and store the samples at -80 . Avoid multiple freeze-thaw cycles. If serum separator tubes are not being used, allow samples to clot overnight at 2-8 . Centrifuge for 10 minutes at 1000×g. Remove serum and assay promptly or aliquot and store the samples at -80 . Avoid multiple freeze-thaw cycles.

c. Plasma: Centrifuge samples at 1,000 x g at 4 °C for 10 min within 30 min of blood collection. Collect the plasma fraction and assay promptly or aliquot and store the samples at -80 . Avoid multiple freeze-thaw cycles.

d. Thaw frozen samples on ice and mix well prior to adding to the assay wells.

e. If there is a high lipid content in serum, plasma or bodily fluid samples, centrifuge at 10,000 x g for 10 min at 2-8 °C. Collect the serum, plasma or bodily fluid fraction for the assays.

f. If samples contain **high analyte concentrations** and need dilution for the assays, use **Sample Dilution Buffer** (**Cat# SDL200**) for sample dilution. The exact dilution must be determined empirically.

6. SOFTWARE / DATA Analysis:

Customers can use FlowJo, FCSExpress, Koluza, Flowlogic or other flow cytometry analysis sw packages to identify bead populations (FSC vs. “APC” or “PC5”) and obtain PE signal (PE vs. “APC” or “PC5” after gating) of each bead population. Once the PE MFIs of each bead set in the standards and samples are obtained, one can use Excel or other data analysis SW package to conduct curve fitting and calculation of pg/mL according to the standards. The FCAP Array just makes the data analysis part easier.

This is very much similar to ELISA. One can obtain ODs from any plate reader then calculate the results. Many plate readers have SW to help the calculation. Plate readers with analysis SW usually cost a lot more than those only give OD reading.