SOP, Cytokine Tests

Materials & Equipment

Media and Reagents

1.1 BD Cytometric Bead Array (CBA) Human Soluble Protein Master Buffer Kit (BD, 558264)
1.2 Human IFN-γ Flex Set (560111)
1.3 Human TNF Flex Set (560112)

2. Consumables

2.1. Centrifuge Tubes (15mL)
2.2. Serological pipette 10 mL

3. Equipment

3.1. BD Flow Cytometry System FACSCalibur
3.2. TOMY Refrigerated Centrifuge-5810R
3.3. GRANT Mini Vortex Mixer PV-1
3.4. NEW BRUNSWICK CO2 Incubator GALAXY 170R
3.5. OLYMPUS Research Inverted Microscope
3.6. SAMSUNG Refrigerator RL40EGPS

Procedure

1. Preparing Human Flex Set Standards

1.1 Open one vial of lyophilized standard from each BD CBA Human Soluble Protein Flex Set that will be tested.
1.2 Pool all lyophilized standard spheres into one 15-mLpolypropylene tube. Label the tube "Top Standard."
1.3 Reconstitute the standards with 4 mL of Assay Diluent.
- Allow the reconstituted standard to equilibrate for at least 15 minutes at room temperature.
- Gently mix the reconstituted standard by pipet only. Do not vortex or mix vigorously.

1.4 Label eight 12 × 75-mm (EP) tubes and arrange them in the following order: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64,1:128, and 1:256.
1.5 Pipette 500 µL of Assay Diluent into each of the12 × 75-mm tubes.
1.6 Perform a serial dilution.
- Transfer 500 µL from the Top Standard to the1:2 dilution tube and mix thoroughly by pipet only. Do not vortex.
- Continue making serial dilutions by transferring500 µL from the 1:2 tube to the 1:4 tube and soon to the 1:256 tube.

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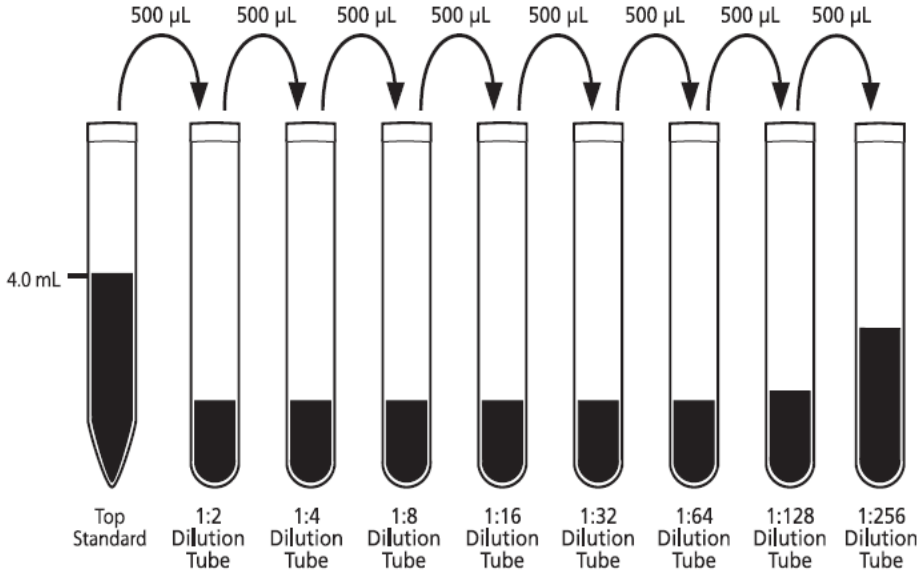

1.7 Prepare one 12 × 75-mm tube containing Assay Diluent to serve as the 0-pg/mL negative control.

Note: We recommend that the first 10 wells or tubes in the experiment be the standards. Standards should be run in order from least concentrated (0 pg/mL) to most concentrated (Top Standard) to facilitate analysis in FCAP Array software.

2. Concentration Standards

2.1 Open one vial of lyophilized standard from each BD CBA Human Soluble Protein Flex Set that will be tested.

	Dilution Tube								
	Top Stand.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Protein (pg/mL)	2,500	1,250	625	312.5	156	80	40	20	10

3. Mixing Human Soluble Protein Flex Capture Beads

Procedure for supernatants to mix the Capture Beads when testing supernatants:

3.1 Open one vial of lyophilized standard from each BD CBA Human Soluble Protein Flex Set that will be tested.

3.2 Determine the number of tests in the experiment.
Note: Extra tests of Capture Beads should be mixed to ensure that the necessary number of tests will be recovered from the mixed Capture Beads tube. Add an additional 2 to 3 assay tubes to the number determined.

3.3 Vortex each Capture Bead stock vial for at least 15 seconds to resuspend the beads thoroughly.

3.4 Determine the total volume of diluted beads needed for the experiment. Each tube/well requires 50 µL of the diluted beads. The total volume of diluted beads can be calculated by multiplying the number of tests (determined in step 2) by 50 µL.

Example: 35 tests × 50 µL = 1,750 µL total bead volume

3.5 Determine the volume needed for each Capture Bead. Beads are supplied so that 1.0 µL = 1 test. Therefore, the required volume (µL) of beads is equal to the number of tests.

Example: 35 tests require 35 µL of each Capture Bead included in the assay

3.6 Determine the volume of Capture Bead Diluent needed to dilute the beads. Calculate the Diluent volume by subtracting the volume for each bead tested from the total volume of diluted beads needed to perform the assay. See Capture Bead and PE Detection Reagent Diluent Calculations.

Example: 1,750 µL total volume of beads − 35 µL for each bead = volume of Capture Bead Diluent

- if testing one analyte: 1,750 µL − (35 µL × 1) =1,715 µL diluent
- if testing five analytes: 1,750 µL − (35 µL × 5) =1,575 µL diluent

3.7 Pipette the Capture Beads and Capture Bead Diluent into a tube labelled “Mixed Capture Beads.”

4. Procedure for serum and plasma samples

To mix the Capture Beads when testing serum or plasma samples:

4.1 Determine the number of BD CBA Human Soluble Protein Flex Sets to be used in the experiment (size of the multiplex).

4.2 Determine the number of tests in the experiment. Beads are supplied so that 1.0 µL = 1 test. Therefore, the required volume (µL) of beads is equal to the number of tests.
Note: Extra tests of Capture Beads should be mixed to ensure that the necessary number of tests will be recovered from the mixed Capture Beads tube. Add an additional 2 to 3 assay tubes to the number determined.

4.3 Vortex each Capture Bead stock vial for at least15 seconds to resuspend the beads thoroughly.

4.4 Pipette the appropriate volume (determined instep 2) of each capture bead into a tube labelled Mixed Capture Beads.

4.5 Add 0.5 mL Wash Buffer and centrifuge at 200g for5 minutes.

4.6 Carefully discard the supernatant by aspiration. Avoid aspirating the bead pellet.

4.7 Resuspend the beads in Capture Bead Diluent for Serum/Plasma to a final concentration of 50 µL/test.

4.8 Vortex the Capture Beads and incubate for15 minutes at room temperature prior to use.

Example: 35 tests × 50 µL = 1,750 µL Capture Bead Diluent for Serum/Plasma

5. Preparing Human Soluble Protein Flex Set PE Detection Reagents

5.1 Determine the number of BD CBA Human Soluble Protein Flex Sets to be used in the experiment (size of the multiplex).

5.2 Determine the number of tests to be run in the experiment. Prepare a few additional tests than necessary to ensure that there is enough material prepared for the experiment.

5.3 Determine the total volume of diluted PE Detection Reagent needed for the experiment. Each tube/well requires 50 µL of the diluted PE Detection Reagent. The total volume can be calculated by multiplying the number of tests (determined in step 2) by 50.

Example: 35 tests × 50 µL = 1,750 µL total volume

5.4 Determine the volume needed for each PE Detection Reagent. The PE Detection Reagent is supplied so that 1.0 µL = 1 test. Therefore, the required volume (µL) of Detection Reagent is equal to the number of tests.

Example: 35 tests require 35 µL of each Detection Reagent included in the assay

5.5 Determine the volume of Detection Reagent Diluent needed to dilute the PE Detection Reagents. Calculate the Detection Reagent Diluent volume by subtracting the volume for each PE Detection Reagent tested from the total volume of diluted PE needed to perform the assay. See Capture Bead and PE Detection Reagent Diluent Calculations.
Example: 1,750 µL total volume PE – 35 µL for each Detection Reagent = volume of Detection Reagent Diluent

- if testing one analyte: 1,750 µL – (35 µL × 1) =1,715 µL diluent
- if testing five analytes: 1,750 µL – (35 µL × 5) =1,575 µL diluent

5.6 Pipette the Detection Reagents and Detection Reagent Diluent into a tube labeled "Mixed PE Detection Reagents." Store at 4°C, protected from light until ready to use.

6. Assay procedure for tubes

6.1 Add 50 µL of Flex Set Standard dilutions to the first 10 tubes as listed in the following table.

Tube Label	Standard Dilution	Concentration (pg/ml)
1	No standard dilution (Assay Diluent only)	0 (Negative control)
2	1:256	10
3	1:128	20
4	1:62	40
5	1:32	80
6	1:16	156
7	1:8	312.5
8	1:4	625
9	1:2	1,250
10	Top Standard	2,500

6.2 Add 50 µL of each unknown sample to the appropriate assay tubes.

6.3 Vortex the mixed Capture Beads for at least 5 seconds.

6.4 Add 50 µL of the Mixed Capture Beads to each assay tube. Gently mix the tubes.

6.5 Incubate the tubes for 1 hour at room temperature.

6.6 Add 50 µL of the mixed PE Detection Reagent to each assay tube. Gently mix the tubes.

6.7 Incubate the tubes for 2 hours at room temperature.
Note: If you have not yet performed cytometer setup, you may wish to do so during this incubation.

6.8 Add 1 mL of Wash Buffer to each assay tube and centrifuge at 200g for 5 minutes.

6.9 Carefully aspirate and discard the supernatant from each assay tube.

6.10 Add 300 µL of Wash Buffer to each assay tube. Vortex assay tubes briefly to resuspend the beads.

6.11 Analyze the samples with flow cytometry.