Video Article

Measurement of Low Concentration Human Serum Cytokines using a Millipore High-Sensitivity Milliplex Assay - ADVERTISEMENT

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Abstract

Video Link

The video component of this article can be found at http://www.jove.com/video/5088/

Protocol

VIII. PREPARATION OF REAGENTS FOR IMMUNOASSAY

A. Preparation of Antibody-Immobilized Beads

Sonicate the premixed antibody-bead bottle for 30 seconds and then vortex for 1 minute prior to use. No dilution is necessary. Unused portions may be stored at 2-8°C for up to one month.

B. Preparation of High Sensitivity Human Cytokine Standard

1.) Prior to use, reconstitute one vial of the High Sensitivity Human Cytokine Standard with 250 µl Deionized Water to give a 2,000 pg/ml concentration of standard for each analyte. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to set for 5-10 minutes and then transfer the standard to appropriately labeled polypropylene microfuge tube. This will be used as the 2,000 pg/ml standard; the unused portions of the reconstituted standard should be discarded.

Standard Concentration (pg/ml) Volume of Deionized Water to Add Volume of Standard to Add 2,000 250 µl 0 Standard Concentration (pg/ml) Volume of Assay Buffer to Add Volume of Standard to Add 400 200 µl 50 µl of 2,000 pg/ml 80 200 µl 50 µl of 400 pg/ml 16 200 µl 50 µl of 80 pg/ml 3.2 200 µl 50 µl of 16 pg/ml 0.64 200 µl 50 µl of 3.2 pg/ml 0.13 200 µl 50 µ of 0.64 pg/ml

Standard Concentration (pg/ml)	Volume of Deionized Water to Add	Volume of Standard to Add	
2,000	250 µl	0	
Standard Concentration (pg/ml)	Volume of Assay Buffer to Add	Volume of Standard to Add	
400	200 µl	50 µl of 2,000 pg/ml	
80	200 µl	50 µl of 400 pg/ml	
16	200 µl	50 µl of 80 pg/ml	
3.2	200 µl	50 µl of 16 pg/ml	
0.64	200 µl	50 µl of 3.2 pg/ml	
0.13	200 µl	50 µ of 0.64 pg/ml	

2). Preparation of Working Standards The following description and the Plate Map illustrate 1:5 dilution of standards. However, the end user has an option to select 1:4 or 1:3 dilution of standards with lowest concentration point at ~0.1 pg/ml. Label six polypropylene microfuge tubes 400, 80, 16, 3.2, 0.64, and 0.13 pg/ml. Add 200 µl of Assay Buffer to each of the six tubes. Prepare serial dilutions by adding 50 µl of the 2,000 pg/ml reconstituted standard to the 400 pg/ml tube, mix well and transfer 50 µl of the 400 pg/ml standard to the 80 pg/ml tube, mix well and transfer 50 µl of the 80 pg/ml standard to the 16 pg/ml tube, mix well and transfer 50 µl of the 16 pg/ml standard to 3.2 pg/ml tube, mix well and transfer 50 µl of the 3.2 pg/ml standard to the 0.64 pg/ml tube, mix well and transfer 50 µl of the 0.64 pg/ml standard to the 0.13 pg/ml tube and mix well. The 0 pg/ml standard (Background) will be Assay Buffer.

VIII. PREPARATION OF REAGENTS FOR IMMUNOASSAY (continued)

C. Preparation of High Sensitivity Human Cytokine Quality Controls

Before use, reconstitute one vial of High Sensitivity Human Cytokine Control 1 and High Sensitivity Human Cytokine Control 2 each with 250 µl Deionized Water. Invert the vials several times to mix and vortex. Allow the vial to set for 5-10 minutes and then transfer the controls to an appropriately labeled polypropylene microfuge tube. Unused portions of reconstituted Quality Controls should be discarded.

D. Preparation of Wash Buffer

Bring the 10X Wash Buffer to room temperature and mix to bring all salts into solution.

Dilute 30 ml of 10X Wash Buffer with 270 ml deionized water. Store unused portions at 2-8°C for up to one month.

E. Preparation of Serum Matrix

This step is required for serum or plasma samples only.

Add 5.0 mL deionized water to the bottle containing lyophilized Serum Matrix. Mix well. Allow at least 10 minutes for complete reconstitution. Left-over reconstituted Serum Matrix should be stored at α -20°C for up to one month.

IX. IMMUNOASSAY PROCEDURE

Prior to beginning this assay, it is imperative to read this protocol completely and to thoroughly understand the Technical Guidelines outlined in Section VII.

Allow all reagents to warm to room temperature (20-25°C) before use in the assay.

1. Diagram placement of Standards, 0 (Background), 0.13, 0.64, 3.2, 16, 80, 400, and 2,000 pg/ml, Controls I and II, and samples on Well Map Worksheet in a vertical configuration. (Note: the instrument will only read the 96-well plate vertically). It is recommended to run the assay in duplicate.
2. Pre-wet the filter plate by pipetting 200 µL of **1X Wash Buffer** into each well of the microtiter plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20--25°C).

IX. IMMUNOASSAY PROCEDURE (continued)

9. Add 50 µL of appropriate matrix solution to the Background, Standards, and Control wells. When assaying serum or plasma, use Serum Matrix provided in the kit. When assaying Tissue Culture supernatant samples, use proper control culture medium as the matrix solution.

10. Add 50 µL of Sample into the appropriate sample wells. Before addition to wells, the samples should be centrifuged to remove any precipitates or denatured proteins occurred during storage and handling.

11. Seal, cover with aluminum foil, and incubate with agitation on a plate shaker overnight (16-18 hr) at 4°C.

12. Gently remove fluid by vacuum. **(NOTE: DO NOT INVERT PLATE)**.

13. Wash plate 2 times with 200 µL/well of Wash Buffer, removing Wash Buffer by vacuum filtration after each wash. Remove any excess Wash Buffer from the bottom of the plate by blotting on an absorbent pad or paper towels.

14. Add 50 µL of Detection Antibody Cocktail into each well. (Note: allow the Detection Antibody to warm to room temperature prior to addition.)

15. Seal, cover with aluminum foil, and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C). **DO NOT VACUUM AFTER INCUBATION.**

16. Add 50 µL Streptavidin-Phycoerythrin to each well containing the 50 µL of Detection Antibody Cocktail.

17. Seal, cover with aluminum foil, and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).

18. Gently remove all contents by vacuum. **(NOTE: DO NOT INVERT PLATE)**.

19. Wash plate 2 times with 200 µL/well Wash Buffer, removing Wash Buffer by vacuum filtration after each wash. Wipe any excess buffer on the bottom of the plate with a tissue.

20. Add 100 µL of Sheath Fluid to all wells. Seal, cover with aluminum foil and resuspend the beads on a plate shaker for 5 minutes.

21. Read plate on Luminex Instrument.

X. EQUIPMENT SETTINGS

Select the following equipment settings:

Events: 100, per bead Sample Size: 50 µl Bead Set: 01 for IL-1

03 for IL-2 09 for IL-4 10 for IL-5 12 for IL-6 13 for IL-7 20 for IL-8 23 for IL-10 25 for IL-12(p70) 26 for IL-13 35 for IFNβ 39 for GM-CSF 40 for TNFa

**Gate (for IS System): 7,500 to 15,000 **Gate (for 1.7 System): 8,060 to 13,000

**These specifications are for the Luminex100 or Luminex200 with software v. 1.7 or IS. Luminex instruments with other software (e.g. Masterplex, Starstation, LiquiChip, Bioplex, LABScan100) would need to follow instrument instructions for gate settings and additional specifications.

Disclosures

No conflicts of interest declared.