The Bio-Plex cytokine assay employs a liquid suspension array for quantification of cytokines in tissue culture supernatants or serum. Using this 96-well microtiter plate-formatted assay, it is possible to profile the level of multiple cytokines in a single well. The principle of the Bio-Plex cytokine assay is similar to a capture sandwich immunoassay. An antibody directed against each desired cytokine is covalently coupled to a different color-coded polystyrene bead. The conjugated beads are allowed to react with a sample containing a known (standard) or unknown amount of cytokines. After unbound cytokines are removed, biotinylated detection antibodies directed against a different epitope on each cytokine are added to the reaction. The result is the formation of a sandwich of antibodies around each cytokine. The complexes are detected by the addition of streptavidin-phycocerythrin (streptavidin-PE), which has fluorescence characteristics distinct from the beads. A specialized microtiter plate reader, which allows for analysis of multiplexed bead-capture immunoassays in a single microtiter well, carries out quantification. By reading beads individually in the mixture, the system can detect each cytokine separately. The Bio-Plex software automatically calculates the concentration of cytokines from standard curves derived from a mixture of cytokine standards of a known amount.

**Preparation for the Assay**

1. Bring buffers and diluents in the reagent kit to room temperature prior to use. The kit includes wash buffer A, assay buffer A, and detection antibody diluent A. The following components should be kept on ice: streptavidin-PE, anti-mouse cytokine 18-plex conjugated beads, mouse cytokine 18-plex detection antibody, and mouse cytokine standard. If frozen, thaw experimental samples and keep all on ice as well.

2. Design experiment for a 96-well plate. Reserve 16 wells for the standard curve (designated S1-S8, in table below) and 2 wells for the blank (culture medium). Use the remaining wells for analyzing experimental samples (indicated by X followed by a number, in table below).

<table>
<thead>
<tr>
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<th>1</th>
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<td>X15</td>
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<td>S6</td>
<td>X1</td>
<td>X9</td>
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<td>S6</td>
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<td>X26</td>
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<tr>
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<td>S7</td>
<td>X4</td>
<td>X12</td>
<td>X20</td>
<td>X28</td>
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<td>X6</td>
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<td>X46</td>
<td>X54</td>
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</table>
   - Reconstitute lyophilized multiplex cytokine standards to a stock concentration of 500,000 pg/ml by adding 50 µl of sterile distilled water. Vortex solution gently for 5 sec and incubate on ice for 30 min. Note: Bio-Rad recommends not using the standards >4 hr after reconstitution.
   - Prepare serial dilutions of the 500,000 pg/ml standard stock solution. Suggested concentrations, designated S1 to S8, are listed in the table below. Dilutions should be made in culture medium such as RAW 264.7 growth medium 1 (RAWGM1). Keep dilutions on ice.

<table>
<thead>
<tr>
<th>Standard sample</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
<th>S7</th>
<th>S8</th>
</tr>
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<tbody>
<tr>
<td>Concentration (pg/ml)</td>
<td>1.95</td>
<td>7.8</td>
<td>31.25</td>
<td>125</td>
<td>500</td>
<td>2,000</td>
<td>8,000</td>
<td>32,000</td>
</tr>
</tbody>
</table>

4. Dilute samples in culture medium if necessary. Keep the diluted samples on ice.

5. Prepare the MultiScreen vacuum manifold following AfCS Procedure Protocol PP00000221, Assembly and Use of MultiScreen Vacuum Manifold for Bio-Plex Assay of Cytokines or Phosphoproteins.

6. Dilute the suspension of anti-mouse cytokine 18-plex conjugated beads.
   - Vortex the anti-mouse cytokine 18-plex conjugated beads (25X) stock solution at medium speed for 20 sec.
   - Prepare a 25-fold working dilution of the suspension in Bio-Plex assay buffer A.

7. Cover all unneeded wells with the sealing tape provided with the kit. Employ razor blade to cut the plastic sealer between those columns of the 96-well plate that will be used and those that will not.
   - Pour Bio-Plex assay buffer A into a pipettor solution basin (labeled “assay”).
   - Pre-wet the filter plate with 100 µl per well of Bio-Plex assay buffer A using an 8-channel pipettor.
   - Vacuum filter.
   - Blot the bottom of the filter plate on a stack of paper towels.

**Cytokine Assay**

8. Add bead suspension.

9. Vortex the working bead suspension at medium speed for 20 sec and pour into a pipettor solution basin (labeled “beads”).

10. Add 50 µl of bead suspension to each well using an 8-channel pipettor.

11. Vacuum filter the plate and blot it.

12. Filter wash the beads twice with 100 µl Bio-Plex wash buffer A. Blot the bottom of the filter plate after each filtration.

13. Vortex each standard dilution and immediately add 50 µl to the appropriate wells (in duplicate). Add the appropriate blank, which is culture medium.

14. Vortex each sample for 20 sec and immediately add 50 µl to the appropriate wells.

15. Cover the wells of the used columns in the 96-well plate with the rest of the sealing tape used in step 7. Blot the bottom of the plate.
16. Cover with aluminum foil to protect from light and incubate for 30 min at room temperature with shaking (using a plate stirrer).
17. Shake the plate at 1,100 rpm for 30 sec, by slowly ramping up to this high speed.
18. Reduce speed to 300 rpm for the remainder of the incubation.
19. Turn on power to the Bio-Plex suspension array system and operate start-up procedure (according to manufacturer’s instructions). Do not turn on more than 2 hr before use or it will go into sleeping mode, in which case, you will need to operate start-up procedure again, which may take about 30 min.
20. Prepare mouse cytokine 18-plex detection antibody (during incubation and 10 min prior to use).
21. Gently vortex the cytokine 18-plex detection antibody stock solution.
22. **Note**: the concentration of the detection antibody stock solution will vary as a function of the degree of multiplexing of each panel.
   - For single analytes, the detection antibody is provided in a 100X stock solution.
   - For 2 to 9 analytes, the detection antibodies are provided in a 50X stock solution.
   - For 10 or more analytes, the detection antibodies are provided in a 25X stock solution.
23. Prepare the appropriate working dilution of the cytokine 18-plex detection antibody stock solution in detection antibody diluent A.
24. Remove the sealing tape. Filter and blot the plate.
25. Wash 3 times with 100 µl Bio-Plex wash buffer A using an 8-channel pipettor.
26. Vortex the working dilution of the detection antibody gently, pour it into pipettor solution basin (labeled “detection”), and add 25 µl to each well using an 8-channel pipettor.
27. Cover the wells with sealing tape, and blot the bottom of the plate.
28. Cover plate with aluminum foil and incubate for 30 min at room temperature with shaking.
29. Initiate shaking as described in steps 17 and 18.
30. Calibrate the Bio-Plex suspension array system as described in the manufacturer’s manual.
31. Prepare streptavidin-PE (during incubation of the beads, step 28, and 10 min prior to use)
32. Vortex the streptavidin-PE (100X) stock solution vigorously.
33. Prepare a 100-fold working dilution of the streptavidin-PE (100X) stock solution in Bio-Plex assay buffer A.
34. Remove the sealing tape. Filter and blot the plate.
35. Wash 3 times with 100 µl Bio-Plex wash buffer A using an 8-channel pipettor.
36. Vortex the streptavidin-PE working dilution, pour it into pipettor solution basin (labeled “PE”), and add 50 µl to each well using an 8-channel pipettor.
37. Cover the wells with sealing tape, and blot the bottom of the plate.
38. Cover with foil and incubate for 10 min at room temperature with shaking.
39. Initiate shaking as described in steps 17 and 18.
40. Remove the sealing tape. Filter and blot the plate.
41. Wash 3 times with 100 µl wash buffer A using an 8-channel pipettor.
42. Resuspend the beads in each well with 125 µl of Bio-Plex assay buffer A using an 8-channel pipettor.
43. Cover the wells with sealing tape, and thoroughly blot the bottom of the plate.
44. Shake plate by slowly ramping up to 1100 rpm, maintain at that speed for 30 sec, and slowly ramp down the speed to stop.
45. Remove the sealing tape and read the plate with Bio-Plex manager.

Reagents and Materials

Bio-Plex cytokine reagent kit: Bio-Rad; catalog no. 171-304000
- Includes assay buffer A, wash buffer A, detection antibody diluent A, streptavidin-PE, 96-well filter plate, 4 rolls sealing tape, and instructions

Bio-Plex mouse cytokine 18-plex panel: Bio-Rad; catalog no. 171-F11181
- Includes anti-mouse cytokine 18-plex conjugated beads, mouse cytokine 18-plex detection antibody, and mouse cytokine standard. 18-Plex cytokine standard includes: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-12(p40), IL-12(p70), IL-17, G-CSF, GM-CSF, INF-γ, KC, MIP-1α, RANTES, and TNF-α.

RAW 264.7 growth medium 1 (RAWGM1): AfCS Solution Protocol ID PS00000510

MultiScreen vacuum manifold (MultiScreen filtration system): Millipore; catalog no. MAVM0960R
- Includes manifold base, quick disconnect body, plastic ring (standard size), manifold support grid, vacuum manifold gaskets, bleeder valve, plate alignment tabs, vacuum pressure gauge, on/off valve, three-way connector, vacuum control valve, hex key, straight connector, and FEP-lined PVC tubing ¼” I.D.

MultiScreen–BV plate (filter plates for high-throughput separations): Millipore; catalog no. MABVN1210 10/PK
- Includes non-sterile clear plates, lid, 1.2-µm hydrophilic, and low protein binding Durapore membrane

Pipet-Lite multichannel pipette: Rainin; catalog no. L8-200

Pipettor solution basin: VWR; catalog no. 21007-970

IKA MTS2/4 digital stirrer (plate stirrer), 115V: VWR; catalog no. 82006-096

Bio-Plex suspension array system: Bio-Rad; catalog no. 171-000005
- Includes array reader, microplate platform, computer, monitor, Bio-Plex manager software, high-throughput fluidics (HTF) system, calibration kit, validation kit, maintenance, calibration and validation (MCV) plate II, 20 L sheath fluid, instructions