

SAMPLE PROTOCOL FOR COMPETITIVE IMMUNOASSAY FOR ANTIBODY-COUPLED MICROSPHERES

Microspheres should be protected from prolonged exposure to light throughout this procedure.

1. Select the appropriate antibody-coupled microsphere sets.
2. Resuspend the microspheres by vortex and sonication for approximately 20 seconds.
3. Prepare a Working Microsphere Mixture by diluting the coupled microsphere stocks to a final concentration of 200 microspheres of each set/ μL in PBS-1% BSA. (Note: 25 μL of Working Microsphere Mixture is required for each reaction.) See **Technical Note 1**.
4. Dilute the biotinylated competitor to the $[\text{IC}_{80}]$ in PBS-1% BSA. (Note: 25 μL of diluted competitor is required for each reaction.) See **Technical Note 2**.
5. Add 25 μL of PBS-1%BSA to each background well in a round-bottom microtiter plate.
6. Add 25 μL of standard or sample to the appropriate wells.
7. Add 25 μL of the diluted, biotinylated competitor to each well.
8. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
9. Add 25 μL of the Working Microsphere Mixture to the appropriate wells of a round-bottom microtiter plate.
10. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
11. Cover the plate and incubate for 60 minutes at room temperature on a plate shaker.
12. Dilute the streptavidin-R-phycoerythrin reporter to 4 $\mu\text{g}/\text{mL}$ in PBS-1% BSA. (Note: 25 μL of diluted streptavidin-R-phycoerythrin is required for each reaction). See **Technical Note 3**.
13. Add 25 μL of the diluted streptavidin-R-phycoerythrin to each well.
14. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
15. Cover the plate and incubate for 30 minutes at room temperature on a plate shaker.
16. OPTIONAL – Include the following steps if high backgrounds occur:
 - a. Pre-wet a 1.2 μm Millipore filter plate with 100 $\mu\text{L}/\text{well}$ of PBS-1% BSA and aspirate by vacuum manifold.
 - b. Add 50 μL of PBS-1% BSA to the appropriate wells of the filter plate.
 - c. Transfer the contents of round-bottom plate to the filter plate.
 - d. Aspirate the supernatant by vacuum manifold.

- e. Wash each well twice with 100 μ L of PBS-1% BSA and aspirate by vacuum manifold.
- f. Resuspend the microspheres in 100 μ L of PBS-1% BSA by gently pipetting up and down five times with a multi-channel pipettor.

17. Analyze 50-75 μ L on the Luminex analyzer according to the system manual.

Technical Note 1: Either PBS-1% BSA or PBS-BN (PBS, 1% BSA, 0.05% Azide, pH 7.4) may be used as Assay Buffer.

Technical Note 2: The $[IC_{80}]$ is the concentration of biotinylated competitor that yields 80% of the maximum obtainable signal. The $[IC_{80}]$ should be determined by titration in PBS-1% BSA (or PBS-BN).

Technical Note 3: Concentrations should be optimized for specific reagents, assay conditions, level of multiplexing, etc. in use.