

## SAMPLE PROTOCOL FOR COMBINED CAPTURE SANDWICH/COMPETITIVE IMMUNOASSAY

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

1. Select the appropriate antibody- and/or antigen-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 1000 microspheres of each set/ $\mu\text{L}$  in PBS-1% BSA. (Note: 5  $\mu\text{L}$  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: 5  $\mu\text{L}$  of diluted competitor is required for each reaction.) See **Technical Note 2**.
5. Add 10  $\mu\text{L}$  of PBS-1%BSA to each background well of a round-bottom microtiter plate.
6. Add 10  $\mu\text{L}$  of standard or sample to the appropriate wells.
7. Add 5  $\mu\text{L}$  of the diluted competitor to each well.
8. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
9. Aliquot 5  $\mu\text{L}$  of the Working Microsphere Mixture to each well.
10. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
11. Cover the filter plate and incubate for 60 minutes at room temperature on a plate shaker.
12. Dilute the biotinylated detection antibody to the appropriate concentration in PBS-1% BSA. (Note: 10  $\mu\text{L}$  of diluted detection antibody is required for each reaction). See **Technical Note 3**.
13. Add 10  $\mu\text{L}$  of the diluted detection antibody 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 60 minutes at room temperature on a plate shaker.
16. Dilute the streptavidin-R-phycoerythrin reporter to 10-12  $\mu\text{g}/\text{mL}$  in PBS-1% BSA. (Note: 10  $\mu\text{L}$  of diluted streptavidin-R-phycoerythrin is required for each reaction). See **Technical Note 3**.
17. Add 10  $\mu\text{L}$  of the diluted streptavidin-R-phycoerythrin to each well.

18. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
19. Cover the plate and incubate for 30 minutes at room temperature on a plate shaker.
20. Bring final volume of each reaction to 100  $\mu$ L with PBS-1% BSA.
21. OPTIONAL – Include the following steps if high backgrounds occur:
  - a. Pre-wet a 1.2  $\mu$ m Millipore filter plate with 100  $\mu$ L/well of PBS-1% BSA and aspirate by vacuum manifold.
  - b. Add 50  $\mu$ L 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  $\mu$ L of PBS-1% BSA and aspirate by vacuum manifold.
  - f. Resuspend the microspheres in 100  $\mu$ L of PBS-1% BSA by gently pipetting up and down five times with a multi-channel pipettor.
22. Analyze 50-75  $\mu$ 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 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.