

## SAMPLE PROTOCOL FOR DIRECT DNA HYBRIDIZATION – WASHED ASSAY FORMAT

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

1. Select the appropriate oligonucleotide-coupled microsphere sets.
2. Resuspend the microspheres by vortex and sonication for approximately 20 seconds.
3. Prepare a Working Microsphere Mixture by diluting coupled microsphere stocks to 150 microspheres of each set/ $\mu\text{L}$  in 1.5X TMAC Hybridization Solution. (Note: 33  $\mu\text{L}$  of Working Microsphere Mixture is required for each reaction.)
4. Mix the Working Microsphere Mixture by vortex and sonication for approximately 20 seconds.
5. To each sample or background well, add 33  $\mu\text{L}$  of Working Microsphere Mixture.
6. To each background well, add 17  $\mu\text{L}$  TE, pH 8.
7. To each sample well add amplified biotinylated DNA and TE, pH 8.0 to a total volume of 17  $\mu\text{L}$ . (Note: 2-5  $\mu\text{L}$  of a robust PCR reaction is usually sufficient for detection.)
8. Mix reaction wells gently by pipetting up and down several times.
9. Cover the reaction plate to prevent evaporation and incubate 95-100°C for 5 minutes to denature the amplified biotinylated DNA. \*
10. Incubate the reaction plate at hybridization temperature for 15 minutes. \*
11. Centrifuge the sample plate at  $\geq 2,250 \times g$  for 3 minutes to pellet the microspheres. See **Technical Note**.
12. During centrifugation, prepare fresh Reporter Mix by diluting streptavidin-R-phycoerythrin to 2-4  $\mu\text{g}/\text{mL}$  in 1X TMAC Hybridization Solution. (Note: 75  $\mu\text{L}$  of Reporter Mix is required for each reaction.)
13. After centrifugation, carefully remove the supernatant. (Note: An 8-channel pipettor can be used to extract the supernatant in 8 wells simultaneously.)
14. Return the sample plate to hybridization temperature.
15. Add 75  $\mu\text{L}$  of Reporter Mix to each well and mix gently by pipetting up and down several times.
16. Incubate the reaction plate at hybridization temperature for 5 minutes.
17. Analyze 50  $\mu\text{L}$  at **hybridization temperature** on the Luminex analyzer according to the system manual.

\* *These steps can be combined with the use of a thermal cycler programmed as follows –*

*Hold at 95°C, 5 minutes*

*Hold at hybridization temperature, FOREVER*

**Technical Note:** Alternatively, pre-wet a 1.2  $\mu\text{m}$  Millipore filter plate with 1X TMAC Hybridization Buffer and filter by vacuum manifold. Transfer the reactions to the pre-wetted filter plate and remove the supernatant by vacuum filtration. Resuspend the reactions in 75  $\mu\text{L}$  of Reporter Mix by gently pipetting up and down several times. Quickly transfer the reactions back into a 96-well V-bottom PCR plate and proceed with step 16.