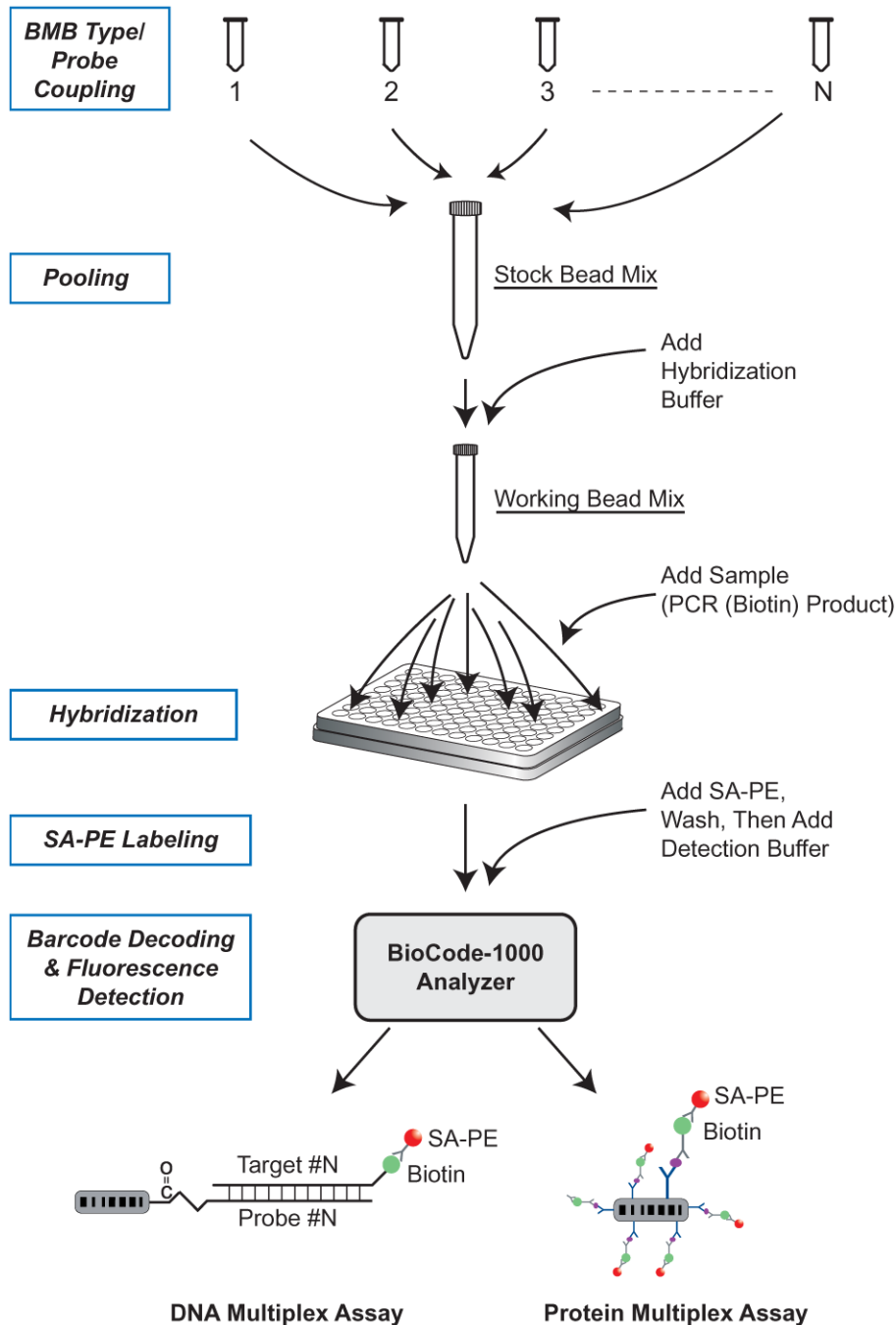


## Protocols for Capture Probe Coupling, Hybridization with Biotinylated Target, and SA-PE Reaction

### Procedure Overview



**Materials:**

1. 25 mM MES buffer (pH 6.0)
2. Tris-HCl 50mM, Ph 7.4
3. 1X PBS (pH 7.4); 1xPBS/1% BSA
4. 1x PBS-T (Tween-20, 0.05%); 1xPBS-T/1% BSA
5. Nuclease-free water
6. Beads: Carboxyl Barcode Magnetic Beads (BMB)
7. Capture Oligo: 5'-Amino C6/C12 modified (NH<sub>2</sub>): (100pM/μl)
8. Analyte: Target Oligo: 5'-Biotin modified-PCR product (or any other targets)
9. Streptavidin-R-Phycoerthrin Conjugate (SA-PE) (1mg/ml)-Invitrogen
10. Hybridization buffer: TMAC 3M, Sarkosyl 0.1%, Tris-HCl 50mM pH8.0, EDTA 4mM pH8.0 (or 5xSSC, 0.1% SDS, or others)
11. Detection buffer (Applied Biocode, Catalog No. 43-B0001)
12. Filtred 96-well plate if used (Milliport), Flat bottom-clear 96-well microplate (Thermo)

**Equipments:**

1. Microplate Incubator-Shaker Vortemp 56 (Labnet International, Woodbridge, NJ)
2. VWR Rotator Multimix (VWR)
3. Magnetic stand, and Magnetic block
4. Tube Vortexer

**Preparation:**

1. 25mM MES buffer: 0.53g of MES buffer (2-(N-morpholinoethane sulfonic acid)), dissolve in 90ml water; adjust pH to 6.0, and final volume to 100ml.
2. Sample or Analyte: PCR products

**A. Capture Oligo Probe Coupling to BMB:**

1. Wash and vortex ~5000 beads once with 500μl nuclease free H<sub>2</sub>O, and once with MES buffer in 2ml tube. Quick spin and use magnetic stand to hold the beads. Remove the supernatant.
2. Add 116μl MES buffer to the beads, vortex 5 seconds.
3. Add 24μl Capture oligo (5' NH<sub>2</sub> C6/C12 modified oligo: 100pM/μl, 20μM final in 120μl Volume), vortex 5 second.
4. Freshly prepare EDC 10mg/ml solution (10mg EDC in 1 ml of cold MES buffer), immediately add 60μl of fresh prepared EDC solution (200μl final), vortex for 5 seconds, then incubate for two hours at RT, shaking at 300 rpm in Vortemp. Vortex 5 second from time to time.
5. Remove the supernatant using magnetic stand to hold the beads, and incubate the BMB with 500μl Tris-HCl 50mM, pH 7.4 for 15 minutes at RT, rotating in VWR Multimix.

6. Wash the BMBs once with 500µl PBS pH 7.4, vortex 5-10 second. Quick spin and use magnetic stand to hold the beads, and remove the supernatant.
7. Block samples with 500µl PBS/1% BSA, incubate for 1 hr at RT rotating in VWR Multimix.
8. Remove the blocking buffer using the magnetic stand. Resuspend the beads in 500µl PBS-T, and store the beads at 4°C, or process for the hybridization.

**B. Prepare Stock Bead Mix for Multiplex Assay:**

In general, BioCode-1000 Analyzer can detect 100 - 700 beads per microwell by using a flat bottomed 96 well microplate. So, depending upon the number of multiplex for each test, you can mix 20-100 beads per type of BMB in PBS-T stock solution. For example, to make 1ml of a Stock Bead Mix for 100 tests with 12 different probes, 2,500 probe coupled beads of each type can be pooled together in one 2ml tube. After removing the supernatant, the beads will be resuspended in 1ml PBS-T. Store the beads at 4°C. Table 1 shows the suggested bead numbers per test depend upon the number of multiplex.

**Table 1:** Suggested bead number based on the number of multiplex.

Number of Multiplex	Number of Beads/Type/Test in 10µl PBS-T	Number of Beads/Type For 100 Test in 1ml PBS-T	Number of Beads in Stock Bead Mix for 100 Test in 1ml PBS-T
Single-Plex	50-100	5,000-10,000	5,000-10,000
2-Plex	50	5,000	10,000
3-Plex	25-50	2,500-5,000	7,500-15,000
4-Plex	25-50	2,500-5,000	10,000-20,000
5-Plex	25-50	2,500-5,000	12,500-25,000
10-Plex	25-35	2,500-3,500	25,000-35,000
20-Plex	20-25	2,000-2,500	40,000-50,000
30-Plex	20-25	2,000-2,500	60,000-75,000

**C. Hybridization Procedure:**

Three Hybridization methods, depending on your choice, are described in detail in the following sections:

- C-1. Using 2ml Tubes
- C-2. Using Filtered 96 well plate
- C-3. Using 96 well flat bottomed plate

**C-1: Hybridization: Using 2ml Tubes**

1. Vortex the Stock Bead Mix for >10 second, and transfer sufficient volume of Stock Bead Mix with desired number of bead/type/test (for example: 10 $\mu$ l of stock mix per test or 100 $\mu$ l for 10 tests, plus 10% extra) into a new 2ml tube. Use magnetic stand to hold the beads, and remove the supernatant. Add 45 $\mu$ l per test (or 450  $\mu$ l for 10 tests) of TMAC hybridization buffer (or 5xSSC, 0.1% SDS buffer, or others) into the tube as your Working Bead Mix.
2. Vortex the Working Bead Mix 10 seconds, and then aliquot 45 $\mu$ l Working Bead Mix into each hybridization tube (2 ml) marked with sample name; pipetting up and down several times before each aliquoting.
3. Add 5 $\mu$ l sample or biotinylated asymmetric PCR product to its corresponding sample tube, and vortex for 5 seconds (For normal PCR products, denature the PCR samples first, and then mix with BMB).
4. Incubate for **10-15 minutes** at 52°C and shaking at 700 rpm in Vortemp 56.

**SA-PE Treatment:**

1. Prepare SA-PE solution: 60 $\mu$ g/ml in 50% TMAC hybridization buffer, and 50% PBS-T.
2. Add 10 $\mu$ l SA-PE solution per tube, vortex 5 seconds, and continue shaking at 700 rpm for **5 – 10 minutes at 52°C**.
3. Add pre-warmed H<sub>2</sub>O 120 $\mu$ l to stop the hybridization reaction.
4. Vortex 5 seconds, quick spin down, and remove supernatant using the magnetic stand.
5. Wash the beads once with 350 $\mu$ l PBS-T buffer by vortexing 5 seconds, spin down, and remove supernatant using magnetic stand.
6. Resuspend the BMB with 200 $\mu$ l Detection buffer, vortex 5 second, pipetting up and down >10 time, and transfer it into 96-well plate.
7. Place the plate in BioCode 1000 Analyzer for decoding and fluorescence detection.

**C-2: Hybridization: Using Filtered 96-Well Plate**

1. Pre-wet the filtered plate well with 150 $\mu$ l PBS-T buffer. Remove the supernatant with vacuum.
5. Vortex the Stock Bead Mix for >10 second, and transfer sufficient volume of Stock Bead Mix with desired number of bead/type/test (for example: 10 $\mu$ l Stock Bead Mix per test or 100 $\mu$ l for 10 tests, plus 10% extra) into a new 2ml tube. Use the magnetic stand to hold the beads, and remove the supernatant. Add 45 $\mu$ l per test (or 450  $\mu$ l for 10 tests) of TMAC hybridization buffer (or 5xSSC, 0.1% SDS buffer, or others) into the tube as your Working Bead Mix.
2. Vortex the Working Bead Mix 10 seconds, and then aliquot 45 $\mu$ l Working Bead Mix into each pre-wetted hybridization well; pipetting up and down several time before each aliquoting.

3. Add 5µl biotinylated asymmetric PCR Product per well (For normal PCR products, denature the PCR samples first, then mix with BMB), mix by pipetting up and down 5 times.
4. Incubate for **10-15 minutes** at 52°C and shaking at 700 rpm in Vortemp 56.

**SA-PE Treatment:**

1. Prepare SA-PE solution: 60µg/ml in 50% TMAC hybridization buffer, and 50% PBS-T.
2. Add 10µl pre-warmed SA-PE solution per well, mix by pipetting up and down 5 times, and continue shaking at 700 rpm for **5 – 10 minutes at 52°C**.
3. Add pre-warmed H<sub>2</sub>O 120µl to stop the hybridization reaction, mix by pipetting up and down 5 times.
4. Remove supernatant using the vacuum.
5. Wash the beads twice with 200µl PBS-T buffer, and remove supernatant using the vacuum. Resuspend the BMB in 200µl Detection buffer by pipetting up and down >10 time, and transfer to 96-well plate.
6. Place the plate in BioCode-1000 Analyzer for decoding and fluorescence detection.

**C-3: Hybridization: Using 96-Well Flat Bottomed Plate**

1. Vortex the Stock Bead Mix for >10 seconds, and transfer sufficient volume of Stock Bead Mix with desired number of bead/type/test (for example: 10µl per test or 100µl for 10 tests, plus 10% extra) into a new 2ml tube. Use the magnetic stand to hold the beads, and remove the supernatant. Add 45µl per test (or 450 µl for 10 tests) of TMAC hybridization buffer (or 5xSSC, 0.1% SDS buffer, or others) into the tube as Working Bead Mix.
2. Vortex the Working Bead Mix for 10 seconds, and then aliquot 45µl Working Bead Mix into each micro-well; pipetting up and down several times before each aliquoting.
3. Add 5µl biotinylated asymmetric PCR product into corresponding well (for normal PCR products, denature the PCR samples first, then mix with BMB), and mix by pipetting up and down 5 times
4. Incubate for **10-15 minutes** at 52°C and shaking at 700 rpm in Vortemp 56.

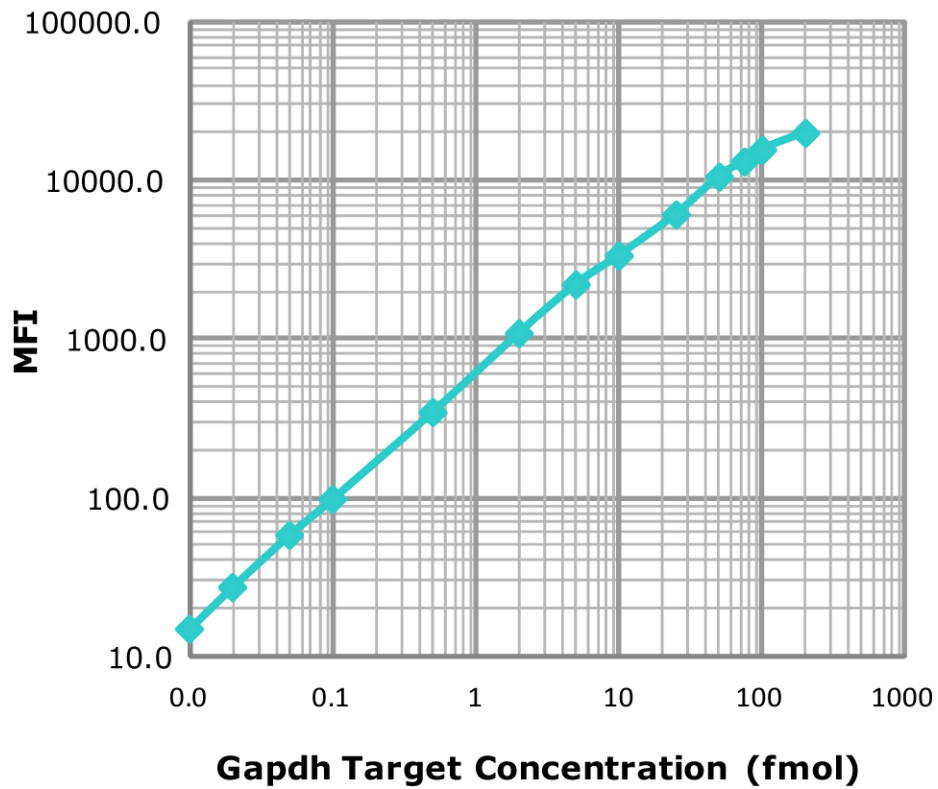
**SA-PE Treatment:**

1. Prepare SA-PE solution: 60µg/ml in 50% TMAC hybridization buffer, and 50% PBS-T.
2. Add 10µl pre-warmed SA-PE solution per well, mix by pipetting up and down 5 times. Continue shaking at 700 rpm for **5-10 minutes at 52°C**.
3. Add pre-warmed H<sub>2</sub>O 120µl to stop the hybridization reaction. Mix by pipetting up and down 5 times
4. Remove supernatant using wide orifice tips, and magnetic block.

5. Wash the beads once by adding 180µl PBS-T buffer per well, and remove supernatant just like step 4. Resuspend the BMB in 200µl Detection buffer, mix by pipetting up and down >10 times.
6. Place the plate in BioCode-1000 Analyzer for barcode decoding and fluorescence detection.

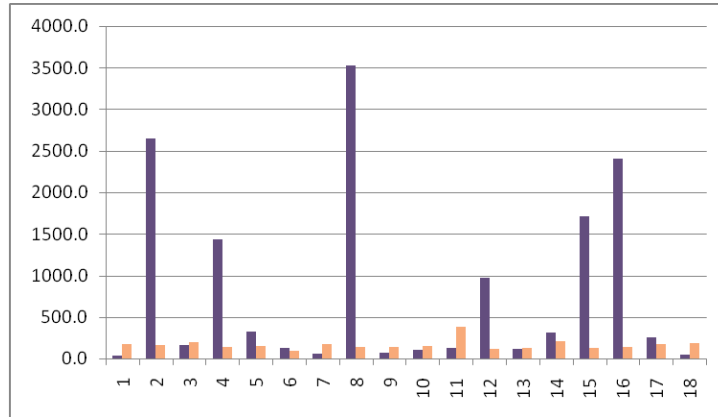
DNA Hybridization

**Gapdh Hybridization Test**

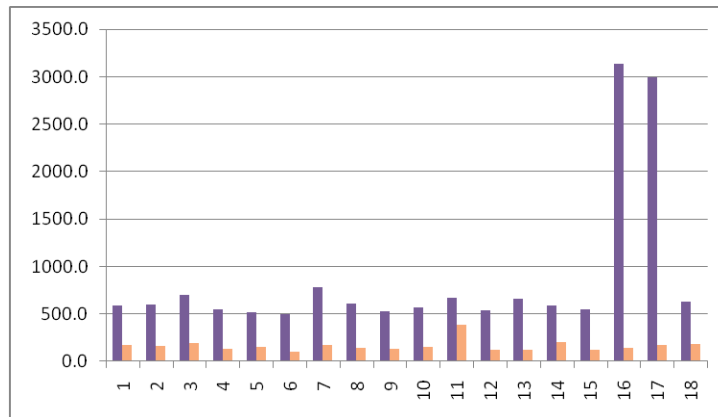


(carboxyl DMB) – (GAPDH Probe) – (GAPDH Target (50 mer)–Biotin) – (SA–PE)

**18-Plex Hybridization, Sample 1 (Positive Targets: 2, 4, 8, 12, 15 and 16)**



**Sample 2 (Positive Targets: 16 and 17)**



**Sample 3 (Positive Target: 18)**

