

MATERIALS

- Carboxylated fluorescent microspheres with covalently attached FlexMAP anti-TAG sequences
- PCR amplification primers for each target resuspended in sterile ddH₂O. PCR primers are reconstituted to 1 mM (1 nanomole/μL)
- ASPE primers with 5' TAG modification resuspended in sterile ddH₂O. ASPE primers are reconstituted to 1 mM (1 nanomole/μL)
- Qiagen HotStarTaq 2X Master Mix (Qiagen Cat. No. 203445) **or equivalent**
- ExoSAP-IT (USB Cat. No. 78200)
- Platinum Tsp, ASPE 10X Buffer, 50 mM MgCl₂ (Invitrogen Cat. No. 11448-024)
- dNTPs at 100 mM each (Invitrogen Cat. No. 10297-018)
- Biotin-14-dCTP at 0.4 mM (Invitrogen Cat. No. 19518-018)
- 2X Tm Hybridization Buffer – 0.4 M NaCl, 0.2 M Tris, 0.16% Triton X-100, pH 8.0
- 1X Tm Hybridization Buffer – 0.2 M NaCl, 0.1 M Tris, 0.08% Triton X-100, pH 8.0
- Streptavidin-R-phycoerythrin See [Solution: 10225](#) (Recommended Reagent List)
- 96 well V-bottom PCR plate and cover See [Solution: 10053](#) (Recommended Consumable List)
- Pipettors, tips, microfuge tubes, etc. See [Solution: 10053](#) (Recommended Consumable List)
- genomic DNA samples

PROCEDURES

Multiplexed PCR Reaction – PCR should be performed under optimized conditions. The parameters listed below are for example purposes only.

Each final reaction contains:

1X Qiagen PCR reaction buffer
1.5 mM MgCl₂
200 μM each dNTP
0.2 μM each primer
2.5 Units Qiagen HotStarTaq polymerase
50 ng template

PCR Cycling Parameters:

HOLD: 95°C, 15 minutes (for enzyme activation)

CYCLE: 94°C, 30 seconds

55°C, 30 seconds

72°C, 30 seconds

35 CYCLES

HOLD 72°C, 7 minutes

HOLD 4°C, FOREVER

EXO/SAP Treatment – OPTIONAL – Perform this step when high background for ASPE of PCR negative control is observed.

Treat 7.5 µL of each PCR reaction with ExoSAP-IT according to the following procedure:

PCR reaction	7.5 µL
ExoSAP-IT	<u>3.0 µL</u>
	10.5 µL

The following steps should be performed on a thermal cycler:

Mix and incubate at 37°C for 30 minutes.
Inactivate ExoSAP-IT by heating to 80°C for 15 minutes.

Hold the treated reactions at 4°C.

Multiplex ASPE Reaction:

Each 20 µL final reaction contains:

1X ASPE Buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl)
1.25 mM MgCl₂
25 nM each TAG-ASPE primer
0.75 U *Tsp* DNA polymerase
5 µM dATP, dTTP, dGTP – **Include when PCR reaction is EXO/SAP treated**
5 µM biotin-dCTP
5 µL treated PCR reaction
dH₂O (to 20 µL)

2X ASPE Master Mix (10 µL/reaction):

10X ASPE reaction buffer	2 µL
50 mM MgCl ₂	0.5 µL
20X TAG-ASPE primer mix (500 nM each)	1.0 µL (dilute 1 mM stocks 1:2000 for 20X mix)
5 U/µL <i>Tsp</i> DNA polymerase	0.15 µL
20X dNTP mix (-dCTP) (100 µM each)	1.0 µL (dilute 100 mM stocks 1:1000 for 20X mix)
400 µM biotin-dCTP	0.25 µL
dH ₂ O	<u>5.1 µL</u>
	10.0 µL

ASPE Cycling Parameters:

HOLD: 96°C, 2 minutes
CYCLE: 94°C, 30 seconds
55°C, 1 minute
74°C, 2 minutes
30 CYCLES
HOLD 4°C, FOREVER

Hybridization to FlexMAP Microspheres:

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

Complete the following steps in order to achieve this objective:

1. Select the appropriate FlexMAP microsphere sets and resuspend by vortex and sonication for approximately 20 seconds.
2. Combine 2500 microspheres of each set per reaction.
3. Concentrate the FlexMAP microsphere mixture by centrifugation at ≥ 8000 x g for 1-2 minutes.
4. Remove the supernatant and resuspend to 100 of each microsphere set per μL in 2X Tm Hybridization Buffer by vortex and sonication for approximately 20 seconds.
5. Aliquot 25 μL of the FlexMAP microsphere mixture to each well.
6. Add 25 μL of dH₂O to each background well.
7. Add 5 to 25 μL of each ASPE reaction to appropriate wells. (Note: 5 μL is usually sufficient.)
8. Adjust the total volume to 50 μL by adding the appropriate volume of dH₂O to each sample well.
9. Cover the plate to prevent evaporation and denature at 96°C for 90 seconds.
10. Hybridize at 37°C for 60 minutes.
11. Pellet the FlexMAP microspheres by centrifugation at ≥ 2250 x g for 3 minutes and remove the supernatant.
12. Resuspend the pelleted FlexMAP microspheres in 75 μL of 1X Tm Hybridization Buffer.
13. Pellet the FlexMAP microspheres by centrifugation at ≥ 2250 x g for 3 minutes and remove the supernatant.
14. Repeat steps 12. and 13. for a total of two washes.
15. Resuspend microspheres in 75 μL of 1X Tm Hybridization Buffer containing 2 $\mu\text{g}/\text{mL}$ streptavidin-R-phycoerythrin.
16. Incubate at 37°C for 15 minutes.
17. Analyze 50 μL at 37°C on the Luminex200 analyzer according to the system manual.