

Sample Protocols for Nucleic Acid Detection using Luminex xMAP Technology

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DIRECT HYBRIDIZATION

- Recommendations for Probe/Primer Design
- Carbodiimide Coupling of Amine-Modified Oligonucleotides to Carboxylated Microspheres
- Direct Hybridization of Oligonucleotide Targets
- Direct Hybridization of PCR-Amplified Targets
- DNA Buffers

FLEXMAP MICROSPHERES

- Hybridization Protocol for FlexMAP Microspheres
- Oligonucleotide Synthesis for Allele-Specific Primer Extension (ASPE)
- Allele-Specific Primer Extension (ASPE) and Hybridization to FlexMAP Microspheres
- Oligonucleotide Synthesis for Oligonucleotide Ligation Assay (OLA)
- Oligonucleotide Ligation Assay (OLA) and Hybridization to FlexMAP Microspheres

RECOMMENDED MATERIALS

- Source List
- Equipment
- Consumables
- Reagents

DIRECT HYBRIDIZATION

Recommendations for Probe/Primer Design

Carbodiimide Coupling of Amine-Modified Oligonucleotides to Carboxylated
Microspheres

Direct Hybridization of Oligonucleotide Targets

Direct Hybridization of PCR-Amplified Targets

DNA Buffers

Recommendations for Probe/Primer Design for Direct Hybridization

Oligonucleotide Synthesis

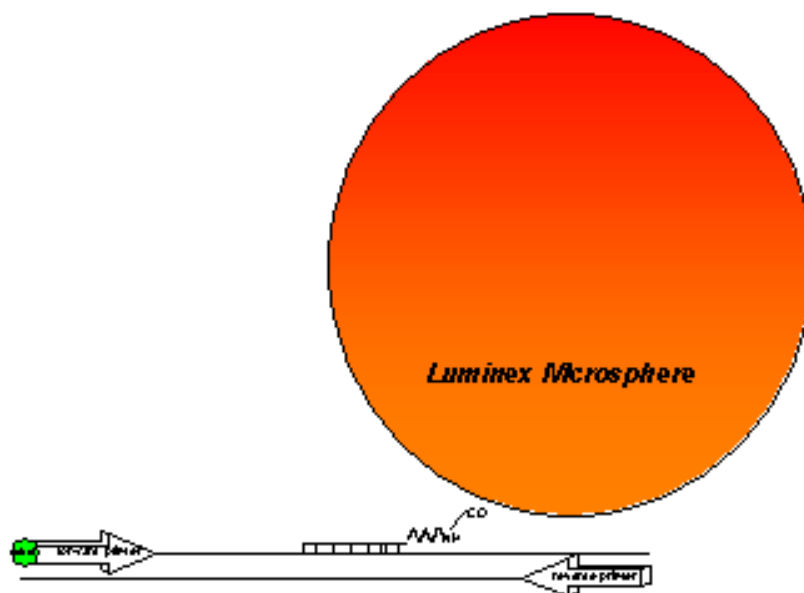
Probe Design Strategy

1. All probes should be exactly the same length per target sequence (using TMAC hybridization buffer).
2. For detection of point mutations, we typically use probes between 18 and 24 nucleotides in length. 20 nucleotides is a good starting point.
3. Point mutations (or SNPs) should be positioned at the center of the probe sequence (i.e., position 10 or 11 for a 20 nucleotide probe). Multiple polymorphisms should be equally spaced throughout the probe sequence.
4. Probes should be synthesized for all sequence variants (all mutant and wild type sequences) and should be from the same DNA strand (per target sequence).
5. Probes must have a primary amino group for coupling to the carboxyl group on the microsphere. We suggest synthesizing the oligonucleotide with a 5' amine-spacer (See 6.).
6. Probes must have a spacer between the reacting amine and the hybridizing sequence. We recommend synthesizing capture probes with 5' Amino Modifier C12 or 5' Uni-Link Amino Modifier. 5' Amine-C12 is available from most vendors. 5' Uni-Link can be ordered from Oligos Etc., Inc. and Operon.

PCR Primer Design For Direct Hybridization Format

1. Primers should be designed to yield amplicons in the 100 to 300 basepair range.
2. One PCR primer must be labeled with a 5'-biotin (for streptavidin-phycoerythrin reporter) or 5'-Cy3.
3. The labeled strand of the amplicon must be complementary to the capture probes on the microspheres.

Luminex¹⁰⁰ Direct Capture of a Labeled PCR Product



SAMPLE PROTOCOL FOR ONE-STEP CARBODIIMIDE COUPLING OF AMINE-MODIFIED OLIGONUCLEOTIDES TO CARBOXYLATED MICROSPHERES

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

1. Bring a fresh aliquot of -20°C, desiccated **Pierce** EDC powder to room temperature.
2. Resuspend the amine-substituted oligonucleotide (“probe” or “capture” oligo) to 1 mM (1 nanomole/ μL) in **dH₂O**.
3. Resuspend the stock microspheres by vortex and sonication for approximately 20 seconds.
4. Transfer 5.0×10^6 of the stock microspheres to a **USA Scientific** microfuge tube.
5. Pellet the stock microspheres by microcentrifugation at $\geq 8000 \times g$ for 1-2 minutes.
6. Remove the supernatant and resuspend the pelleted microspheres in 50 μL of 0.1 M MES, **pH 4.5** by vortex and sonication for approximately 20 seconds.
7. Prepare a 1:10 dilution of the 1 mM capture oligo in **dH₂O** (0.1 nanomole/ μL).
8. Add 2 μL (0.2 nanomole) of the 1:10 diluted capture oligo to the resuspended microspheres and mix by vortex.
9. Prepare a fresh solution of 10 mg/mL EDC in **dH₂O**. (Note: Return the EDC powder to desiccant to re-use for the second EDC addition.)
10. Add 2.5 μL of fresh 10 mg/mL EDC to the microspheres (25 μg or $\cong [0.5 \mu\text{g}/\mu\text{L}]_{\text{final}}$) and mix by vortex.
11. Incubate for 30 minutes at room temperature in the dark.
12. Prepare a second fresh solution of 10 mg/mL EDC in dH₂O. (Note: The aliquot of EDC powder should now be discarded. We recommend using a fresh aliquot of EDC powder for each coupling episode.)
13. Add 2.5 μL of fresh 10 mg/mL EDC to the microspheres and mix by vortex.
14. Incubate for 30 minutes at room temperature in the dark.
15. Add 1.0 mL of 0.02% Tween-20 to the coupled microspheres.
16. Pellet the coupled microspheres by microcentrifugation at $\geq 8000 \times g$ for 1-2 minutes.
17. Remove the supernatant and resuspend the coupled microspheres in 1.0 mL of 0.1% SDS by vortex.
18. Pellet the coupled microspheres by microcentrifugation at $\geq 8000 \times g$ for 1-2 minutes.
19. Remove the supernatant and resuspend the coupled microspheres in 100 μL of TE, pH 8.0 by vortex and sonication for approximately 20 seconds.
20. Enumerate the coupled microspheres by hemacytometer:
 - a. Dilute the resuspended, coupled microspheres 1:100 in dH₂O.
 - b. Mix thoroughly by vortex.
 - c. Transfer 10 μL to the hemacytometer.
 - d. Count the microspheres within the 4 large corners of the hemacytometer grid.
 - e. $\text{Microspheres}/\mu\text{L} = (\text{Sum of microspheres in 4 large corners}) \times 2.5 \times 100$ (dilution factor).
 - f. (Note: maximum is 50,000 microspheres/ μL .)
21. Store coupled microspheres refrigerated at 2-8°C in the dark.

RECOMMENDATIONS FOR SCALING OLIGONUCLEOTIDE-MICROSPHERE COUPLING

Number of Microspheres	Reaction Volume	Probe Input ^a	EDC Concentration ^b	Tween-20 Wash Volume	SDS Wash Volume	Final Volume ^c
1 x 10 ⁶	10 µL	0.04-0.1 nmol	0.5-2.5 mg/mL	0.5 mL	0.5 mL	20 µL
2.5 x 10 ⁶	25 µL	0.1-0.2 nmol	0.5-1 mg/mL	0.5 mL	0.5 mL	50 µL
5 x 10 ⁶	50 µL	0.2-1 nmol	0.5-1 mg/mL	1.0 mL	1.0 mL	100 µL
10 x 10 ⁶	50 µL	0.5-1 nmol	0.5-1 mg/mL	1.0 mL	1.0 mL	200 µL
50 x 10 ⁶	50-100 µL	1-4 nmol	0.5-1 mg/mL	1.0 mL	1.0 mL	1000 µL
100 x 10 ⁶	100 µL	1-4 nmol	0.5-1 mg/mL	1.0 mL	1.0 mL	2000 µL

^a We recommend titrating the probe input to optimize coupling for the particular application.

^b EDC input was not adjusted for reactions containing less than 5x10⁶ microspheres.

^c Resuspension volume of TE, pH 8.0 for 50,000 microspheres/µL assuming 100% recovery.

SAMPLE PROTOCOL FOR OLIGONUCLEOTIDE HYBRIDIZATION

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

1. Select appropriate oligonucleotide-coupled microsphere sets.
2. Resuspend 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/ μL in 1.5X TMAC Hybridization Buffer. (Note: 33 μL 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 μL of Working Microsphere Mixture.
6. To each background well, add 17 μL TE, pH 8.
7. To each sample well, add biotinylated complementary oligonucleotide (5 to 200 femtomoles) and TE, pH 8.0 to a total volume of 17 μL .
8. Mix reaction wells gently by pipetting up and down several times.
9. Cover the reaction plate to prevent evaporation and incubate at 95-100°C for 1 to 3 minutes to denature any secondary structure in the sample oligonucleotides. *
10. Incubate the reaction plate at hybridization temperature for 15 minutes. *
11. Prepare fresh Reporter Mix by diluting streptavidin-R-phycoerythrin to 10 $\mu\text{g}/\text{mL}$ in 1X TMAC Hybridization Buffer. (Note: 12 to 25 μL of Reporter Mix is required for each reaction.)
12. Add 12 to 25 μL of Reporter Mix to each well and mix gently by pipetting up and down several times.
13. Incubate the reaction plate at hybridization temperature for 5 minutes.
14. Analyze 50 μL **at hybridization temperature** on the Luminex 100 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, 1 (to 3) minutes
Hold at hybridization temperature, FOREVER

SAMPLE PROTOCOL FOR DIRECT DNA HYBRIDIZATION

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/ μL in 1.5X TMAC Hybridization Buffer. (Note: 33 μL 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 μL of Working Microsphere Mixture.
6. To each background well, add 17 μL TE, pH 8.
7. To each sample well add amplified biotinylated DNA and TE, pH 8.0 to a total volume of 17 μL . (Note: 2-5 μL 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.
12. During centrifugation, prepare fresh reporter mix by diluting streptavidin-R-phycoerythrin to 2 to 4 $\mu\text{g}/\text{mL}$ in 1X TMAC Hybridization Buffer. (Note: 75 μL 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 μL 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 μL **at hybridization temperature** on the Luminex 100 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

DNA BUFFERS

0.1 M MES, pH 4.5 (COUPLING BUFFER) 250 mL

Reagent	Catalog Number	Final Concentration	Amount/ 250 mL
MES (2[N-Morpholino] ethanesulfonic acid)	Sigma M-2933	0.1 M	4.88 g
5 N NaOH	Fisher SS256-500	-----	~ 5 drops

Filter Sterilize and store at 4°C

0.02% TWEEN (WASH BUFFER I) 250 mL

Reagent	Catalog Number	Final Concentration	Amount/ 250 mL
TWEEN 20 (Polyoxyethylenesorbitan monolaurate)	Sigma P-9416	0.02%	50 µL

Filter Sterilize and store at Room Temperature

0.1% SDS (WASH BUFFER II) 250 mL

Reagent	Catalog Number	Final Concentration	Amount/ 250 mL
SDS (Lauryl Sulfate) 10% solution	Sigma L-4522	0.1%	2.5 mL

Filter Sterilize and store at Room Temperature

20% Sarkosyl 250 mL

Reagent	Catalog Number	Final Concentration	Amount/ 250 mL
Sarkosyl (N-Lauroylsarcosine)	Sigma L-9150	20%	50g

Filter Sterilize and store at Room Temperature

TE, pH 8.0 (SAMPLE DILUENT) 250 mL

Reagent	Catalog Number	Final Concentration	Amount/ 250 mL
Tris EDTA Buffer pH 8.0 100X	Sigma T-9285	1 X	2.5 mL

Filter Sterilize and store at Room Temperature

1.5 X TMAC Hybridization Solution (MICROSPHERE DILUENT) 250 mL

Reagent	Catalog Number	Final Concentration	Amount/ 250 mL
5 M TMAC	Sigma T-3411	4.5 M	225 mL
20% Sarkosyl	-----	0.15%	1.88 mL
1 M Tris-HCl , pH 8.0	Sigma T-3038	75mM	18.75 mL
0.5 M EDTA pH 8.0	Gibco 15575-038	6mM	3.0 mL
H ₂ O	-----	-----	1.37 mL

Store at Room Temperature

1 X TMAC Hybridization Solution (DETECTION BUFFER) 250 mL

Reagent	Catalog Number	Final Concentration	Amount/ 250 mL
5 M TMAC	Sigma T-3411	3 M	150 mL
20% Sarkosyl	-----	0.1%	1.25 mL
1 M Tris-HCl , pH 8.0	Sigma T-3038	50mM	12.5 mL
0.5 M EDTA pH 8.0	Gibco 15575-038	4mM	2 mL
H ₂ O	-----	-----	84.25 mL

Store at Room Temperature

6X SSPET (STRINGENT WASH BUFFER) 250 mL

Reagent	Catalog Number	Final Concentration	Amount/ 250 mL
SSPE (20X concentrate) – Saline, Sodium Phosphate, EDTA	Sigma S-2015	6X concentrate	75 mL
Triton X-100	Sigma T-9284	0.005%-0.01%	12.5-25 µL

Filter Sterilize and store at Room Temperature

FLEXMAP MICROSPHERES

Hybridization Protocol for FlexMAP Microspheres

Oligonucleotide Synthesis for Allele-Specific Primer Extension (ASPE)

Allele-Specific Primer Extension (ASPE) and Hybridization to FlexMAP Microspheres

Oligonucleotide Synthesis for Oligonucleotide Ligation Assay (OLA)

Oligonucleotide Ligation Assay (OLA) and Hybridization to FlexMAP Microspheres

SAMPLE PROTOCOL FOR HYBRIDIZATION TO FlexMAP UNIVERSAL ARRAY MICROSPHERES

MATERIALS

- Carboxylated fluorescent microspheres with covalently attached anti-TAG sequences
- Biotin-labeled targets with appropriate TAG sequence modification
- 2X T_m Hybridization Buffer – 0.4 M NaCl, 0.2 M Tris, 0.16% Triton X-100, pH 8.0
- 1X T_m Hybridization Buffer – 0.2 M NaCl, 0.1 M Tris, 0.08% Triton X-100, pH 8.0
- Streptavidin-R-phycoerythrin (see REAGENTS list)
- 96 well V-bottom PCR plate & cover (see CONSUMABLES list)
- Pipettors, tips, microfuge tubes, plates, etc. (see CONSUMABLES list)

PROCEDURE

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

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 $\geq 8000 \times g$ for 1-2 minutes.
4. Remove the supernatant and resuspend to 100 of each microsphere set per μL in 2X T_m 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 biotinylated, TAGged targets to the sample wells. (Note: For synthetic biotin-TAG oligonucleotide targets, use 5 to 200 femtomoles per reaction.)
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 30-60 minutes.
11. Pellet the FlexMAP microspheres by centrifugation at $\geq 2250 \times g$ for 3 minutes and remove the supernatant.
12. Resuspend the pelleted FlexMAP microspheres in 70 μL of 1X T_m Hybridization Buffer. *
13. Pellet the FlexMAP microspheres by centrifugation at $\geq 2250 \times g$ for 3 minutes and remove the supernatant. *
14. Repeat steps 11. and 12. for a total of two washes. *
15. Resuspend the pelleted FlexMAP microspheres in 70 μL of 1X T_m 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 Luminex 100 analyzer according to the system manual.

* ***These steps can be omitted when hybridizing synthetic biotin-TAG oligonucleotide targets.***

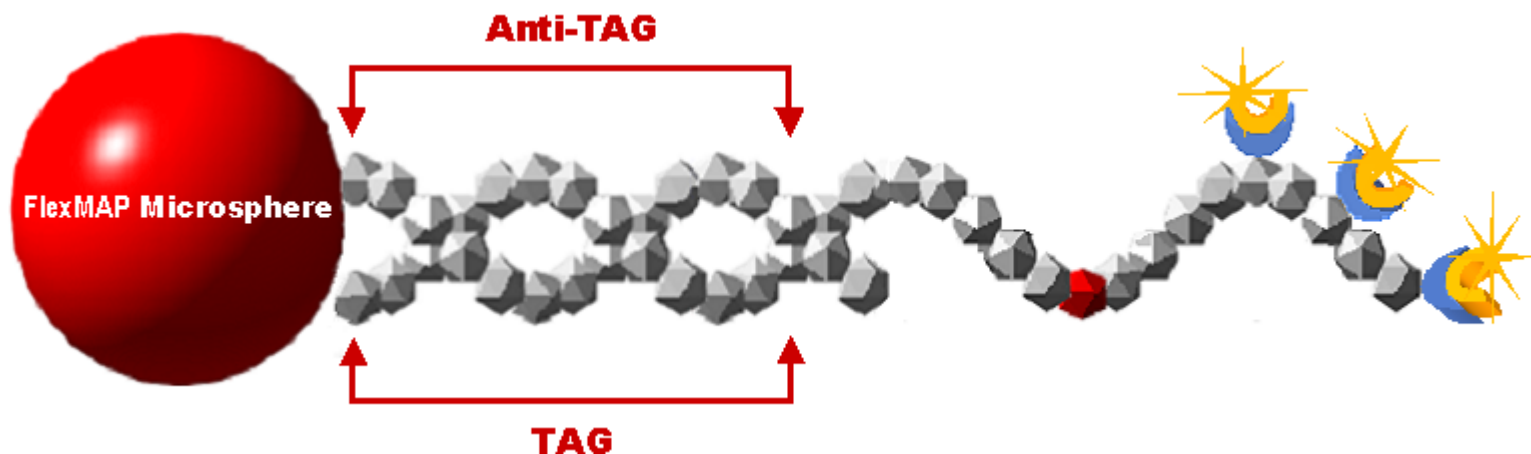
Oligonucleotide Synthesis for Allele-Specific Primer Extension (ASPE)

PCR Primer Design

1. PCR primers should be designed to amplify a region containing the SNP of interest.
2. PCR primers should not be labeled.
3. Amplicon size is not restricted.

ASPE Primer Design

1. ASPE primers should be synthesized for all sequence variants and should be from the same DNA strand (per target sequence).
2. ASPE primers should be matched for melting temperature at 51-56°C.
3. ASPE primers should extend out to and include the SNP as the 3' nucleotide.
4. Use Tag IT™ oligo design software to select an appropriate TAG sequence.
5. The primer is synthesized with the TAG sequence incorporated at the 5' end.



SAMPLE PROTOCOL FOR ALLELE-SPECIFIC PRIMER EXTENSION (ASPE) AND HYBRIDIZATION TO FlexMAP UNIVERSAL ARRAY MICROSPHERES

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/ μ L).
- ASPE primers with 5' TAG modification resuspended in sterile ddH₂O. ASPE primers are reconstituted to 1 mM (1 nanomole/ μ L).
- Qiagen HotStarTaq 2X Master Mix (Qiagen Cat. No. 203445) **or equivalent**
- ExoSAP-IT (USB Cat. No. 78200)
- Platinum Tsp DNA polymerase, 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 REAGENTS list)
- 96 well V-bottom PCR plate & cover (see CONSUMABLES list)
- Pipettors, tips, microfuge tubes, etc. (see CONSUMABLES 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 μ M each dNTP
0.2 μ M 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 μ L of each PCR reaction with ExoSAP-IT according to the following procedure:

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

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.

Multiplexed ASPE Reaction

Each 20 μ L final reaction contains:

1X ASPE Buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl)	
1.25 mM MgCl ₂	
25 nM each TAG-ASPE primer	
0.75 U Tsp DNA polymerase	
5 μ M dATP, dTTP, dGTP	– Include when PCR reaction is EXO/SAP treated
5 μ M biotin-dCTP	
5 μ L treated PCR reaction	
dH ₂ O (to 20 μ L)	

2X ASPE Master Mix (10 μ L/reaction):

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

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.

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 $\geq 8000 \times g$ for 1-2 minutes.
4. Remove the supernatant and resuspend to 100 of each microsphere set per μL in 2X T_m 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 dH_2O to each background well.
7. Add 5 to 25 μL of each ASPE reaction to the appropriate sample wells. (Note: 5 μL is usually sufficient.)
8. Adjust the total volume to 50 μL by adding the appropriate volume of dH_2O 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 30-60 minutes.
11. Pellet the FlexMAP microspheres by centrifugation at $\geq 2250 \times g$ for 3 minutes and remove the supernatant.
12. Resuspend the pelleted FlexMAP microspheres in 70 μL of 1X T_m Hybridization Buffer.
13. Pellet the FlexMAP microspheres by centrifugation at $\geq 2250 \times g$ for 3 minutes and remove the supernatant.
14. Repeat steps 11. and 12. for a total of two washes.
15. Resuspend microspheres in 70 μL of 1X T_m 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 Luminex 100 analyzer according to the system manual.

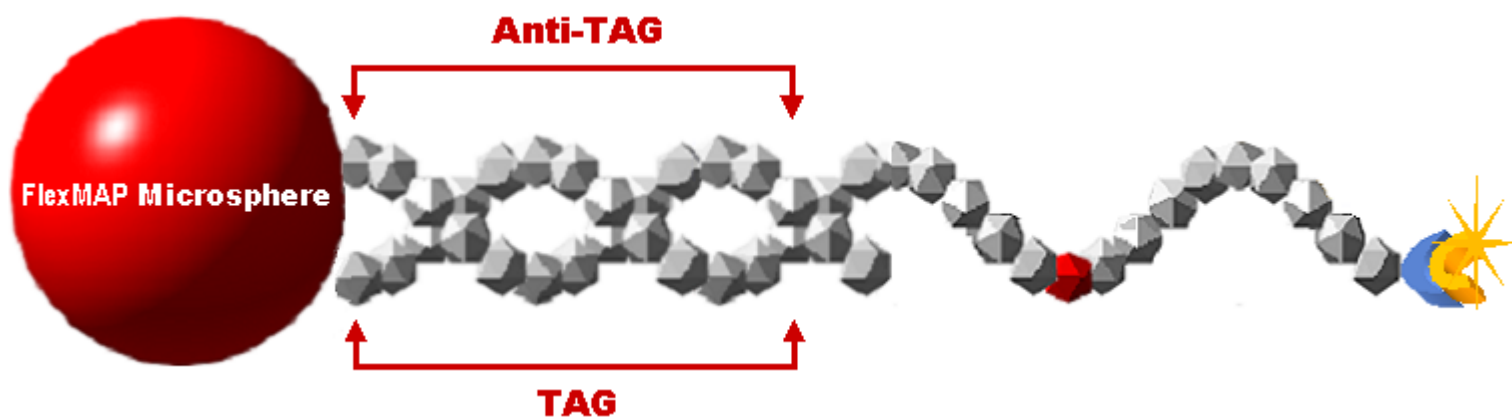
Oligonucleotide Synthesis for Oligonucleotide Ligation Assay (OLA)

PCR Primer Design

1. PCR primers should be designed to amplify a region containing the SNP of interest.
2. PCR primers should not be labeled.
3. Amplicon size is not restricted.

OLA Probe Design

1. Allele-specific probes should be synthesized for all sequence variants and should be from the same DNA strand (per target sequence).
2. Allele-specific probes should be matched for melting temperature at 51-56°C.
3. Allele-specific probes should extend out to and include the SNP as the 3' nucleotide.
4. Use Tag IT™ oligo design software to select an appropriate TAG sequence.
5. The allele-specific probe is synthesized with the TAG sequence incorporated at the 5' end.
6. The reporter probe should have a melting temperature of 51-56°C.
7. The reporter probe should begin with the nucleotide immediately downstream from the SNP as the 5' nucleotide.
8. The reporter probe must be modified with phosphate at the 5' end and with biotin at the 3' end.



SAMPLE PROTOCOL FOR OLIGONUCLEOTIDE LIGATION ASSAY (OLA) AND HYBRIDIZATION TO FlexMAP UNIVERSAL ARRAY MICROSPHERES

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/ μ L).
- Allele-specific probes with 5' TAG modification resuspended in sterile ddH₂O. Allele-specific probes are reconstituted to 1 mM (1 nanomole/ μ L).
- Reporter probes with 5' phosphate and 3' biotin modifications resuspended in sterile ddH₂O. Reporter probes are reconstituted to 1 mM (1 nanomole/ μ L).
- Qiagen HotStarTaq 2X Master Mix (Qiagen Cat. No. 203445) **or equivalent**
- Taq DNA Ligase, 10x Taq DNA Ligase Buffer (New England Biolabs Cat. No. 11448-024)
- 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 REAGENTS list)
- 96 well V-bottom PCR plate & cover (see CONSUMABLES list)
- Pipettors, tips, microfuge tubes, etc. (see CONSUMABLES 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 μ M each dNTP
0.2 μ M 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

Multiplexed OLA ReactionEach 20 μ L final reaction contains:

1X Taq DNA Ligase Buffer (20 mM Tris-HCl, pH 7.6; 25 mM potassium acetate; 10 mM magnesium acetate; 10 mM dithiothreitol; 1 mM NAD; 0.1% Triton X-100)
 10 U Taq DNA Ligase
 5 nM each TAG–allele-specific probe
 250 nM each reporter probe
 3 to 20 ng PCR target (usually 0.5 to 5 μ L)
 dH₂O (to 20 μ L)

2X OLA Master Mix (10 μ L/reaction)

10X Taq DNA Ligase Buffer	2	μ L
Taq DNA Ligase (40,000 U/mL)	0.25	μ L
20X TAG–allele-specific probe mix (100 nM each)	1	μ L (dilute 1 mM stocks 1:10,000 for 20X mix)
20X reporter probe mix (5 μ M each)	1	μ L (dilute 1 mM stocks 1:200 for 20X mix)
dH ₂ O	<u>5.75</u>	<u>μL</u>
	10	μ L

OLA Cycling Parameters:

HOLD: 96°C, 2 minutes
CYCLE: 94°C, 15 seconds
 37°C, 1 minute
30 CYCLES
HOLD: 4°C, FOREVER

Hybridization to FlexMAP Microspheres

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

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 $\geq 8000 \times g$ for 1-2 minutes.
4. Remove the supernatant and resuspend to 100 of each microsphere set per μL in 2X T_m 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 dH_2O to each background well.
7. Add 5 to 25 μL of each OLA reaction to the appropriate sample wells. (Note: 5 μL is usually sufficient.)
8. Adjust the total volume to 50 μL by adding the appropriate volume of dH_2O 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 30-60 minutes.
11. Pellet the FlexMAP microspheres by centrifugation at $\geq 2250 \times g$ for 3 minutes and remove the supernatant.
12. Resuspend the pelleted FlexMAP microspheres in 70 μL of 1X T_m Hybridization Buffer.
13. Pellet the FlexMAP microspheres by centrifugation at $\geq 2250 \times g$ for 3 minutes and remove the supernatant.
14. Repeat steps 11. and 12. for a total of two washes.
15. Resuspend microspheres in 70 μL of 1X T_m 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 Luminex 100 analyzer according to the system manual.

RECOMMENDED MATERIALS

Source List

Equipment

Consumables

Reagents

SOURCE LIST

Company	Phone Number	International Phone	Website
Amersham Biosciences	732-457-8000	See website for regions	www4.amershambiosciences.com
Cole Parmer	1-800-323-4340	847-549-7600	www.coleparmer.com
Costar (Corning)	607-974-9000	607-974-9000	www.corning.com
Fisher	1-800-766-7000	See website for regions	www2.fishersci.com
Millipore	1-800-645-5476	See website for regions	www.millipore.com
MJ Research	1-888-785-8437	See website for distributors	www.mjr.com
Molecular Probes	1-800-438-2209	(541) 465-8338	www.molecularprobes.com
Pall Life Sciences	1-800-521-1520	See website for regions	www.pall.com
Pierce	1-800-874-3723	See website for regions	www.piercenet.com
Promega	1-800-356-9526	See website for regions	www.promega.com
Prozyme	1-800-457-9444	See website for distributors	www.prozyme.com
Roche	1-800-428-5433	See website	biochem.roche.com
Sigma	1-800-325-3010	314-771-5765	www.sigma-aldrich.com
USA Scientific	1-800-522-8477	352-237-6288	www.usascientific.com
VWR	1-800-932-5000	888-897-8031	www.vwrsp.com

EQUIPMENT

Product	Source	Catalog Number
Dry Block Heater (2)	VWR	13259-032
Dry Block 96 well PCR plate block (2)	VWR	13259-260
Thermal cycler (MJ Research PTC-100)	MJ Research or Equivalent	PTC-1196
Microcentrifuge Brinkman 5415D	VWR	77888-2001
Microcentrifuge rotor	VWR	58922-854
Centrifuge Brinkman 5804	VWR Fisher Brinkman	53513-800 05-400-93 22 62 250 1
Microtiter plate rotor A-2-MTP	Fisher Brinkman	05-400-107 22 63 730 4
Vortex Mixer	VWR	58816-121
Sonicator (mini)	Cole Parmer	08849-00
Pipettors P10, P20, P100, P1000, 8 ch.	Rainin or Equivalent	
Hemocytometer (Bright Line)	VWR	15170-172
Rotator	VWR	56264-302
Vacuum Manifold	Millipore	MAVM 096 OR

CONSUMABLES

Product	Source	Catalog Number
1.5 mL microcentrifuge tubes	USA Scientific	1415-2500
2.0 mL screw-cap microcentrifuge tubes for storage	Fisher	05-669-8
0.2 mL PCR tubes - sterile	MJ Research or Various Vendors	
1 mL microtiter tubes	Bio-Rad	223-9391
10 μ L pipette tip refills	Rainin	GPS-10G
250 μ L pipette tip refills	Rainin	GPS-250
1000 μ L pipette tip refills	Rainin	GPS-1000
Spin column	Chemicon Int'l	2152
PD-10 column	Amersham Biosciences	17-1851-01
Thermowell aluminum sealers (to reduce light exposure of samples in a plate)	Costar	6570
Black PCR Plates (recommended for XYP heater block)	Fisher	Polyfiltronics #7703-1902
96-well Thermowell P polycarbonate clear PCR plates - (recommended for XYP heater block)	Costar VWR Fisher	6509 29444-000 07-200-541
Sealers for 96-well Thermowell P plates: Sealing Mat	Costar VWR Fisher	6555 22250-104 07-200-614
Microseal 'A' film	MJ Research	MSA-5001
96-well black half-area plates (recommended for no wash assays, not filtered)	VWR Costar	29444-314 3694
1.2 μ m PVDF filter microtiter plates (recommended for washed assays)	Millipore	MABV N12 50
1 μ m PTFE filter microtiter plates (recommended for washed assays)	Millipore	MAR1 N10 50
1.2 μ m Supor filter microtiter plates (recommended for washed assays)	Pall Life Sciences VWR	5039 28148-668
Half area flat bottom assay plates (black, non-treated)	Costar	3693
96-Well Polycarbonate Solid Plates Flat Bottom	Costar	3912 (white) 3915 (black)
96-Well Polycarbonate Solid Plates Round Bottom	Costar	3789 (white) 3792 (black)

REAGENTS

Product	Source	Catalog Number
EDC (1-ethyl-3-[3dimethylaminopropyl] carbodiimide hydrochloride)	Pierce	22980
Sulfo-NHS (N-hydroxysulfosuccinimide)	Pierce	24510
Streptavidin, ALEXA [®] 532	Molecular Probes	S-11224
Streptavidin-R-phycoerythrin (1 mg/ml)	Molecular Probes	S-866
Streptavidin-R-phycoerythrin (1 mg)	Prozyme	PJ31S
2-Propanol	VWR	MK304306
MES (2-[N-Morpholino]ethanesulfonic acid)	SIGMA	M-2933
TWEEN [®] 20 (Polyoxyethylenesorbitan monolaurate)	SIGMA	P-9416