Protocol for the mtSNP 11-plex Primer Extension Assay on the ABI 3100 CE

Reagents Included:
-11-plex **PCR** primer mix (~400 uL or 250 reactions)
-11-plex **ASPE** primer mix (~240uL or 240 reactions)

Materials Needed:
- PCR buffer and Mg++ (e.g. supplied with TaqGold)
- TaqGold DNA polymerase (5 U/µL)
- dNTPs (10 mM)
- BSA (fraction V) (3.2 mg/mL)
- Capillary Array
- POP-6 polymer
- Matrix standards for ABI 3100
- Genetic Analyzer Buffer
- GeneScan and Genotyper software programs

Eleven mtSNP sites for separating the most common Caucasian HV1/HV2 type. The primary sequence variants are listed, with the rCRS variant listed first. Site 14470 has been observed to also have a C variant.

Position	Sequence Variation
477	T/C
3010	G/A
4580	G/A
4793	A/G
5004	T/C
7028	C/T
7202	A/G
10211	C/T
12858	C/T
14470	T/A
16519	T/C

See flow chart on next page for workflow

Multiplex PCR

	Stock conc	mtPCR 11plex	Desired PCR conc	Volumes to add		Number of Reactions
		Total volume of Reaction	15			16
mM	25	Mg concentration (micromolar)	2	1.2	uL	19.2
uM	4.5	Primer concentration (micromolar)	0.45	1.5	uL	24
U/uL	5	units of Taq (units)	1.5	0.3	uL	4.8
mM	10	dNTP concentration (micromolar)	250	0.375	uL	6
x	10	PCR Buffer	1	1.5	uL	24
	3.2	BSA	0.16	0.75	uL	12
		Water to add		7.375	uL	118
		Master Mix volume		13		208
		Volume of added template (uL)	2			

Above is an example a PCR set up sheet for 16 reactions. Typically we run 15 uL reactions. Approximately 0.5 to 1 ng of *genomic* DNA (Nuclear DNA quantified using Quantifiler) is added. *The mtDNA concentration will of course be much higher.*
Note: volumes may change for differing concentrations of dNTPs, Mg^{++}, BSA, etc

Thermal Cycling

Thermal cycling was performed with the GeneAmp 9700 (Applied Biosystems) using the following conditions in 9600-emulation mode (i.e., ramp speeds of 1 °C/s):
95 °C C for 10 min
3 cycles of 95 °C for 30 s, 50 °C for 55 s, 72 °C for 30 s
19 cycles of 95 °C for 30 s, 50 °C for 55 s +0.2 °C per cycle, 72 °C for 30 s
11 cycles of 95 °C for 30 s, 55 °C for 55 s, 72 °C for 30 s
72 °C for 7 min
4 °C hold

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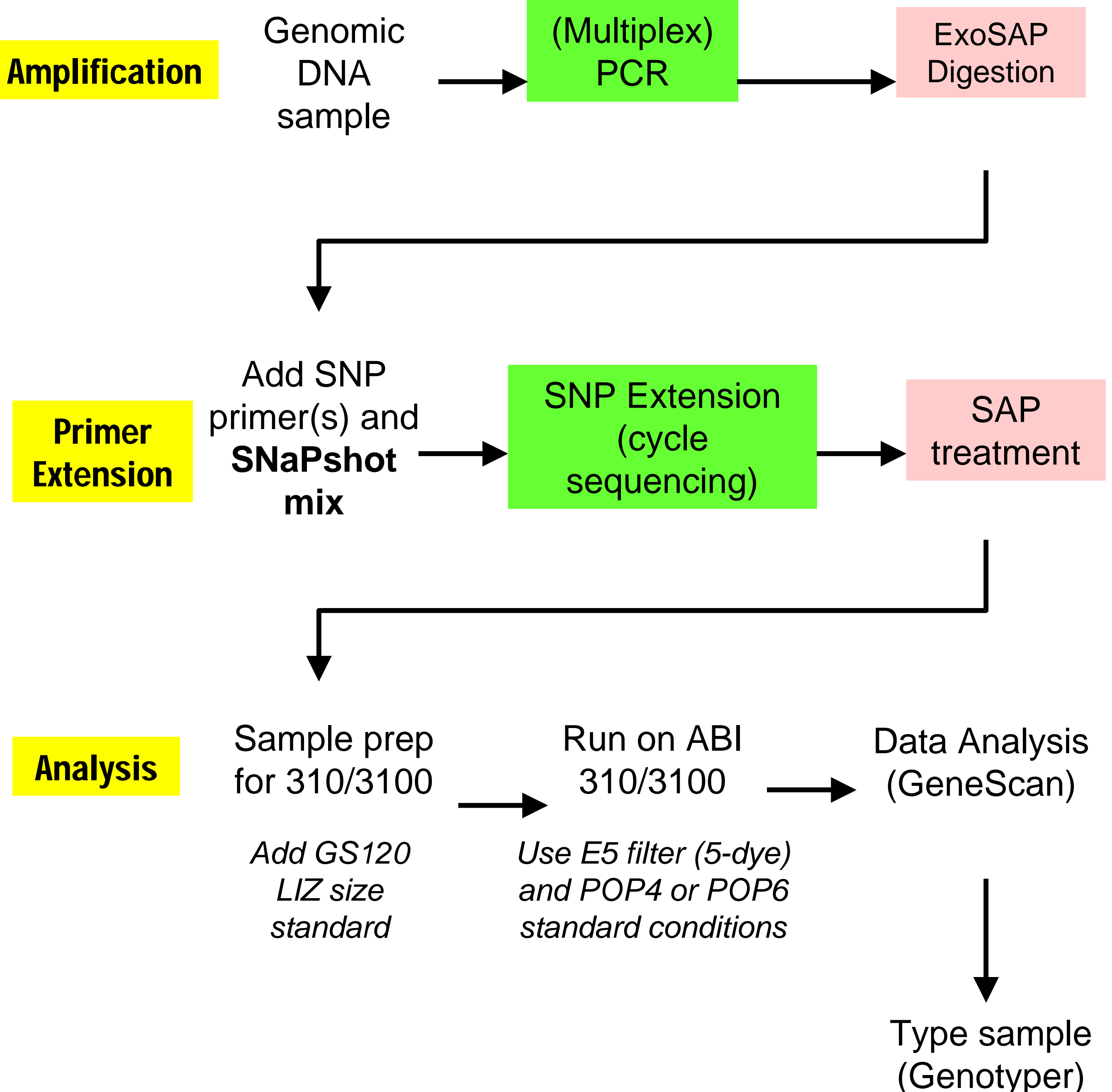
Protocol for ASPE Assay



Exo-SAP

Post PCR, amplicons can be confirmed on an agarose gel or the Agilent Bioanalyzer 2100. Exo-SAP treatment is required to degrade leftover PCR primers and unincorporated dNTPs.

Each reaction is treated with Exonuclease I and Shrimp Alkaline Phosphatase. Pre-mixed Exo-SAP-IT can be used *or* you can make your own: 1.4 uL Exo I (5U/µL) + 2.6 µL of SAP (1U/µL) per 15µL reaction. Add enzymes to plate/tubes and spin down before incubating at 37°C.

Thermal cycling for Exo-SAP

90min at 37°C – (**the 90 min is needed to ensure that all leftover PCR primers are degraded**)
20 min at 80°C – (this is needed to kill the enzymes)
5 min at 25°C

ASPE with SNaPshot

	Stock conc	mtSNP ASPE	Desired SNP conc	Volume (uL)		Number of Rxns
		Total volume of Reaction	10			16
uM	10	Primer concentration (micromolar)	1	1	uL	16
x	2	SNaPshot kit	10	5	uL	80
		Water to add		1	uL	16
		Master Mix volume		7		112
		Volume of added PCR amplicons (uL)	3			

The ASPE mix can be set up as shown above. Spin down after adding all components.

Thermal cycling for ASPE
25 cycles of:
96 °C for 10 s
50 °C for 5 s
60 °C for 30 s

Thermal cycling for SAP
Add 1.5 µL (1U/µL) of SAP to each reaction and spin down. The SAP is required to inactivate the fluorescently labeled ddNTPs.

40min at 37°C
5 min at 90°C
5 min at 25°C

CE run on the ABI 3100

<u>LIZ-120 mix</u>
For each reaction prepare:
14.5 µL HiDi formamide
0.4 µL LIZ-120 sizing ladder

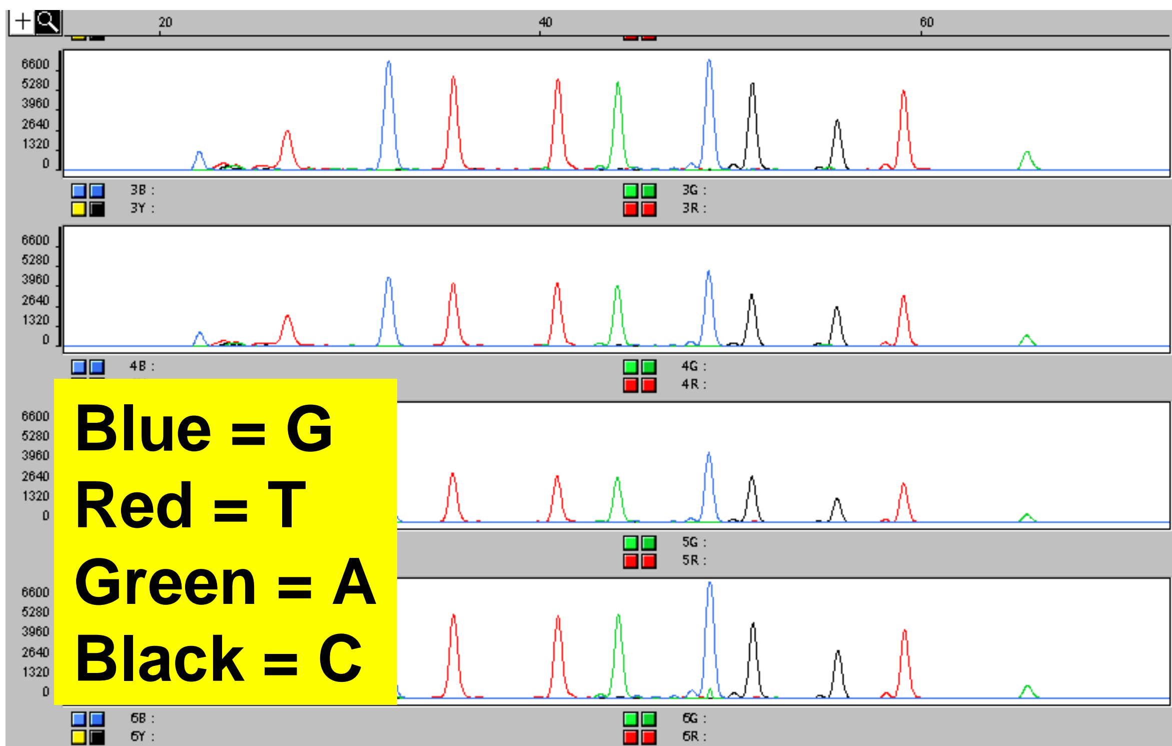
Add 1.0 µL of the SAP treated ASPE reaction to the CE plate containing the HiDI-LIZ and spin down to mix.

<u>Analysis on ABI 3100</u>
The ABI 3100 was used with filter set E5 in order to process the data from the 5 dyes dR110, dR6G, dTAMRA, dROX, and LIZ after an appropriate spectral matrix had been created using materials from the matrix standard set DS-02.

A 36 cm capillary array filled with either denaturing POP6 or POP4 performance optimized polymer was utilized for DNA fragment separation.

Typical run module parameters were: Run temperature = 60 °C, capillary fill volume = 184 steps, pre-run voltage = 15 kV, pre-run time = 60 sec, **injection voltage = 1kV, injection time = 13 sec**, run voltage = 15 kV, data delay = 200 sec, and run time = 1200 sec.

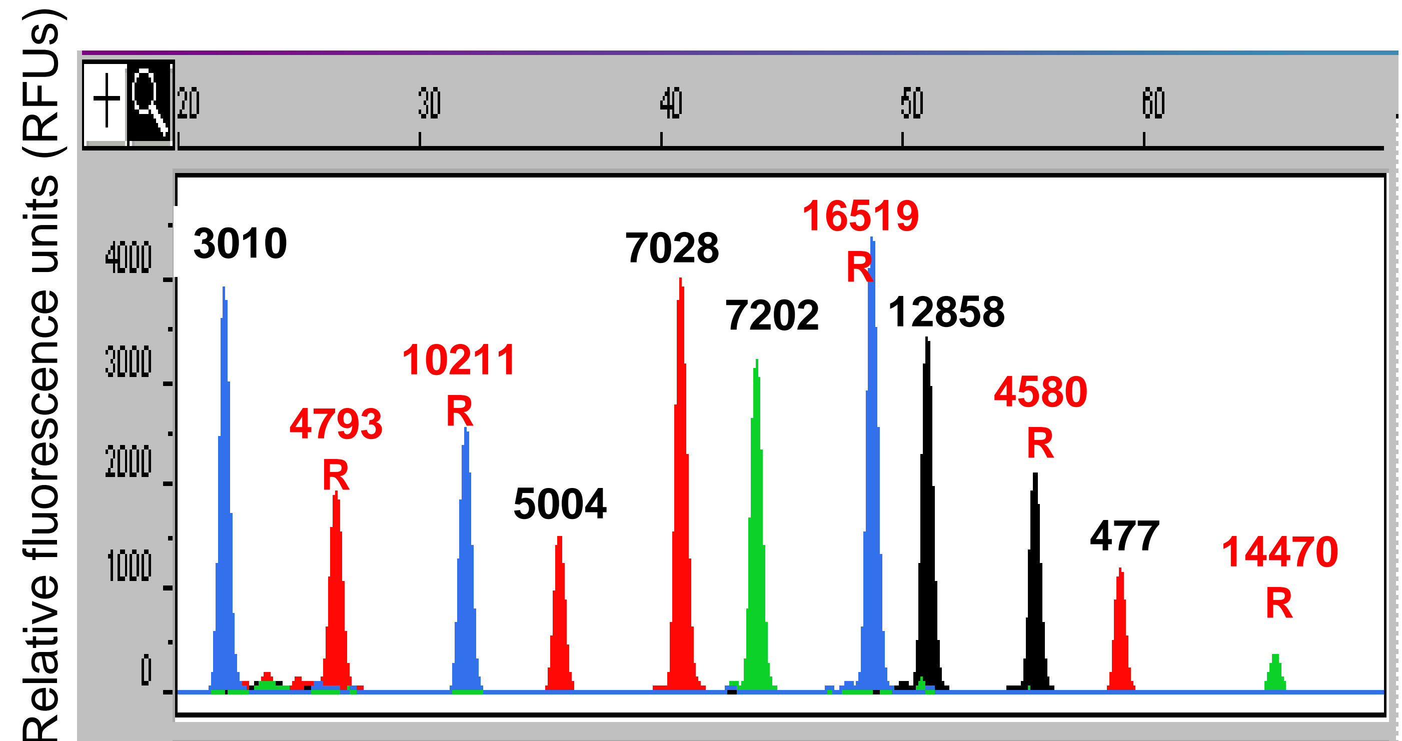
Data analysis was performed using GeneScan 3.7 and Genotyper 3.7 software. A macro based on fragment size and dye color was implemented using the Genotyper 3.7 software for automated allele calls.



mtSNP 11-plex run on ABI 3100

Multiplex PCR and Multiplex SNP Detection

Measured size (nt) (relative to GSLIZ-120 size standard)



Loci highlighted in red with a R are “reverse” orientation ASPE primers. A correction needed to be applied. For example 4793 is A/G. The red peak (T) corresponds to an A allele call.

Primer Information

Primer Sequences from Vallone et al.,. *Int. J. Legal Med.,* 118: 147-157.

http://www.cstl.nist.gov/biotech/strbase/pub_pres/Vallone_IJLM2004.pdf

Locus	PCR Primer Sequence	Amplicon (bp)
477-F	CTTTTGGCGGTATGCACTTT	122
477-R	GGTGTGTGTGTGCTGGGTA	
3010-F	GCGCAATCCTATTCTAGAGTCC	124
3010-R	TCACGTAGGACTTTAATCGTTGA	
4580-F	TCTTTGCAGGCACACTCATC	130
4580-R	GCAGCTTCTGTGGAACGAG	
4793-F	CAACCGCATCCATAATCCTT	186
4793-R	ATGTCAGAGGGGTGCCTTG	
5004-F	TCCATCATAGCAGGCAGTTG	124
5004-R	TGGTTATGTTAGGGTTGTACGG	
7028-F	GGCCTGACTGGCATTGTATT	125
7028-R	AAGCCTCCTATGATGGCAAA	
7202-F	ACGCCAAAATCCATTTCACT	126
7202-R	TTCATGTGGTGTATGCATCG	
10211-F	ACCACAACTCAACGGCTACA	143
10211-R	GGAGGGCAATTTCTAGATCAAA	
12858-F	ATGATACGCCCGAGCAGA	126
12858-R	TGTGGGTCTCATGAGTTGGA	
14470-F	CAAGACCTCAACCCCTGACC	129
14470-R	GGGGGAGGTTATATGGGTTT	
16519-F	ACCACCATCCTCCGTGAAAT	183
16519-R	AGACCTGTGATCCATCGTGA	

The multiplex primer mix concentration is ~4.5 uM (for each primer).

Locus	ASPE Primer Sequence	w/ T tail	[μM]
3010-F	TGTTGGATCAGGACATCCC	19	0.4
4793-R	$(T)_{4}$ - TCAGAAGTGAAAGGGGGC	22	11.5
10211-R	$(T)_{10}$ - ACTAAGAAGAATTTTATGGA	30	15.5
5004-F	$(T)_{14}$ - AGACCCAGCTACGCAAAATC[b]	34	12.4
7028-F	$(T)_{18}$ –GACACGTACTACGTTGTAGC	38	5.8
7202-F	$(T)_{22}$ –CCACAACACTTTCTCGGCCT	42	1.0
16519-R	$(T)_{24}$ -TGTGGGCTATTTAGGCTTTATG	46	5.4
12858-F	$(T)_{27}$ -GCAGCCATTCAAGCAATCCTATA	50	5.2
4580-R	$(T)_{29}$ -TGGTTAGAACTGGAATAAAAGCTAG	54	6.0
477-F	$(T)_{38}$ –CCCTCCCACTCCCATACTAC	58	5.6
14470-R	$(T)_{41}$ –GGGAATGATGGTTGTCTTTGG	62	10.0