Multiplex SNaPshot for Age Estimation Using Saliva

Multiplex PCR

Reagents Needed:

5 X Primer Mix
AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Foster City, CA)
Gold ST*R 10 X Buffer (Promega, Madicine, WI)

5 X Primer Mix for Multiplex PCR:

Target ID	Sequence (5'→3')	Conc. (uM)	Amplicon size (bp)
cg18384097_PTPN7	TTG TTT TAG TAA GTA TTT GAA GGG G	1.0	157
	CAT CAA ATC TAT AAA CAC CCA TAC C	1.0	
cg00481951_SST	AGG TGA GTT TTT ATT TGG TAT TTA AGA AA	3.0	198
	TTT AAA TTA CCC CTT TAC CCT AAT C	3.0	
cg19671120_CNGA3	GGA GAG GGA GGT TAT AGG TTT TTT	3.0	162
	TCC TTA CCC TAC CAA AAT TTA AAC TT	3.0	
cg14361627_KLF14	AGG TTG TTG TAA TTT AGA AGT TT	3.0	114
	ATA TTT AAC AAC CTC AAA AAT TAT CTT ATC	3.0	
cg08928145_TSSK6	AGG GAA GYG GAA GGG AAA AAG	5.0	141
	ACT AAA AAC CRA ATA ATT CCA ACC ATT CCT	5.0	
cg12757011_TBR1	GGG TGG GTT TAG GTT TTA GAG TTA	5.0	188
	ATA AAA TTA TCC TCC TAC AAT TCC C	5.0	
cg07547549_SLC12A5	GGT TTA GTT AAT TTA AGT TAG TT	15.0	129
	AAA CTC AAC TCC ATT AAA ATA CTC C	15.0	

PCR Mixture:

PCR Component	Vol. (ul)
dH_2O	~12.4
10 X Gold ST*R Buffer	2
5 X Primer Mix	4
AmpliTaq Gold (5 U/μL)	0.6 (3 U)
Bisulfite converted DNA	1 (~4)*
Total	20

Thermal Cycling:

95℃ for 11 minutes, then:

94℃ for 20 seconds
56℃ for 60 seconds
72℃ for 30 seconds
for 35 cycles, then:

72℃ for 7 minutes
4℃ soak

* Please be aware that you should not use more than 1/5 PCR volume of bisulfite converted DNA when using Sigma's Imprint ® DNA modification kit because, in our experience, it may cause PCR failure.

Post-PCR Reaction

Enzyme Purification of the PCR Product

Reagents Needed:

PCR product	5 µL
ExoSAP-IT® (USB, Cleveland, OH)	1 µL

Thermal Cycling:

37℃ for 45 minutes

80℃ for 15 minutes

Multiplex SNaPshot

Reagents Needed:

10 X SBE Primer Mix

5 X Sequencing buffer_BigDye Termination (Applied Biosystems, Foster City, CA)

SNaPshot™ Kit (Applied Biosystems, Foster City, CA)

10 X SBE Primer Mix:

Target ID	Sequence (5'→3')	Conc. (uM)	Length (nt)
PTPN7_cg18384097	CAT ACC CCA ACC AAA CAC TAT AAC	4.0	24
SST_cg00481951	(T)9 CCA AAA TCA ACA CCA AAA ATA AAC	10.0	33
CNGA3_cg19671120	(T)17 CTA CCA AAA TTT AAA CTT CTC C	10.0	39
KLF14_cg14361627	(T)19 TTA ACA ACC TCA AAA ATT ATC TTA TCT CC	2.0	48
TSSK6_cg08928145	(T)36 CCA AAA ACA CTA AAC CAA AAC	10.0	57
TBR1_cg12757011	(T)38 ACC TAA ACA ATC CTA TCA AAC AAC AAC	4.0	65
SLC12A5_cg07547549	(T)45 CRA ACR CTA TCC AAA ATA CTA AAA TAC	20.0	72

SBE Reaction Mixture:

Reaction Component	Vol. (ul)
dH_2O	~ 5
10 X SBE Primer Mix	1
5 X Sequencing Buffer	2
SNaPshot Reaction Mix	1
Purified PCR Product	> 1
Total	10

Thermal Cycling:

96℃ for 10 seconds
50℃ for 5 seconds
60℃ for 30 seconds

for 25 cycles

Post-Single Base Extension

Enzyme (SAP or CIP) Treatment

Reagents Needed:

SBE reaction product	10 µL
SAP-Recombinant (USB, Cleveland, OH)	1 µL

Thermal Cycling:

37℃ for 45 minutes

80℃ for 15 minutes

Capillary Electrophoresis

Materials and Reagents Needed:

Dry heating block, water bath or thermal cycler

3130 capillaries, 33 cm x 50 µm (Applied Biosystems, Foster City, CA)

Performance Optimized Polymer (POP4, Applied Biosystems, Foster City, CA)

Matrix Standard Set DS-02 (dR110, dR6G, dTAMRA™, dROX™, LIZ® Dyes)

(Applied Biosystems, Foster City, CA)

Run Module GS STR POP4 (1 mL) E5

GeneScan™ 120 LIZ™ Size Standard

Hi-Di™ Formamide (Applied Biosystems, Foster City, CA)

Creating Matrix:

According to the ABI PRISM®SNaPshot™Multiplex Kit protocol

Reagents Needed:

GeneScan™ 120 LIZ™ Size Standard	0.2 µL
Hi-Di™ Formamide	10 µL
SNaPshot product	1~2 µL

Thermal Cycling:

95℃ for 5 minutes

4℃ soak

3130 Data Collection Software:

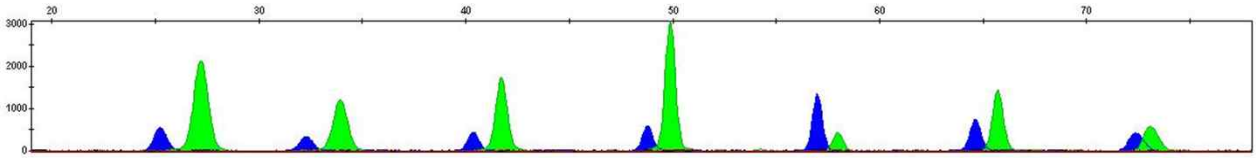
Verify that you have chosen GeneScan Run module E5 and the DS-02 GeneScan Matrix Set.

Run prepared samples under the following conditions: injection time of 3 sec, electrophoresis voltage of 15 kV, collection time of 8 min, EP voltage of 15 kV and heat plate temperature of 60℃.

Detect and calculate peak heights with an analytical threshold of 50 rfu.

Analysis

Electropherogram



Target ID	UCSC RefGene Name	Location
cg18384097	PTPN7	chr1:202129566
cg00481951	SST	chr3:187387650
cg19671120	CNGA3	chr2:98962974
cg14361627	KLF14	chr7:130419116
cg08928145	TSSK6	chr19:19625364
cg12757011	TBR1	chr2:162281111
cg07547549	SLC12A5	chr20:44658225

Representative electropherogram of age estimataion in saliva using multiplex methylation SNaPshot. Because all SBE primers were designed to be in the reverse direction, a blue peak represents the nucleotide G as a methylation signal and a green peak represents the nucleotide A as an unmethylation signal.

Age Estimation

Target ID	Methylation	Coefficients	Estimated Age
(Intercept)	(intercept)	-27.511	
cg18384097		-29.088	**-27.511 + (-29.088) × cg18384097 + 9.285 ×cg00481951 + 46.992 × cg19671120 + 86.268 × cg14361627 + 32.211 × cg08928145 + 58.699 × cg12757011 + 56.384 × cg07547549**
cg00481951		9.285	
cg19671120	$\frac{B}{B+G}$ *	46.992	
cg14361627		86.268	
cg08928145		32.211	
cg12757011		58.699	
cg07547549		56.384	

* B denotes the height of a blue peak and G denotes that of a green peak. This methylation value will be in the range of 0 to 1.