



# Multiplex SNaPshot for Age Estimation Using Semen

## Multiplex PCR

### Reagents Needed:

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

### 5 X Primer Mix for Multiplex PCR:

Target ID	Sequence (5'→3')	Conc. (uM)	Amplicon size (bp)
cg06304190_TTC7B	AAT TTT ATT TTT GGT ATT TAA AGT AG	10.0	195
	AAA CAA AAA CTA CCA CTC TCA CAC	10.0	
cg12837463	AGT TGG TAT TAG GGT TTG AAA TGT A	20.0	209
	TCT CAA AAA CTC TAC AAT AAA AAA AA	20.0	
cg06979108_NOX4	TAG TTA TTT GAG TGA AGT GTG TTG G	1.0	194
	ACC TCC CAA AAT ACT AAA TTA CTC	1.0	

### PCR Mixture:

PCR Component	Vol. (ul)
dH <sub>2</sub> O	~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)*
<b>Total</b>	<b>20</b>

### Thermal Cycling:

95°C for 11 minutes, then:

94°C for 20 seconds

56°C for 60 seconds

72°C for 30 seconds

for 35 cycles, then:

72°C for 7 minutes

4°C soak

\* It is recommended to use at least 10 ng of bisulfite converted DNA for PCR amplification.

# Post-PCR Reaction

## Enzyme Purification of the PCR Product

### Reagents Needed:

PCR product	5 $\mu$ L
ExoSAP-IT® (USB, Cleveland, OH)	2 $\mu$ L

### Thermal Cycling:

37°C for 45 minutes  
80°C 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. ( $\mu$ M)	Length (nt)
cg06304190_TTC7B	AAT AAT CAC CTA CTA TAT ACT AAA C	10.0	25
cg12837463	CCT TCT TTA ACT CAT ATA CTT TAA AAA TAT CTA C	12.0	34
cg06979108_NOX4	(T) <sub>21</sub> TCA ATT AAA TCC TCA ACT AAA TC	6.0	44

### SBE Reaction Mixture:

Reaction Component	Vol. ( $\mu$ l)
dH <sub>2</sub> O	~ 5
10 X SBE Primer Mix	1
5 X Sequencing Buffer	2
SNaPshot Reaction Mix	1
Purified PCR Product	> 1
<b>Total</b>	<b>10</b>

### Thermal Cycling:

96°C for 10 seconds  
50°C for 5 seconds  
60°C for 30 seconds  
  
for 25 cycles

\* Please keep the SNaPshot mixture on ice before putting it into the thermal cyclor. Leaving the mixture at ambient temperature may result in a higher background because the SNaPshot kit does not support Hot Start PCR.

# Post-Single Base Extension

## Enzyme (SAP or CIP) Treatment

### Reagents Needed:

SBE reaction product	10 $\mu$ L
SAP-Recombinant (USB, Cleveland, OH)	1 $\mu$ L

### Thermal Cycling:

37°C for 45 minutes  
80°C for 15 minutes

## Capillary Electrophoresis

### Materials and Reagents Needed:

Dry heating block, water bath or thermal cycler  
3130 capillaries, 33 cm x 50  $\mu$ m (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 $\mu$ L
Hi-Di™ Formamide	10 $\mu$ L
SNaPshot product	1–2 $\mu$ L

### Thermal Cycling:

95°C for 5 minutes  
4°C soak

### 3130 Data Collection Software:

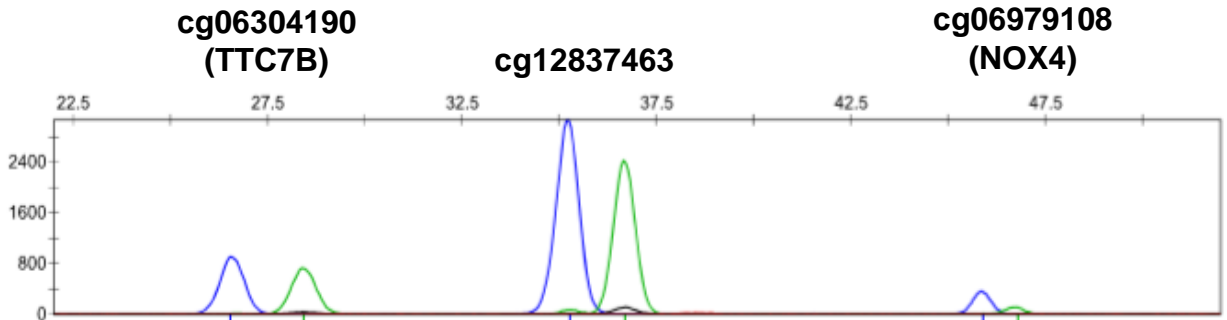
Verify that you have chosen GeneScan Run module E5 and the DS-02 GeneScan Matrix Set.

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°C.

Detect and calculate peak heights with an analytical threshold of 30 rfu.

# Analysis

## Electropherogram



Target ID	UCSC RefGene Name	Location
cg06304190	TTC7B	chr14:91283606
cg12837463		chr7:35300228
cg06979108	NOX4	chr11:89322851

Representative electropherogram of age estimation in semen 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 (n=125)	Estimated Age
(Intercept)	(intercept)	46.24	
cg06304190		-51.9	46.240 + (-51.9) × cg06304190
cg12837463	$\frac{B}{B+G}$ *	-17.8	+ (-17.8) × cg12837463
cg06979108		54.1	+ 54.1 × cg06979108

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