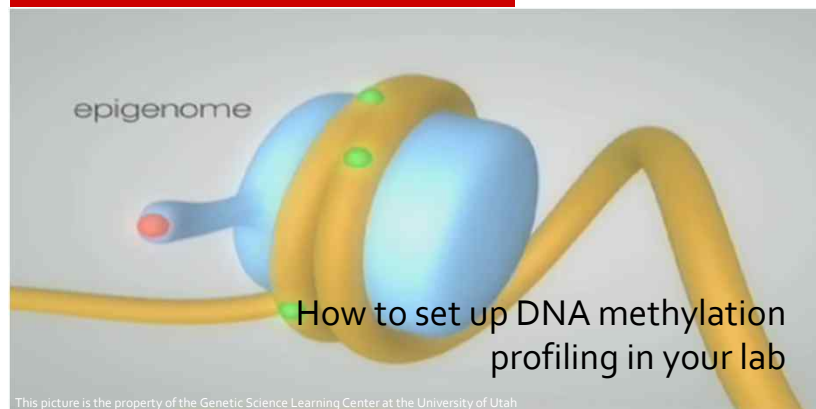


DNA Methylation Profiling Using SNaPshot Multiplex



Introducing DNA methylation profiling in your lab

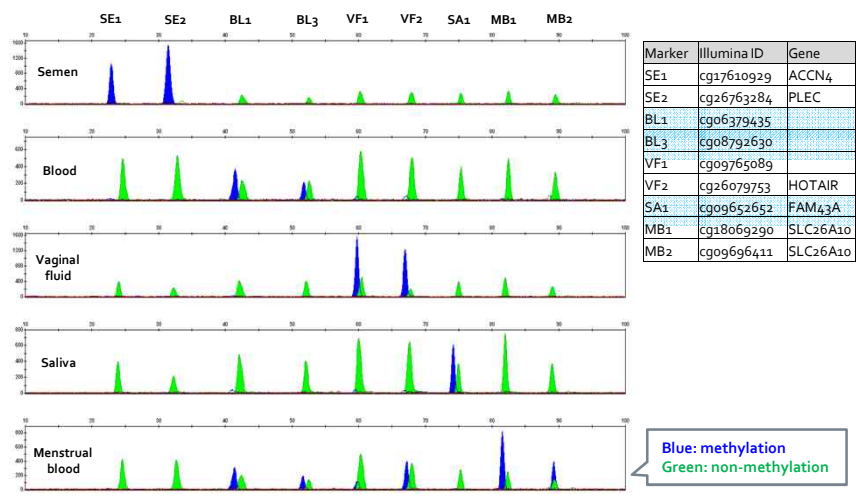
1. Refer to previous articles e.g.:

- Jung SE et al. A collaborative exercise on DNA methylation-based body fluid typing. *Electrophoresis*. 2016;21:2759-2766.
- Lee HY et al. DNA methylation profiling for a confirmatory test for blood, saliva, semen, vaginal fluid and menstrual blood. *Forensic Sci Int Genet*. 2016;24:75-82.

2. Prepare reagents and kits such as:

- Bisulfite modification Kit: Imprint® DNA Modification Kit (Sigma-Aldrich Inc. Cat. No. MOD50) or EpiTect Fast Bisulfite Conversion Kit (Qiagen, Cat. No. 59104)
- Primers (bisulfite converted DNA-specific): multiplex PCR and multiplex SBE
- PCR product purification: ExoSAP-IT® (USB, Cat. No. 97067-402)
- SBE kit: SNaPshot™ Kit (Applied Biosystems, Cat. No. 4323159)
- Post SBE: SAP or CIP
- CE: Hi-Di Formamide, GeneScan™ 120 LIZ™ Size Standard, Matrix Standard Set DS-02, Run Module GS STR POP4 E5, POP4 (Applied Biosystems)

A SNaPshot multiplex for body fluid identification



Lee HY et al., Forensic Sci Int Genet (2016)

SNaPshot multiplexes for body fluid identification

forensic.yonsei.ac.kr/protocols.html

DNA methylation analysis

- Body fluid identification
SNaPshot multiplex for 7 CpGs (Semen, blood, vaginal fluid)
SNaPshot multiplex for 9 CpGs (Semen, blood, vaginal fluid)
- Age prediction from saliva
SNaPshot multiplex
Age calculator
- Age prediction from semen
SNaPshot multiplex
Age calculator

ABO genotyping

- Rapid direct PCR for ABO blood typing
- ABO blood typing for degraded DNA

PCR product purification

- Using the QIAquick® PCR Purification Kit
- Using the ExoSAP-IT® for PCR Product Cleanup

Multiplex PCR

Reagents needed:

- 4 X Primer Mix
- AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Foster City, CA)
- Goat BSA® 10 X Buffer (Promega, Madison, WI)

4 X Primer Mix for Multiplex PCR:

Target ID	Sequence (F-R)	Conc. (µM)	Amplification rate (%)
SE1	cg17610929 TTC TTA ACA TGT TTT GGA TTA ATA AG ATA ACT TTC CTT ATT AAC AGC AAC	2.4	174
SE2	cg26763284 TGA TTT ATA ATT ATT AAG GAG GGA AAT AG GCT AAA AGA ACC BAT TCC CAA C	8.0	100
BL1	cg06379435 TTC ATT GGG GAA TTT TTA TGT GTT AG AAA ATA GAA GTT ACT GCT AAA GAC C	8.0	137
BL3	cg08792630 TGT TTY AAG AGG ATG ATA AGG AA GAA GCT GAA TCC AAA GTA AAT AGA	2.4	200
VF1	cg09765089-2216 TTC GTA GTT TTT GGA TTT TGG AG AAA GAT AAG AAT ACC GAA AC	9.2	137
VF2	cg26079753-74 TCT TGG AAG TGT GAG AGA TTT TTA AGA AAA AGC TCC AAA ACA AAA CCT GTA	1.8	176
SA1	cg09652652-26 GGG GAT TTT TTT TGT TAT GT GGA TTT GCG GGT TGG TAA AA	9.0	163
MB1	cg18069290 GTT TTT TAG GGG AGA GTA GGA AT ATA ATA AAA GAA GCA ATA CAA C	8.0	160
MB2	cg09696411 ATA ATA AAA GAA GCA ATA CAA C	8.0	160

PCR Mixtures

PCR Component	Vol. (µL)	Final Conc.
dH ₂ O	-11.3	
10 X Goat BSA® Buffer	2	
4 X Primer Mix	8	
AmpliTaq Gold® (5 U/µL)	0.7 (3.6 U)	
Template (genomic DNA)	1 (100 ng)	
Total	20	

Thermal Cycling

Step	Time
95°C for 11 minutes, Heat	
95°C for 20 seconds	
95°C for 40 seconds	
95°C for 20 seconds	
For 30 cycles, then	
95°C for 7 minutes	
4°C cool	

DNA methylation and bisulfite modification

Each modification kit has a different capacity in the optimal or minimum DNA quantity that it can deal with

- Procedure takes 2 to 12 hours depending on the kits
- Most kits provide less than 5 hours of processing time



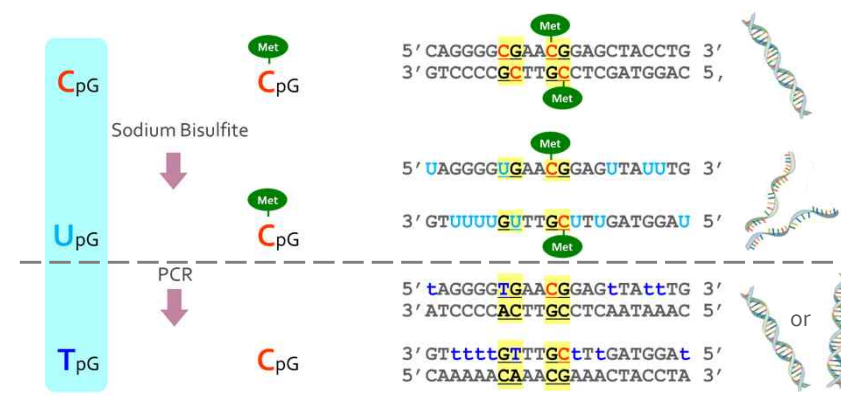
Kits and manufacturers	Recommended Input	Minimum Input
Imprint® DNA modification kit (Sigma)	50 pg-200 ng	> 50 pg
EpiTect Bisulfite kit (Qiagen)	1 ng-1 ug	> 1 ng
EZ DNA Methylation™ kit (Zymo Research)	0.5 ng-2 ug	> 500 pg
EZ DNA Methylation-Direct™ kit	DNA, cells, tissue	> 50 pg

The above characters are from Kakao Friends own by Kakao corp.

DNA methylation and bisulfite modification

DNA methylation detection

- Bisulfite treatment converts non-methylated C to U, which changes into T by PCR



Target sequences of the Illumina beadchip array

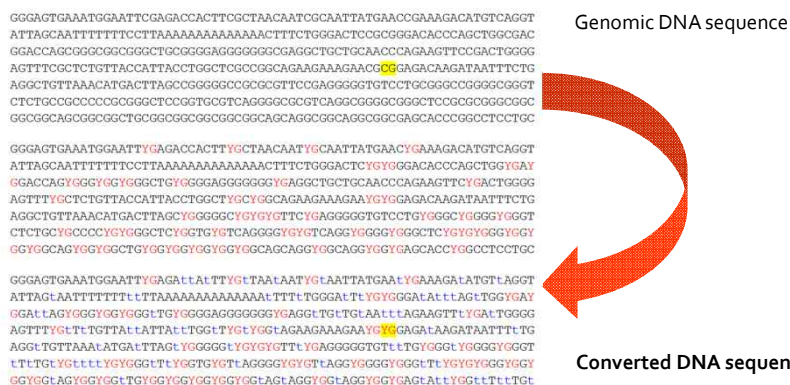
Which sequence was targeted?

AI	Color_Channel	Forward Sequence	Genome_Build	CHR	MAPINFO	SourceSeq	Chromosome 36	Coordinate 36	Strand
		TGGGGTGAAGTGAAGTTC	37	16	28890100	CGCCCTCAATGTA	16	28797601	F
		GTACACTGTCTTTT	37	6	25282779	CGCCATGAAGTTT	6	25390758	R
		ATGGCCCTCTTCTG	37	15	59785306	TGCCCTCTGTGGG	15	57527598	R
		CTCCTTGGGTGGGA	37	8	49890609	TGCCAAGGTGGG	8	50053162	F
		GGTTTGAAGGATT	37	8	87081553	CGCCTGAGGTTCC	8	87150669	F
		CCACCTCACCCG	37	1	166958439	CAAGCGGGCAGCA	1	165225063	R

DNA methylation and PCR primer design

PCR primer design

- Targets bisulfite-converted DNA



DNA methylation and SBE primer design

SBE primer design

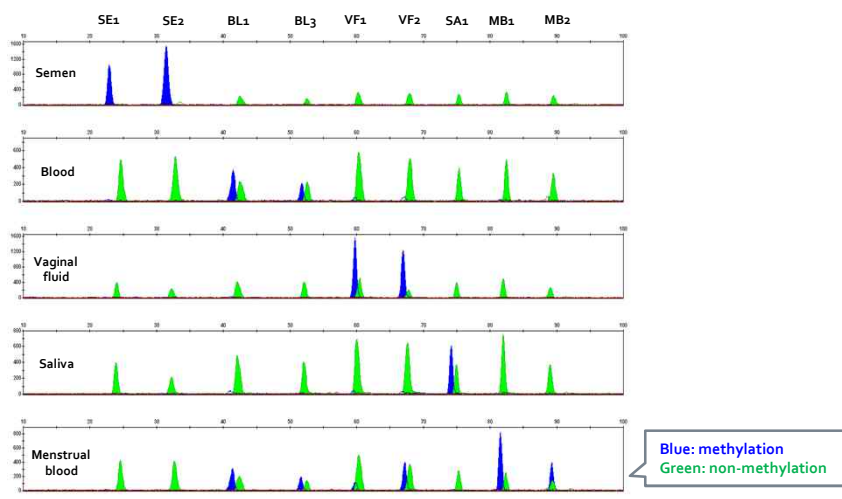
- BatchPrimer3 program (<https://probes.pw.usda.gov/batchprimer3/>)

SBE primers:

Orientation	Start	Len	Tm	GC%	Any compl	3' compl	Score	SNP	Pos	Primer Seq
1 FORWARD	60	21	59.5	33.33	2	0	84.28	T/C	81	TYGTYGGTAGAAGAAAGAAAYG
2 REVERSE	112	29	59.68	27.59	5	0	71.93	T/C	81	TTAACACCTCAAAAATTATCTATCTCC

450K probe sequence:
 RACCCCCRACATAAATCATATTTAACACCTCAAAAATTATCTATCTCC

A SNaPshot multiplex for body fluid identification



Introducing DNA methylation profiling in your lab

3. Bisulfite conversion of genomic DNA

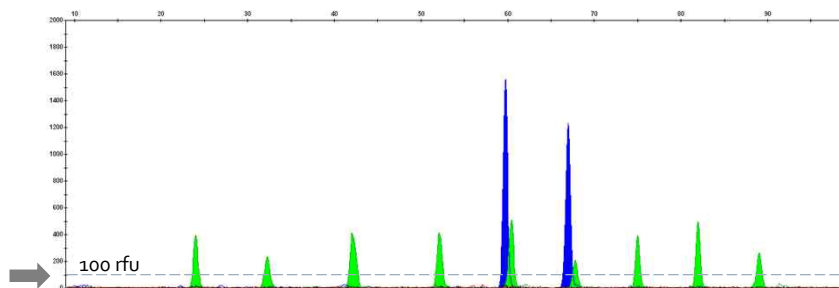
- For various body fluids: semen, blood, saliva, vaginal fluid and menstrual blood
- Extract and quantify DNA
- Select optimal input DNA amount (> 50 ng) and perform bisulfite conversion
- Bisulfite converted DNA is present as a single strand
- The converted DNA is stable for one day at room temperature, one week at 4°C, and two to four months at -20°C
- Recommend on storing your converted DNA below -70°C whenever possible

4. Perform multiplex PCR followed by multiplex SBE

- Determine optimal amount of bisulfite converted DNA (> 10 ng)
- Perform multiplex PCR followed by multiplex SBE according to the protocol
- Analyze on CE

Interpretation

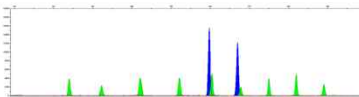
- Determine detection threshold for methylation, non-methylation signal, e.g. 100 rfu
- Put peak height values into the excel sheet and calculate methylation percentages
- Determine analytical threshold for methylation percentage at each marker, e.g. 10%
- Compare with reference DNA methylation profiles to determine body fluid type



Interpretation

- Put peak height values into the excel sheet and calculate methylation percentages : $100 \times \text{nucleotide G} / (\text{nucleotide G} + \text{A intensities})$
- Determine analytical threshold for methylation percentage at each marker, e.g. 10%
- Compare with reference DNA methylation profiles to determine body fluid type

Dye/Sample Peak	Sample File Name	Size	Height	Area	Data Point
G,8	BF_SE_BSM31E6	1449.76	153	1047	2554
B,1	BF_VT_XDP15022	59.78	1558	12660	1326
B,2	BF_VT_XDP15022	68.97	1232	10123	1422
G,1	BF_VT_XDP15022	24.01	394	4163	840
G,2	BF_VT_XDP15022	32.25	238	2167	947
G,3	BF_VT_XDP15022	42.01	412	4847	1074
G,4	BF_VT_XDP15022	52.09	418	4352	1217
G,5	BF_VT_XDP15022	80.43	509	4777	1535
G,6	BF_VT_XDP15022	67.74	212	1866	1432
G,7					
G,8					




Sample	Body fluid	B	G	METHYL %	B	G	METHYL %	B	G	METHYL %	B	G	METHYL %	B	G	METHYL %	B	G	METHYL %	B	G	METHYL %	B	G	METHYL %	B	G	METHYL %	B	G	METHYL %	B	G	METHYL %	B	G	METHYL %																			
20W1313	Semen	2062	0	100.00	1733	0	100.00	0	951	0.00	0	685	0.00	0	704	0.00	0	710	0.00	0	411	0.00	0	814	0.00	0	437	0.00	0	1190	0.00	0	100.00	104	485	31.96	0	827	0.00	0	824	0.00	0	845	0.00	0	846	0.00	0	1112	0.00	0	544	0.00		
40W1313	Semen	1152	0	100.00	3761	0	100.00	0	545	0.00	0	699	0.00	0	817	0.00	0	820	0.00	0	426	0.00	0	1117	0.00	0	569	0.00	0	1190	0.00	0	100.00	104	485	31.96	0	827	0.00	0	824	0.00	0	845	0.00	0	846	0.00	0	1112	0.00	0	544	0.00		
20W1304	Blood	0	382	0.00	0	483	0.00	0	347	192	54.62	278	220	45.82	0	130	0.00	0	689	0.00	0	429	0.00	0	441	0.00	0	254	0.00	0	1190	0.00	0	100.00	104	485	31.96	0	827	0.00	0	824	0.00	0	845	0.00	0	846	0.00	0	1112	0.00	0	544	0.00	
40W1304	Blood	0	424	0.00	0	480	0.00	0	366	207	56.41	296	217	50.26	0	140	0.00	0	670	0.00	0	427	0.00	0	468	0.00	0	289	0.00	0	1190	0.00	0	100.00	104	485	31.96	0	827	0.00	0	824	0.00	0	845	0.00	0	846	0.00	0	1112	0.00	0	544	0.00	
40W1307	Blood	0	491	0.00	0	512	0.00	0	376	224	61.64	214	229	48.77	0	160	0.00	0	512	0.00	0	397	0.00	0	488	0.00	0	239	0.00	0	1190	0.00	0	100.00	104	485	31.96	0	827	0.00	0	824	0.00	0	845	0.00	0	846	0.00	0	1112	0.00	0	544	0.00	
10P15075	Vaginal fluid	0	329	0.00	0	348	0.00	0	292	206	70.53	149	111	42.72	0	111	0.00	0	414	0.00	0	364	0.00	0	482	0.00	0	264	0.00	0	1190	0.00	0	100.00	104	485	31.96	0	827	0.00	0	824	0.00	0	845	0.00	0	846	0.00	0	1112	0.00	0	544	0.00	
10P15023	Vaginal fluid	0	358	0.00	0	348	0.00	0	424	0.00	0	444	0.00	100.00	104	485	31.96	0	827	0.00	0	824	0.00	0	845	0.00	0	846	0.00	0	1112	0.00	0	544	0.00																					
10P15009	Vaginal fluid	0	654	0.00	0	533	0.00	0	118	356	75.28	0	693	0.00	0	581	793	40.35	582	742	42.18	0	362	0.00	0	1127	0.00	0	545	0.00	0	1190	0.00	0	100.00	104	485	31.96	0	827	0.00	0	824	0.00	0	845	0.00	0	846	0.00	0	1112	0.00	0	544	0.00
20W1302	Saliva	0	138	0.00	0	419	0.00	0	248	0.00	0	179	0.00	0	271	0.00	0	546	0.00	0	139	249	63.98	0	347	0.00	0	221	0.00	0	1190	0.00	0	100.00	104	485	31.96	0	827	0.00	0	824	0.00	0	845	0.00	0	846	0.00	0	1112	0.00	0	544	0.00	
20W1303	Saliva	0	483	0.00	0	516	0.00	0	119	709	85.39	0	585	0.00	0	801	0.00	0	859	0.00	0	186	712	65.41	0	848	0.00	0	437	0.00	0	1190	0.00	0	100.00	104	485	31.96	0	827	0.00	0	824	0.00	0	845	0.00	0	846	0.00	0	1112	0.00	0	544	0.00
20W1307	Saliva	0	473	0.00	0	754	0.00	0	467	0.00	0	393	0.00	0	653	0.00	0	1123	0.00	0	585	110	79.28	0	489	0.00	0	292	0.00	0	1190	0.00	0	100.00	104	485	31.96	0	827	0.00	0	824	0.00	0	845	0.00	0	846	0.00	0	1112	0.00	0	544	0.00	
10P15001_23	Menstrual Blood	0	347	0.00	0	409	0.00	0	151	0.00	0	144	0.00	0	411	247	36.86	363	116	31.46	0	381	0.00	0	278	0.00	0	107	0.00	0	1190	0.00	0	100.00	104	485	31.96	0	827	0.00	0	824	0.00	0	845	0.00	0	846	0.00	0	1112	0.00	0	544	0.00	
20P15003_22	Menstrual Blood	0	464	0.00	0	644	0.00	0	471	379	54.24	239	212	48.54	102	614	14.29	533	494	48.19	0	404	0.00	0	1231	342	71.28	605	148	11.61	0	1190	0.00	0	100.00	104	485	31.96	0	827	0.00	0	824	0.00	0	845	0.00	0	846	0.00	0	1112	0.00	0	544	0.00
20P15005_23	Menstrual Blood	0	633	0.00	0	712	0.00	0	148	724	78.36	110	212	68.34	123	612	21.87	187	684	19.61	0	602	0.00	0	466	223	69.68	191	141	17.14	0	1190	0.00	0	100.00	104	485	31.96	0	827	0.00	0	824	0.00	0	845	0.00	0	846	0.00	0	1112	0.00	0	544	0.00
10P15009_24	Menstrual Blood	0	737	0.00	0	737	0.00	0	498	0.00	0	225	0.00	0	225	0.00	0	1004	456	31.34	614	872	47.94	0	773	0.00	0	227	0.00	0	1190	0.00	0	100.00	104	485	31.96	0	827	0.00	0	824	0.00	0	845	0.00	0	846	0.00	0	1112	0.00	0	544	0.00	

Interpretation

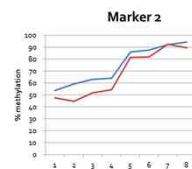
- Nucleotide G intensity higher than nucleotide A intensity by 1.7 fold is beneficial to detect positive signal

Methyl	Unmethyl	1* methyl%	1.7* methyl	Difference
5	95	5	9	4
10	90	10	17	7
15	85	15	24	9
20	80	20	31	11
25	75	25	38	13
30	70	30	44	14
35	65	35	49	14
40	60	40	55	15
45	55	45	60	15
50	50	50	64	14
55	45	55	69	14
60	40	60	73	13
65	35	65	77	12
70	30	70	81	11
75	25	75	84	9
80	20	80	88	8
85	15	85	91	6
90	10	90	94	4
95	5	95	97	2

Marker 1			Marker 2		
Pyro	SNaPshot	Difference	Pyro	SNaPshot	Difference
31	38	7	45	59	14
34	48	14	48	54	6
35	58	24	52	63	11
38	56	18	54	64	10
69	84	15	82	86	4
70	82	13	82	88	6
83	93	9	90	94	5
85	92	8	93	92	0



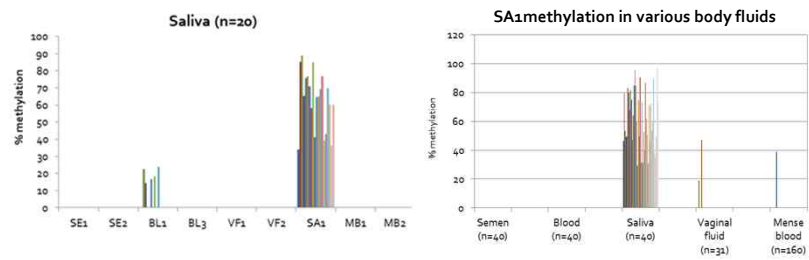
Marker 1



Marker 2

Interpretation

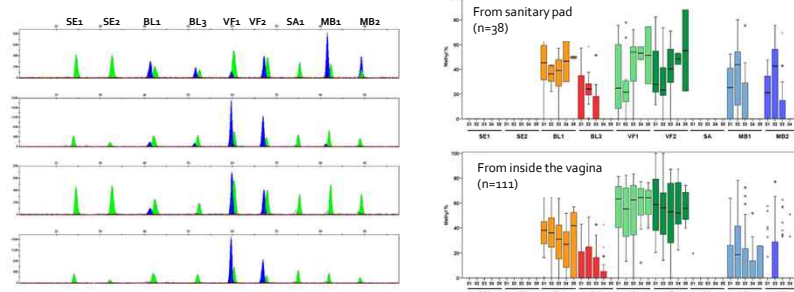
- Compare with reference DNA methylation profiles to determine body fluid type
 - Only one marker positive for saliva → observed
 - One marker positive for saliva but with additional positive signal → need more careful considerations



Interpretation

- Compare with reference DNA methylation profiles to determine body fluid type
 - Menstrual blood samples show different methylation profiles depending on the menstrual cycle and the sampling methods
 - Both of the MB1 and MB2 markers positive → observed
 - One of the MB1 and MB2 markers, BL1 and BL3 markers positive → observed

Various profiles of menstrual bloods



Introducing DNA methylation profiling in your lab

5. Analyze more samples

- More body fluid samples (validation and establishment of interpretational guidelines)
- Lower amount (< 50 ng of g DNA and < 10 ng of bisulfite converted DNA)
- Mixed samples (Joined interpretation with STR and DNA methylation profiles)

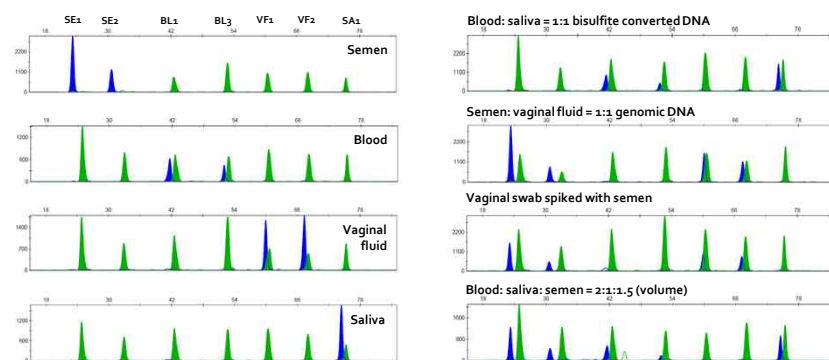
6. Develop reporting guidelines or formats

7. Establish Standard Operating Procedures

8. Inform your clients

More complex samples

e.g. Mixed samples test in a lab of collaborative exercise (Electrophoresis, 2016)

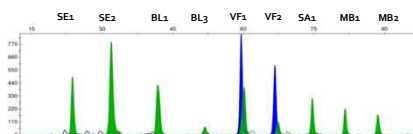


Examples of NFS DNA methylation profiling

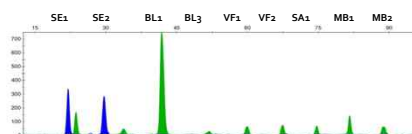
Case 1: Rape

- Vaginal swab from the victim was positive for semen in ACP test and the A-STR profile of the perpetrator was observed
- From a penile swab, STR profile of the victim was obtained
- A stain from the belly of the victim was negative for semen and saliva, but showed STR profile of the perpetrator

A penile swab (victim's STR profile): 0.427 ng/ul



A stain from the belly (perpetrator's STR profile): 0.071 ng/ul



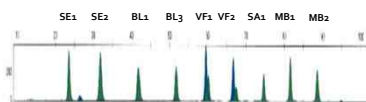
Please see more at poster Nos. P05-297 and P05-299

Examples of NFS DNA methylation profiling

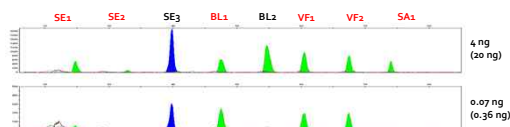
Case 2: Digital penetration on the public transportation

- Vaginal swab was negative for semen and showed only STR profile of the victim
- A swab wiped the fingers of the alleged perpetrator showed STR profile of the victim

A finger swab from the alleged perpetrator (victim's STR profile) : 0.051 ng/ul



Skin swab samples (FSIG 2015): 6 overlapping markers



Examples of NFS DNA methylation profiling

Case 3: Murder in 2004

- A female karaoke worker was found to be dead
- A knife and an unknown stain were found near the victim
- From the test in 2004
 - The knife blade was negative for blood, but showed STR profile of the victim
 - The stain was positive for saliva, and showed unknown male's STR profile

Knife blade (victim's STR profile): 6.7 ng/ul

Unknown stain: 10.7 ng/ul



- The victim's body was damaged by cutting the left breast part and vagina with the knife
- DNA methylation profile was consistent with the scenario of the case
- DNA methylation profiling was successfully done with 13 year-old DNA samples

Examples of NFS DNA methylation profiling

Case 5: Indecent exposure with no suspect

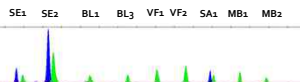
- A stain preliminary positive for semen and saliva
- Two men's mixed STR profile
- Differential extraction and body fluid ID test
 - Supernatant: STR profile of a man
 - Precipitate: STR profile of another unknown man → add. age prediction



Semen (Unknown suspect: male 1 STR)



A man of 61 years old predicted



4 ng bisulfite converted DNA

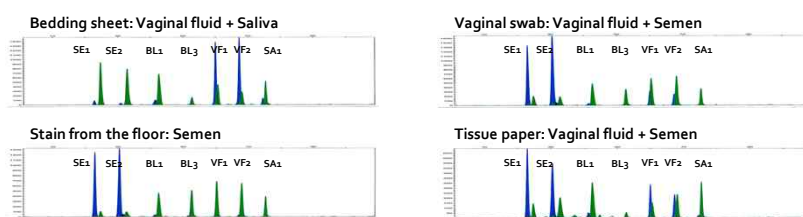
Saliva (Innocent: male 2 STR)



Examples of NFS DNA methylation profiling

Quantification of male and female DNA from sexual abuse samples using the Quantifiler Duo kit

Samples	Human DNA (ng/ul)	Male DNA (ng/ul)	Seminal test (SM test & RSID semen)	Methylation result
UNKNOWN 1 (Bedding sheet)	1.0	0.1	-	Vaginal Fluid + Saliva
UNKNOWN 2 (vaginal swab)	4.1	5.3	Inconclusive	Vaginal Fluid + Semen
Crime Scene Sample (stain from the floor)	9.0	9.2	+	Semen
Crime Scene Sample (tissue paper)	1.8	1.3	+	Vaginal Fluid + Semen



From a poster of Promega HID conference 2016

NEXT
Lunch & Hands-on demo

