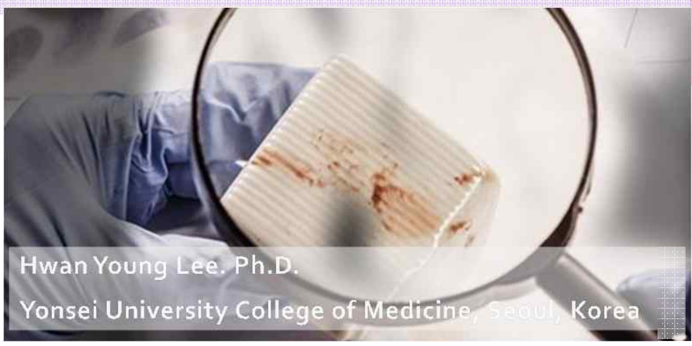
DNA methylation-based body fluid identification



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Presentation Overview

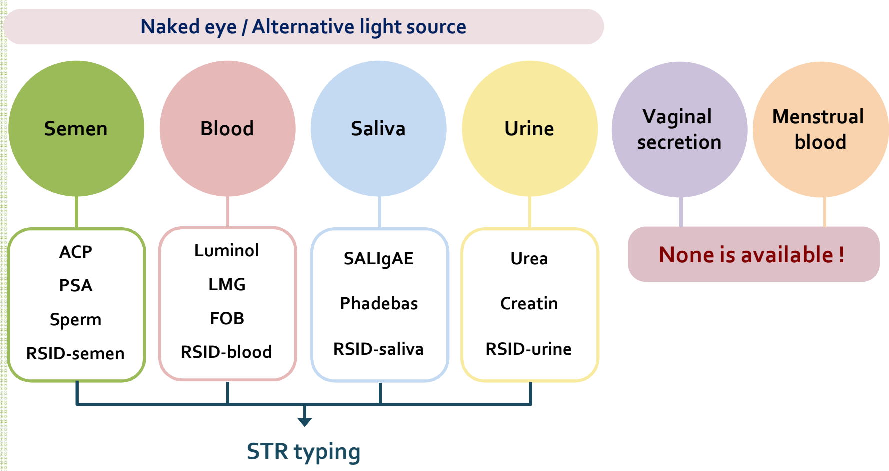
1. Body fluid typing in forensics
2. Identification of body fluid-specific CpGs
3. A SNaPshot multiplex for body fluid identification
4. Examples of casework analysis

Body Fluids and Tissues at a Crime Scene

- Can link sample donors with actual criminal acts

	Type	Forensic relevance
Body fluids	Blood	Violence
	Semen	Sexual assault
	Saliva	Sexual assault e.g. licking, kissing or inoffensive stain
	Vaginal secretion	Sexual assault
	Menstrual blood	Sexual assault or inoffensive alternative scenario to violence
	Urine	Confirmation of sampled area
Touch	Skin	Confirmation of sampled area
Organs	Brain	Head injury
	Heart, lung	Chest injury
	Kidney , liver	Abdominal injury

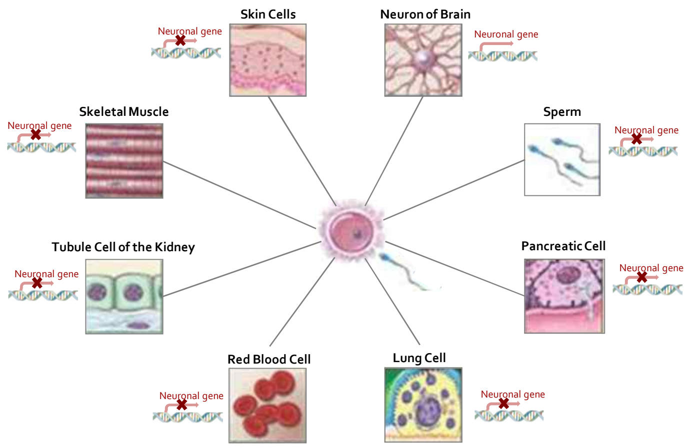
Conventional Body Fluid Typing in Forensics



Limitations of Conventional Body Fluid Typing

- Chemical, catalytic, enzymatic and immunological tests
- Low specificity
- Lack of sensitivity
- Sample destruction during body fluid test
- Instability of biomolecules assayed
- Incompatibility with downstream STR analysis
- Carried out for only one body fluid at a time
- Cannot be applied when only DNA extract remains in an old case

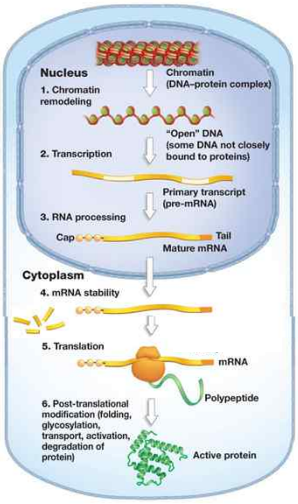
Different Tissues and Cells



How Specialization Achieved

Epigenetic modification

miRNA



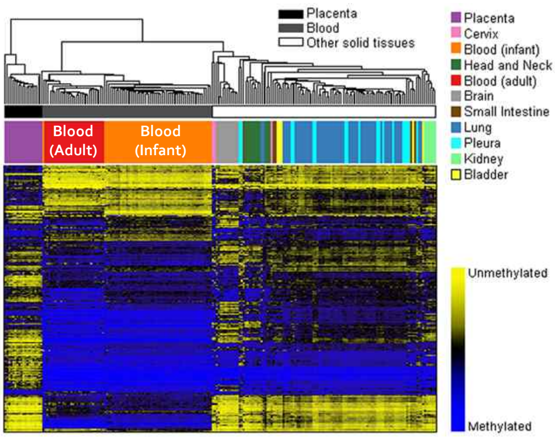
DNA

mRNA

Functional Protein

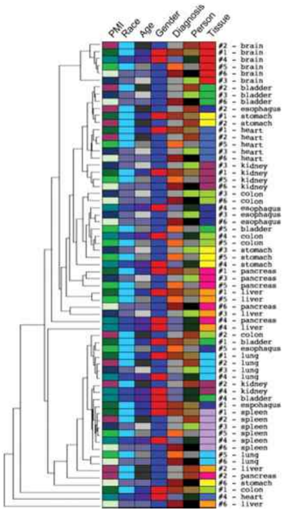
Epigenetic Variation Across Tissues

- DNA methylation profiles are specific to tissue and age

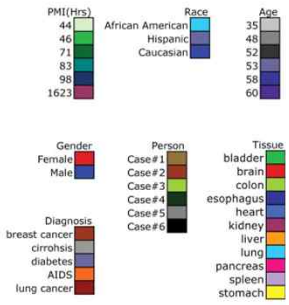


Unsupervised clustering of average beta values in normal human tissues. Christensen et al. PLoS Genet (2009)00

Tissue-Specific DNA Methylation of Somatic Tissues from Human Autopsy Specimens



- DNA methylation patterns were more consistent between the same tissues from different people than between different tissues from the same individual



Intra-tissue and intra-individual variability of DNA methylation. Two-way hierarchical cluster analysis consistent with DNA methylation profiles of 11 organs from 6 individuals. Byun et al. Hum Mol Genet (2009)

Common Features of DNA Methylation Variation

1. **Methylation Variable Position (MVP)**
2. **Variably Methylated Region (VMR)**
3. **Differentially Methylated Region (DMR)**
   - Imprinting-specific DMR (iDMR)
   - Tissue-specific DMR (tDMR)
   - Reprogramming-specific DMR (rDMR)
   - Cancer-specific DMR (cDMR)
   - Aging-specific DMR (aDMR)

Tissue-Specific Differentially Methylated Region (tDMR) for Forensic Application

Int J Legal Med (2012) 126:55–62
DOI 10.1007/s00414-011-0569-2

ORIGINAL ARTICLE

Potential forensic application of DNA methylation profiling to body fluid identification

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Abstract DNA analysis of various body fluid stains at crime scenes facilitates the identification of individuals but does not currently determine the type and origin of the biological material. Recent advances in whole genome epigenetic analysis indicate that chromosome pieces called tDMRs (tissue-specific differentially methylated regions) show different DNA methylation profiles according to the type of cell or tissue. We examined the potential of tissue-specific differential DNA methylation for body fluid identification. Five tDMRs for the genes DACT1, USP49, HOXA4, PFN3, and PRMT2 were selected, and DNA methylation profiles for these tDMRs were produced by bisulfite sequencing using pooled DNA from blood, saliva, semen, menstrual blood, and vaginal fluid. The tDMRs for DACT1 and USP49 showed semen-specific hypomethylation, and the tDMRs for HOXA4, PFN3, and PRMT2 displayed varying degrees of methylation according to the type of body fluid. Preliminary tests using methylation-specific PCR for the DACT1 and USP49 tDMRs showed that these two markers could be used successfully to identify semen samples including sperm cells. Body fluid-specific differential DNA methylation may be a promising indicator for body fluid identification. Because DNA methylation profiling uses the same biological source of DNA for individual identification profiling, the determination of more body fluid-specific tDMRs and the development of convenient tDMR analysis methods will facilitate the broad implementation of body fluid identification in forensic casework.

Keywords Body fluid identification · DNA methylation · Tissue-specific differentially methylated region

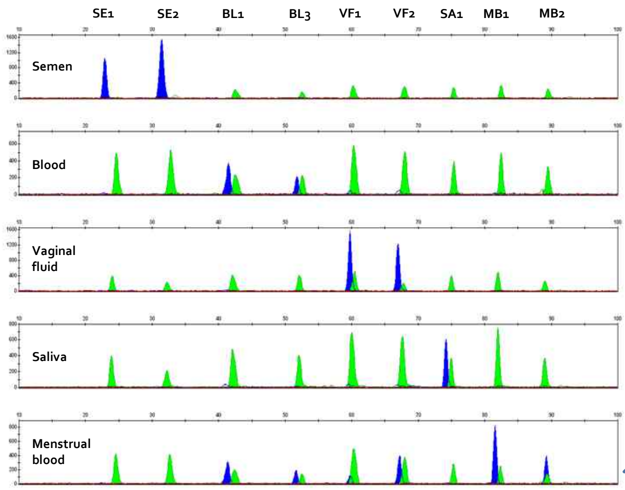
Introduction

Body fluids found at a crime scene provide crucial information to link evidence and the crime. DNA obtained from body fluids such as blood, saliva, and semen can be used to identify the donor of the biological material, and the determination of the type and origin of the biological material can help reconstruct crime scenes. However, conventional body fluid identification using serological or immunological tests cannot positively confirm the presence

Publications

1. Frumkin D *et al.* (2011) DNA methylation-based forensic tissue identification. Forensic Sci Int Genet 5, 517–524
2. Lee HY *et al.* (2012) Potential forensic application of DNA methylation profiling to body fluid identification. Int J Legal Med 126, 55–62
3. Madi T *et al.* (2012) The determination of tissue-specific DNA methylation patterns in forensic biofluids using bisulfite modification and pyrosequencing. Electrophoresis 33, 1736–1745
4. Park JL *et al.* (2014) Identification of body fluid-specific DNA methylation markers for use in forensic science. Forensic Sci Int Genet 13, 147–153
5. Lee HY *et al.* (2015) Genome-wide methylation profiling and a multiplex construction for the identification of body fluids using epigenetic markers. Forensic Sci Int Genet 17, 17–24
6. Forat S *et al.* (2016) Methylation Markers for the Identification of Body Fluids and Tissues from Forensic Trace Evidence. PLoS One 11, e0147973
7. Lee HY *et al.* (2016). DNA methylation profiling for a confirmatory test for blood, saliva, semen, vaginal fluid and menstrual blood. Forensic Sci Int Genet. 24, 75-82
8. Jung SE *et al.* (2016) A collaborative exercise on DNA methylation-based body fluid typing. Electrophoresis. 21, 2759-2766
9. Holtkötter H *et al.* (2017) Independent validation of body fluid-specific CpG markers and construction of a robust multiplex assay. Forensic Sci Int Genet. 29, 261-268

The Latest DNA Methylation-Based Body Fluid ID



Marker	Illumina ID	Gene
SE1	cg17610929	ACCN4
SE2	cg26763284	PLEC
BL1	cg06379435	
BL3	cg08792630	
VF1	cg09765089	
VF2	cg26079753	HOTAIR
SA1	cg09652652	FAM43A
MB1	cg18069290	SLC26A10
MB2	cg09696411	SLC26A10

Blue: methylation
Green: non-methylation

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

Development of a Multiplex Assay for Body Fluid ID

1. **Identification of body fluid-specific CpGs**
   - Epigenome-wide technology, e.g. whole genome bisulfite sequencing, bisulfite microarray, and enrichment methods
   - Enormous data generated using illumina' BeadChip array can be browsed and downloaded from the public databases, such as NCBI GEO database
   - 12 semen, 12 blood, 12 saliva, 6 vaginal fluid, and 9 menstrual blood samples
   - Candidate marker validation with targeted bisulfite sequencing, such as pyrosequencing and methylation SNaPshot
2. **Design of a multiplex assay**
   - Locus-specific analysis based on multiplex PCR of bisulfite-converted DNA
   - Methylation SNaPshot has the same workflow used in SNP analysis
   - Semen (2), blood (2), vaginal fluid(2), saliva (1), menstrual blood (2)
3. **Validation of the multiplex assay**
   - Independent samples
   - Set-up interpretational guidelines

Identification of Body Fluid-Specific CpGs

Comparison of 450K results from various body fluids

- 12 semen, 12 blood, 12 saliva, 3 vaginal fluid, 3 menstrual blood samples (GSE59505)

	Comparison	Cut-off	No. of CpGs
1	SE vs. BL	Abs (delta_mean) ≥ 0.3, fdr. P < 0.05	64,079
	SE vs. SA	Abs (delta_mean) ≥ 0.3, fdr. P < 0.05	64,305
	SE vs. VF	Abs (delta_mean) ≥ 0.3, fdr. P < 0.05	54,062
	SE vs. MB	Abs (delta_mean) ≥ 0.3, fdr. P < 0.05	45,310
	BL vs. SA	Abs (delta_mean) ≥ 0.3, raw P < 0.05	9,100
	BL vs. VF	Abs (delta_mean) ≥ 0.3, raw P < 0.05	442
	BL vs. MB	Abs (delta_mean) ≥ 0.3, raw P < 0.05	556
	SA vs. VF	Abs (delta_mean) ≥ 0.3, raw P < 0.05	620
	SA vs. MB	Abs (delta_mean) ≥ 0.3, raw P < 0.05	371
	VF vs. MB	Abs (delta_mean) ≥ 0.2, raw P < 0.05	0
2	SE vs. (BL, SA, VF, MB)	Abs (delta_mean) ≥ **0.5**, fdr. P < 0.05	20,542
	BL vs. (SA, VF, MB)	Abs (delta_mean) ≥ 0.3, raw P < 0.05	4,252
	SA vs. (BL, VF, MB)	Abs (delta_mean) ≥ 0.3, raw P < 0.05	2,771
	(VF, MB) vs. (BL, SA)	Abs (delta_mean) ≥ **0.2**, raw P < 0.05	604

Identification of Body Fluid-Specific CpGs

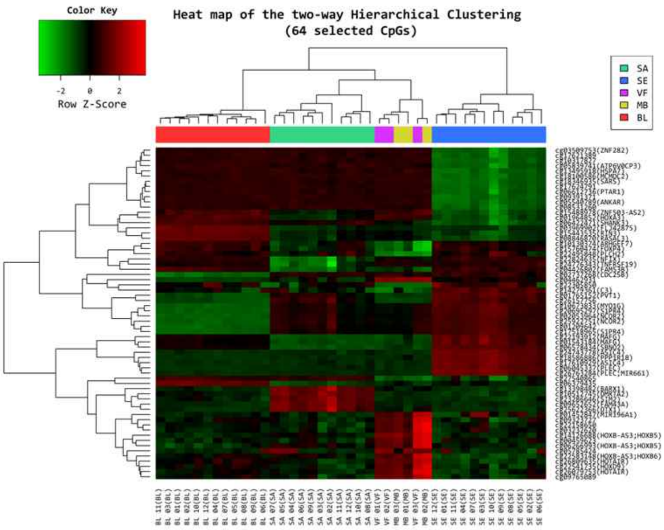
Selection criteria

- CpGs having methylation signal only in a target body fluid with almost complete non-methylation in other body fluids or vice versa.
- Body fluid-specific hyper- or hypo-methylation with a low standard deviation in the same type of body fluid

1	TargetID	SE.Mean	(BL,SA,VF,MB).Mean	SE-(BL,SA,VF,MB).delta_mean	SE-(BL,SA,VF,MB).comment	SE.sd	(BL,SA,VF,MB).sd
2	cg17610929	0.924979074	0.025744487	0.899234587	hyper	0.057049908	0.012295329
3	cg18586886	0.909328143	0.023801124	0.885527019	hyper	0.067048608	0.018397728
4	cg26763284	0.899585963	0.020395829	0.879190133	hyper	0.073013894	0.004682332
5	cg03953626	0.898747975	0.026167513	0.872580461	hyper	0.068058878	0.025062029
6	cg24743778	0.902577848	0.037137072	0.865440776	hyper	0.057582452	0.014577394
7	cg06045337	0.885240066	0.025568726	0.85967134	hyper	0.071210238	0.008583933

	A	B	C	D	E	F	G	H	I	J	K	L	M
13	cg05592911	0.892566022	0.042846956	0.849719066	hyper	0.064561829	0.021922673	0.111101377	0.053970422	0.031527093	0.027807861	0.029100859	0.021807406
14	cg23324953	0.865701504	0.017365552	0.848335952	hyper	0.081984832	0.004824946	0.019949905	0.021883994	0.013385105	0.0111649	0.010352889	0.029966673
15	cg26999505	0.878239085	0.030910069	0.847329015	hyper	0.07444244	0.012530969	0.032068272	0.0242061	0.02220404	0.018192917	0.03017479	0.030319419
16	cg18307912	0.885076701	0.038107633	0.846969068	hyper	0.069207218	0.010171145	0.054503602	0.05015744	0.042018843	0.033910561	0.042328541	0.0466614
17	cg22065733	0.877709087	0.030888944	0.846820143	hyper	0.068831542	0.013698615	0.092880141	0.028496678	0.018381092	0.032571713	0.029969349	0.040171217
18	cg15780398	0.88074547	0.035672607	0.845072863	hyper	0.073420825	0.015143379	0.054163194	0.04330542	0.02885545	0.050443242	0.035427948	0.01855778
19	cg04707822	0.876142074	0.034535505	0.84160657	hyper	0.085658502	0.013698338	0.081480344	0.045443508	0.025650576	0.038827346	0.02760442	0.026995983
20	cg17584970	0.864317819	0.023681094	0.840636725	hyper	0.097325553	0.005898105	0.022324751	0.026204044	0.021241265	0.019302664	0.018740209	0.03579709
21	cg04560810	0.866687351	0.027383744	0.839303606	hyper	0.076055692	0.010098114	0.025728281	0.019926223	0.031046687	0.028226763	0.013066031	0.023598838
22	cg04021962	0.869948976	0.031156729	0.838792247	hyper	0.075757615	0.00751415	0.041483925	0.027279047	0.022668807	0.032960585	0.034518379	0.0258985
23	cg13578134	0.883038562	0.045178614	0.837859948	hyper	0.061919722	0.010382595	0.047952636	0.065267482	0.050919641	0.04850832	0.04956683	0.044502296
24	cg19640166	0.880211995	0.043907849	0.836304146	hyper	0.063784827	0.017410693	0.023239464	0.04193762	0.028010222	0.044112787	0.027615915	0.044209444
25	cg06888746	0.930180186	0.095171209	0.835008977	hyper	0.042987672	0.084010869	0.061336086	0.018293751	0.061444867	0.037267734	0.061139307	0.015885911
26	cg09611472	0.859366472	0.024699901	0.83466657	hyper	0.088506589	0.007336329	0.032005684	0.013741813	0.01946558	0.03197656	0.035084369	0.026074054
27	cg04180929	0.875226916	0.043296419	0.831930497	hyper	0.073616716	0.011631288	0.053331032	0.050372173	0.045932557	0.040063683	0.040813106	0.030567164
28	cg14654324	0.88900978	0.058140773	0.830869008	hyper	0.066430684	0.010098727	0.055835686	0.049712572	0.051001352	0.044417127	0.048015074	0.085724032
29	cg09946832	0.861881853	0.032346177	0.829535676	hyper	0.075002091	0.006120515	0.040606934	0.029534468	0.041591176	0.035802223	0.034741141	0.043713681
30	cg12229555	0.900778537	0.076877074	0.823901463	hyper	0.052625348	0.064818302	0.15456997	0.173967203	0.015364395	0.084846135	0.058112387	0.028584077
31	cg21627589	0.857952758	0.034387302	0.823565456	hyper	0.068207298	0.009183073	0.040687622	0.045791234	0.033449827	0.040254206	0.020156506	0.044536018
32	cg24387367	0.900197574	0.076924396	0.823273178	hyper	0.064611221	0.029093212	0.101689728	0.045655891	0.075164575	0.136736188	0.096274618	0.034869924

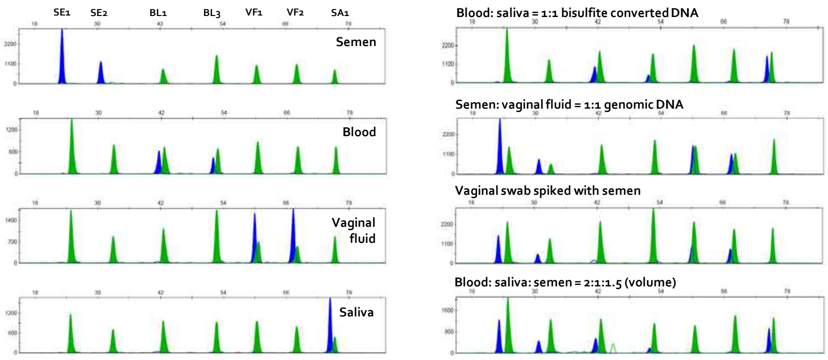
Identification of Body Fluid-Specific CpGs



On-Off Signal in Mixture Analysis

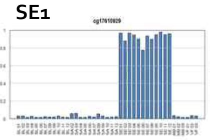
- Methylation only in a target body fluid with almost complete non-methylation in others
- Non-methylation in a target body fluid with almost complete methylation in others

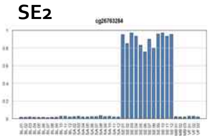
e.g. Mixed sample test in a lab of collaborative exercise (Electrophoresis, 2016)

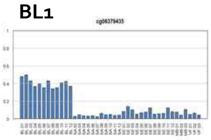


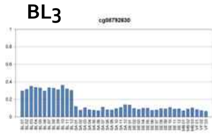
Selected Body Fluid-Specific CpGs from Array Data

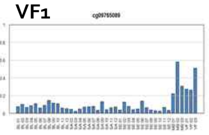
		Mean beta values ± SD					Genome build_37		
Marker	**Target ID**	**SE (n=12)**	**BL (n=12)**	**VF (n=3)**	**MB (n=3)**	**SA (n=11)**	**Chr**	**Map info.**	**Gene symbol**
SE1	cg17610929	**0.92 ± 0.06**	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.02	2	220379044	ACCN4;ASIC4
SE2	cg26763284	**0.90 ± 0.07**	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	8	145018185	PLEC;MIR661
BL1	cg06379435	0.08 ± 0.03	**0.40 ± 0.05**	0.05 ± 0.01	0.07 ± 0.03	0.04 ± 0.01	19	3344273	
BL3*	cg08792630	0.10 ± 0.02	**0.32 ± 0.02**	0.07 ± 0.01	0.09 ± 0.02	0.10 ± 0.02	6	108883909	FOXO3
VF1	cg09765089	0.06 ± 0.04	0.09 ± 0.03	**0.35 ± 0.14**	**0.37 ± 0.19**	0.06 ± 0.03	7	27291346	
VF2	cg26079753	0.07 ± 0.03	0.09 ± 0.02	**0.39 ± 0.21**	**0.39 ± 0.24**	0.07 ± 0.02	12	54355528	HOTAIR
SA1	cg09652652	0.02 ± 0.01	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	**0.49 ± 0.17**	3	194408845	FAM43A

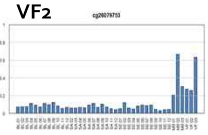
SE1


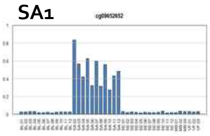
SE2


BL1


BL3


VF1


VF2


SA1


Lee HY et al. Forensic Sci Int Genet (2015) *Park JL et al. Forensic Sci Int Genet (2014)

Identification of Body Fluid-Specific CpGs

Comparison of menstrual blood and vaginal fluid

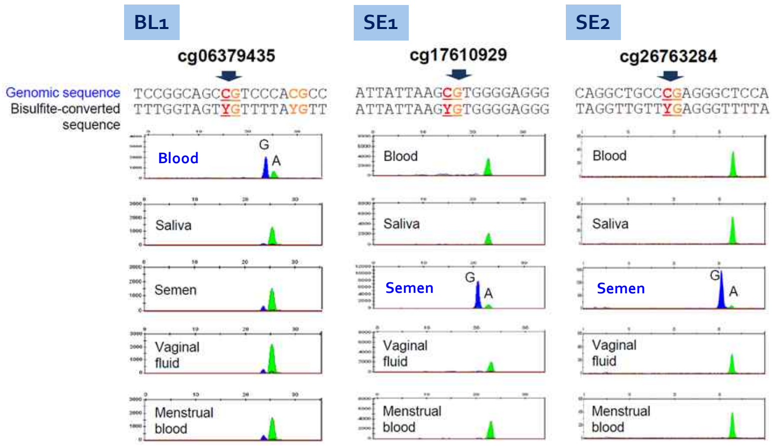
- 450K results of 3 vaginal fluids and 3 of each menstrual bloods obtained from the 1st, 2nd and 3rd days of menstrual bleeding (GSE77283)

Comparison[a]		Cut-off	No. of CpGs
3	MB day 1 vs. VF	Abs (delta_mean) ≥ 0.3, $P < 0.05$, sd < 0.1	165
	MB day 2 vs. VF	Abs (delta_mean) ≥ 0.3, $P < 0.05$, sd < 0.1	31
	MB day 3 vs. VF	Abs (delta_mean) ≥ 0.2, $P < 0.05$, sd < 0.1	15

	Mean beta values ± SD[a]								Genome build_37		
Target ID	SE (n = 12)	BL (n = 12)	SA (n = 12)	SK (n = 19)	VF (n = 6)	MB-1 (n = 3)	MB-2 (n = 3)	MB-3 (n = 3)	Chr	Map info.	Gene
cg05021643	0.02 ± 0.01	0.08 ± 0.03	0.06 ± 0.02	**0.23 ± 0.08**	0.05 ± 0.01	**0.38 ± 0.11**	**0.36 ± 0.06**	**0.31 ± 0.12**	2	177029608	HOXD3
cg02009088	0.03 ± 0.01	0.02 ± 0.01	**0.40 ± 0.21**	**0.70 ± 0.03**	0.02 ± 0.01	**0.44 ± 0.12**	**0.41 ± 0.09**	**0.35 ± 0.14**	5	139228153	NRG2
cg14486338	0.02 ± 0.01	**0.15 ± 0.04**	**0.11 ± 0.03**	**0.17 ± 0.10**	0.07 ± 0.02	**0.41 ± 0.11**	**0.38 ± 0.05**	**0.34 ± 0.20**	8	99440279	KCNS2
cg19893585	**0.80 ± 0.08**	**0.38 ± 0.04**	0.06 ± 0.04	0.05 ± 0.03	0.09 ± 0.04	**0.46 ± 0.13**	**0.36 ± 0.04**	**0.34 ± 0.16**	8	145025064	PLEC1
cg17124583	0.03 ± 0.01	0.09 ± 0.02	**0.37 ± 0.13**	0.05 ± 0.02	0.03 ± 0.01	**0.45 ± 0.09**	**0.38 ± 0.06**	**0.41 ± 0.18**	10	8097641	GATA3
cg04255276	0.02 ± 0.01	0.07 ± 0.04	0.04 ± 0.03	0.05 ± 0.02	0.09 ± 0.07	**0.41 ± 0.11**	**0.36 ± 0.07**	**0.28 ± 0.13**	11	65314021	LTBP3
cg18023065	**0.15 ± 0.10**	**0.39 ± 0.03**	**0.10 ± 0.06**	**0.20 ± 0.05**	0.06 ± 0.02	**0.48 ± 0.13**	**0.43 ± 0.03**	**0.40 ± 0.19**	11	94278603	FUT4
cg09696411	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.05 ± 0.02	0.01 ± 0.00	**0.41 ± 0.10**	**0.35 ± 0.06**	**0.28 ± 0.14**	12	58013517	SLC26A10
cg18069290	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.00	**0.35 ± 0.12**	**0.32 ± 0.10**	**0.22 ± 0.13**	12	58013539	SLC26A10
cg16567290	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	**0.13 ± 0.05**	0.04 ± 0.01	**0.46 ± 0.11**	**0.44 ± 0.05**	**0.37 ± 0.12**	12	58013569	SLC26A10
cg20985399	**0.13 ± 0.12**	**0.15 ± 0.03**	0.07 ± 0.03	**0.36 ± 0.05**	0.07 ± 0.02	**0.50 ± 0.12**	**0.50 ± 0.04**	**0.41 ± 0.18**	15	65689263	IGDCC4
cg22320365	**0.18 ± 0.14**	0.07 ± 0.02	0.09 ± 0.06	**0.26 ± 0.07**	0.06 ± 0.02	**0.37 ± 0.12**	**0.36 ± 0.04**	**0.33 ± 0.17**	17	36718198	SRCIN1
cg12798338	0.06 ± 0.02	0.02 ± 0.00	0.03 ± 0.01	**0.22 ± 0.06**	0.03 ± 0.01	**0.37 ± 0.11**	**0.34 ± 0.08**	**0.27 ± 0.14**	17	76128683	TMC8
cg01032675	**0.81 ± 0.10**	**0.19 ± 0.07**	0.03 ± 0.02	**0.34 ± 0.06**	0.04 ± 0.01	**0.45 ± 0.12**	**0.43 ± 0.07**	**0.39 ± 0.21**	19	3136430	GNA15
cg16606773	0.07 ± 0.06	**0.44 ± 0.04**	0.06 ± 0.05	0.08 ± 0.03	0.06 ± 0.01	**0.38 ± 0.06**	**0.36 ± 0.06**	**0.23 ± 0.12**	20	19955806	RIN2

Candidate Marker Validation

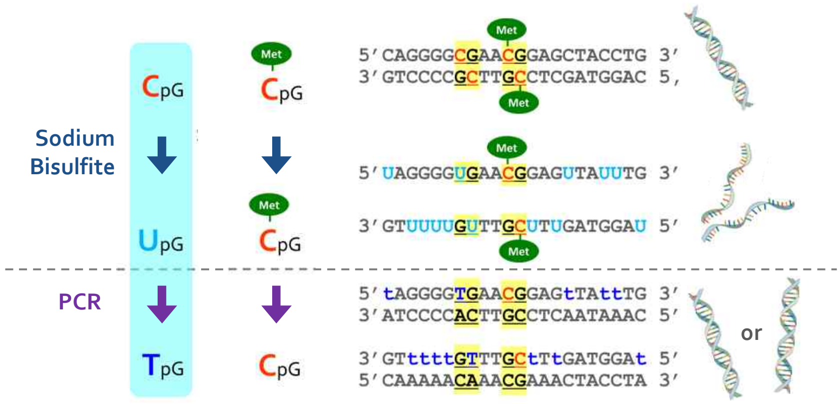
Targeted bisulfite sequencing using methylation SNaPshot



DNA Methylation Detection

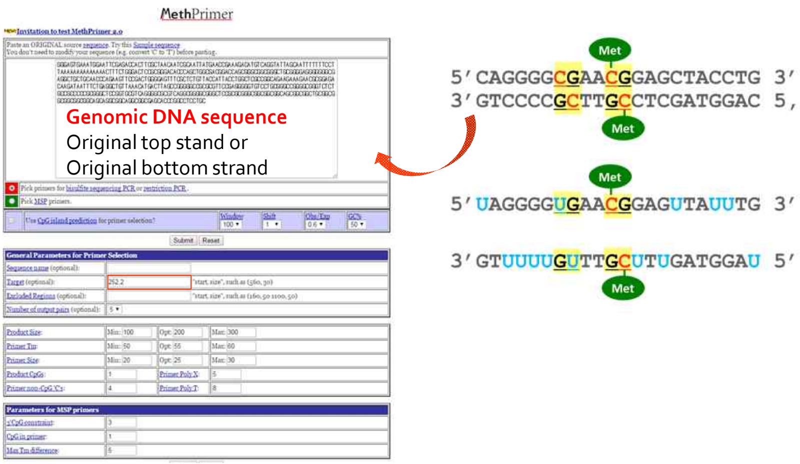
Bisulfite conversion

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

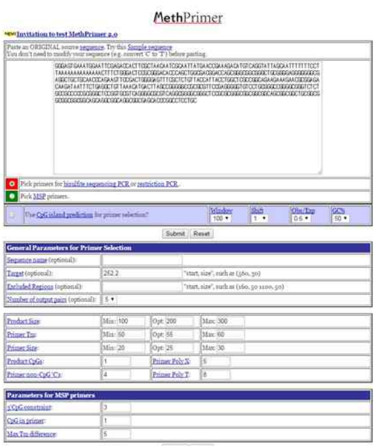


DNA Methylation and PCR Primer Design

- MethPrimer program (http://www.urogene.org/methprimer/)

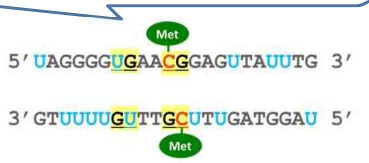


DNA Methylation and PCR Primer Design



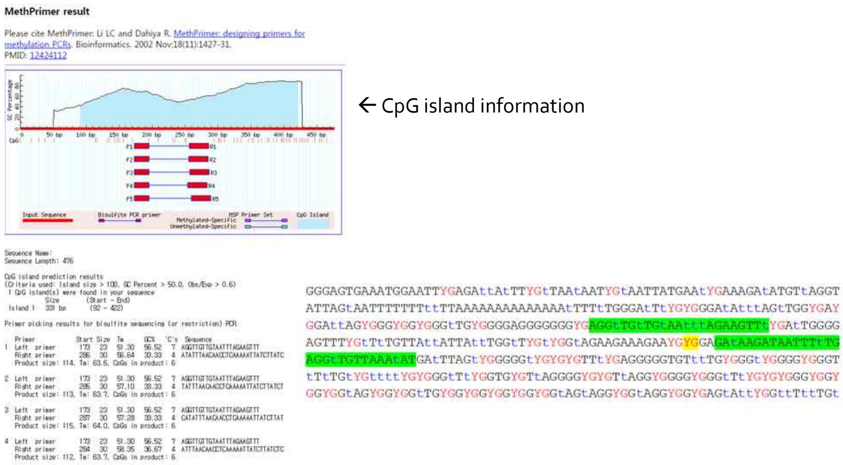
Product Size:	Min: 100	Opt: 200	Max: 300
Primer Tm:	Min: 50	Opt: 55	Max: 60
Primer Size:	Min: 20	Opt: 25	Max: 30
Product CpGs:	1	Primer Poly X:	5
Primer non-CpG 'C's:	4	Primer Poly T:	8

Primer non-CpG 'C's:
The minimum number of non-CpG 'C's in a primer. This is important for discriminating between the bisulfite-modified DNA and unmodified or incompletely modified DNA.



DNA Methylation and PCR Primer Design

- MethPrimer program (http://www.urogene.org/methprimer/)



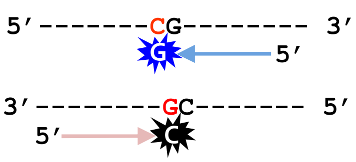
← CpG island information

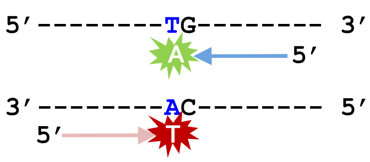
Methylation SNaPshot

Methylation-sensitive single nucleotide primer extension-based approach

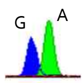
- Dideoxy single-base extension (SBE) of an unlabeled oligonucleotide primer

Converted sequence of methylated CpG | **Converted sequence of non-methylated CpG**





REVERSE



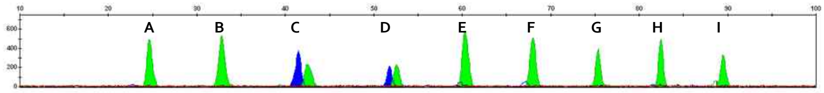
$$\%methyl = \frac{G\ intensity}{(G+A)\ intensity} \times 100$$

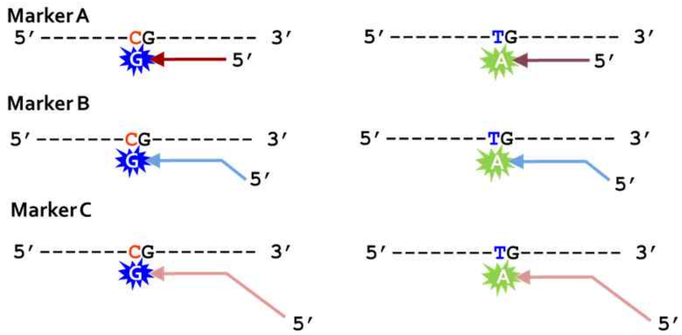
FORWARD

$$\%methyl = \frac{C\ intensity}{(C+T)\ intensity} \times 100$$

SNaPshot Multiplex Following Multiplex PCR

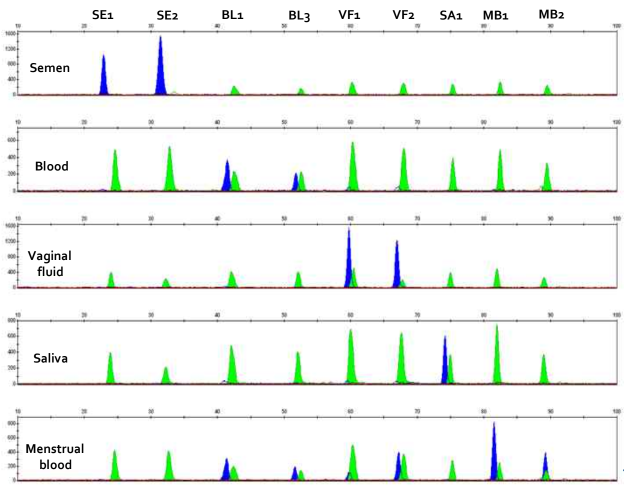
- Its multiplexing capability allows us to incorporate multiple CpG markers in a single reaction
- Multiple primers with different lengths are used for multiplex SBE reaction





SNaPshot Multiplex Construction

Multiplex SNaPshot reaction using SBE primers with different lengths

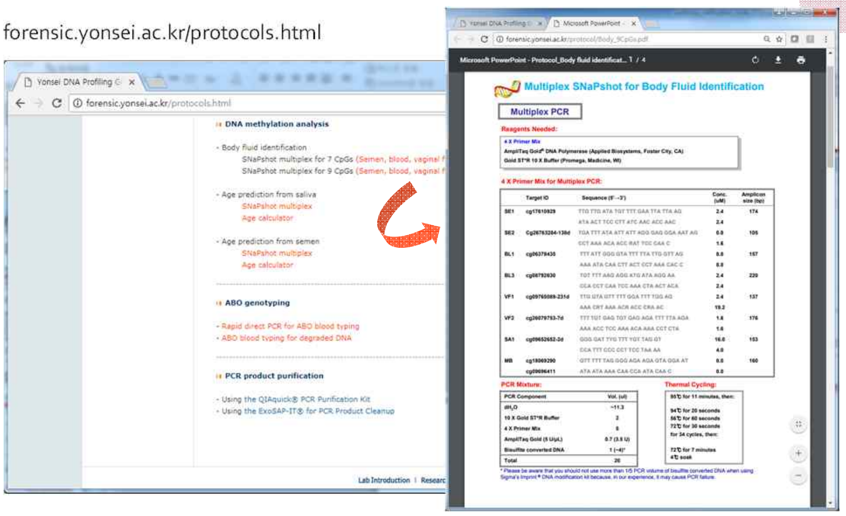


Marker	Illumina ID	Length(nt)
SE1	cg17610929	21
SE2	cg26763284	29
BL1	cg06379435	41
BL3	cg08792630	51
VF1	cg09765089	60
VF2	cg26079753	67
SA1	cg09652652	74
MB1	cg18069290	81
MB2	cg09696411	88

Blue: methylation
Green: non-methylation

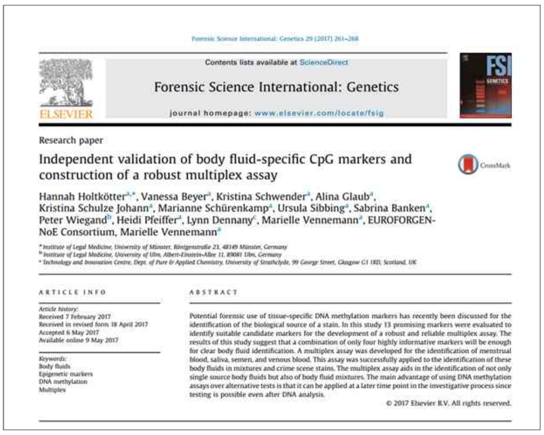
SNaPshot Multiplexes for Body Fluid Typing

forensic.yonsei.ac.kr/protocols.html



Independent Validation in a Different Population

- SE2, BL1, VF2 and SA1 were validated in Germans for the identification of semen, blood, menstrual blood and saliva, respectively, and were used for a multiplex assay construction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Research paper

Independent validation of body fluid-specific CpG markers and construction of a robust multiplex assay

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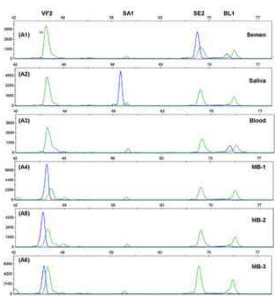
Multiplex

ABSTRACT

Potential forensic use of tissue-specific DNA methylation markers has recently been discussed for the identification of the biological source of a stain. In this study 13 promising markers were evaluated to identify suitable candidate markers for the development of a robust and reliable multiplex assay. The results of this study suggest that a combination of only four highly informative markers will be enough for clear body fluid identification. A multiplex assay was developed for the identification of menstrual blood, saliva, semen, and venous blood. This assay was successfully applied to the identification of these body fluids in mixtures and crime scene stains. The multiplex assay aids in the identification of not only single source body fluids but also of body fluid mixtures. The main advantage of using DNA methylation assays over alternative tests is that it can be applied at a later time point in the investigative process since testing is possible even after DNA analysis.



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)

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

Introducing DNA Methylation Profiling in Your Lab

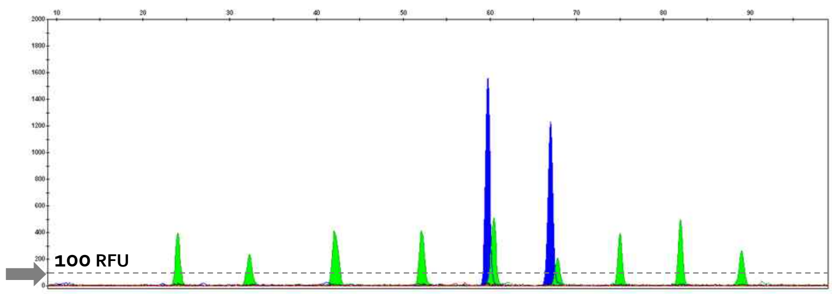
3. **Bisulfite conversion of genomic DNA**
   - For various body fluids: semen, blood, saliva, vaginal fluid and menstrual blood
   - Extract and quantify DNA
   - Select optimal input DNA amount (> 50 ng) and perform bisulfite conversion
   - Bisulfite converted DNA is present as a single strand
   - 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

4. Perform multiplex PCR followed by multiplex SBE (continued)

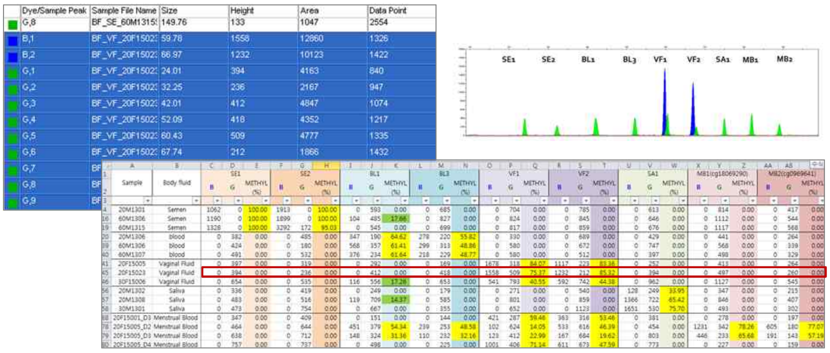
Interpretation

- 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

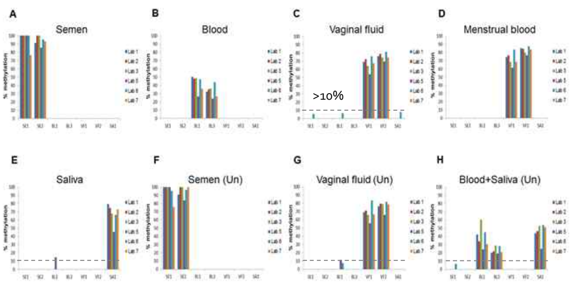
- Put peak height values into the excel sheet and calculate methylation percentages
  : 100*nucleotide G /(nucleotide G+A intensities)
- Determine analytical threshold for methylation percentage at each marker, e.g. 10%
- Compare with reference DNA methylation profiles to determine body fluid type



4. Perform multiplex PCR followed by multiplex SBE (continued)

Interpretation

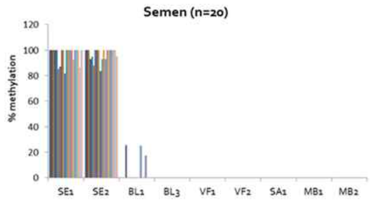
- Determine minimum threshold for positive methylation signal, e.g. 10%
- Compare with reference DNA methylation profiles to determine body fluid type



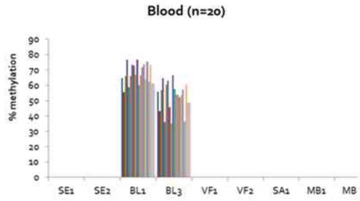
DNA methylation profiling results from 6 laboratories using bisulfite converted DNA from the same batch: a few unexpected signals with <10% methylation

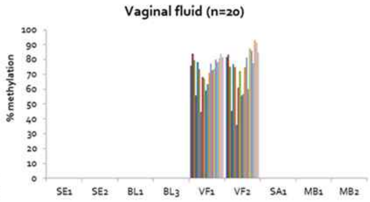
Interpretation

- Compare with reference DNA methylation profiles to determine body fluid type
  - Two markers positive for semen, blood or vaginal fluid → observed



- Only one marker positive for semen, blood or vaginal fluid
  → not reliable

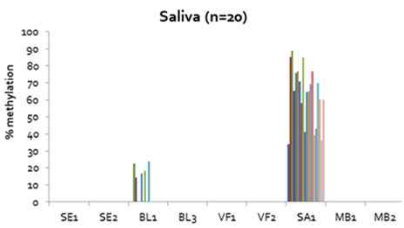


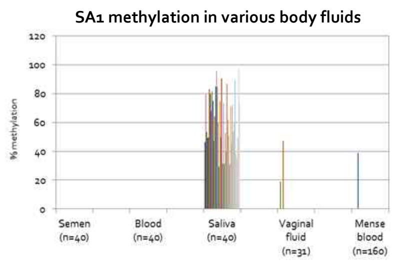


4. Perform multiplex PCR followed by multiplex SBE (continued)

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

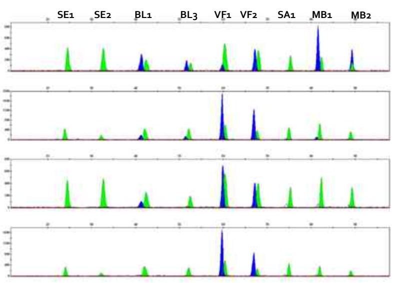


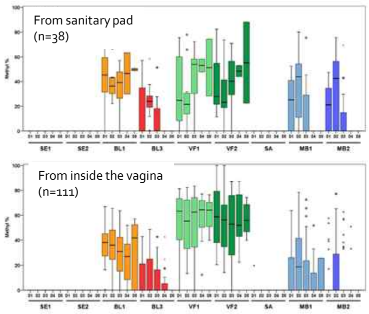


Interpretation

- 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

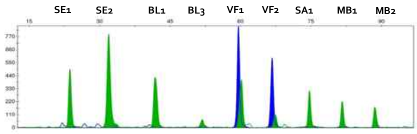
5. **Analyze more samples**
   - More body fluid samples (validation and establishment of interpretational guidelines)
   - Lower amount (< 50 ng of g DNA and < 10 ng of bisulfite converted DNA)
   - Mixed samples (Joined interpretation with STR and DNA methylation profiles)
6. **Develop reporting guidelines or formats**
7. **Establish Standard Operating Procedures**
8. **Inform your clients**

Examples of NFS DNA Methylation Profiling

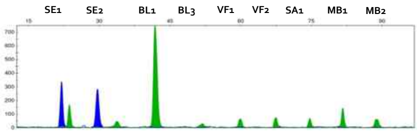
Case 1: Rape

- 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

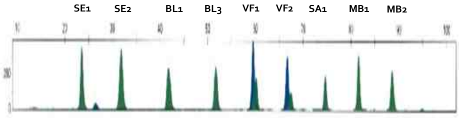


Examples of NFS DNA Methylation Profiling

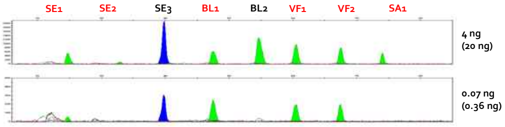
Case 2: Digital penetration on the public transportation

- 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

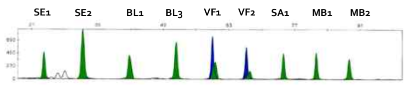


Examples of NFS DNA Methylation Profiling

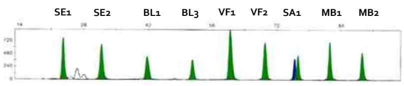
Case 3: Murder in 2004

- 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

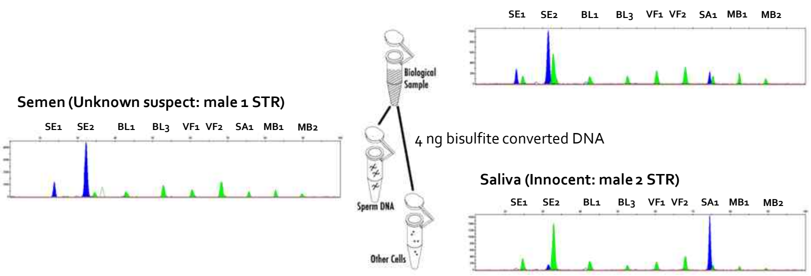


- The victim's body was damaged by cutting the left breast part and vagina with the knife
- DNA methylation profile was consistent with the scenario of the case
- DNA methylation profiling was successfully done with 13 year-old DNA samples

Examples of NFS DNA Methylation Profiling

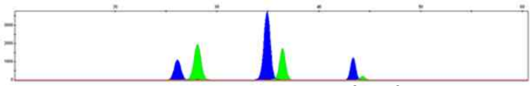
Case 4: Indecent exposure with no suspect

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



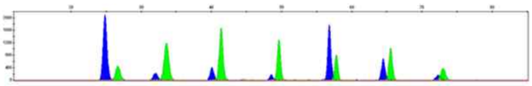
Examples of NFS DNA Methylation Profiling

Semen age prediction (ppt)



61 years old

Saliva age prediction (supernatant)



30 years old

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