DNA methylation-based body fluid typing

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Forensic Body Fluid Identification

Body fluid identification can provide information linking sample donors with actual criminal acts







Semen from rape kit?

Kayser M and de Kniff P, Nat Rev Genet (2011)

Body Fluids and Tissues at a Crime Scene

	Туре	Forensic relevance
Body fluids	Blood	Violence human-specific assay
body halds	Semen fertile	Sexual assault, confirmation sampled area
	Semen, sterile	Sexual assault, confirmation sampled area
	Saliva	Sexual assault e.g. licking, kissing or inoffensive stain
	Vaginal mucosa	Sexual assault, confirmation sampled area
	Menstrual secretion	Sexual assault or inoffensive alternative scenario
Touch	Skin	Confirmation sampled area
Other secretions	Expirated blood	Violence, confirmation bloodstain pattern analysis
	Nasal blood	Thump on the nose or inoffensive alternative scenario
	Nasal secretion	Inoffensive alternative scenario
	Sweat	Confirmation witness report, cross-reactivity?
	Urine	Confirmation sampled area, cross-reactivity?
	Tears	Possible inoffensive scenario if cross-reactive
	Breast milk	Possible inoffensive scenario if cross-reactive
	Vomit	Contains saliva and stomach content, inoffensive scenario
	Faeces	Anal sexual assault
Organs	Brain	Head injury
	Heart, lung	Chest injury
	Kidney, liver	Abdominal injury
	Skeletal muscle	Injury

Different Tissues and Cells



How Specialization Achieved



RNA Expression across Tissues

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RNA expression varies depending on the type of tissues, and may change with environment or physical state

Body Fluid Specific mRNA Markers

Blood	Semen	Saliva	Vaginal Secretions	Menstrual Blood	Skin	Housekeeping Genes	
ALAS2*	PRM1	HTN3*	MUC4	MMP7	LCE1C*	B2M*	
SPTB	PRM2*	STATH*	HBD1	MMP10*	LCE1D	UBC	
HMBS (PBGD)	MSP	PRB4	ESR1	MMP11	LCE2D	UCE	
CD3G	TGM4*	SPRR3	CYP2B7P1*	CK19	CCL27*	GAPDH	
HBB	PSA (KLK3)	SPRR1A	MYOZ1	PR	IL1F7	G6PDH	
CASP2	SEMG1	KRT4	FUT6	LEFTY2*	LOR	TEF	
AM1CA1	SEMG2	KRT6A	DKK4	MSX1	CDSN		
C1QR1		KRT13	SFTA2	SFRP4	KRT9		
ALOX5AP		MUC7	IL19*		1000)		
AQP9			L.cris	TECHNICAL NOTE	1999)		
C5R1			L.gas				
NCFS2			L.jen	Martin Bauer,' M.D.; Alexandra Kraus,' M.D.; and Dieter Patzelt,' M.D.			
MNDA				Detection of Enith	olial Colle i	n Dried Blood Stains	
ARHGAP26				by Reverse Trans	crintase-Po	lymerase Chain	
GYPA				Reaction*	onplase i o	lymerase enam	
ANK1*							
HBA							

*bacteria

*Ballantyne lab - most frequently used markers

Epigenetic Variation across Tissues

Epigenetic profiles are specific to tissue, age, and environmental factors



DNA methylation

Histone modification

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Unsupervised clustering of average beta
values in normal human tissues.
Christensen et al. PLoS Genet (2009)
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DNA Methylation-Based Body Fluid ID

ELSEVIER	Forens	Forensic Science International: Genetics 5 (2011) 517–524 Contents lists available at ScienceDirect ic Science International: Genetics al homepage: www.elsevier.com/locate/fsig	Not until 2011
DNA methyla Dan Frumkin ^{a,*} , ^a Nucleix Ltd., 27 Habarzel St ^b Institute of Investigative Ge A R T I C L E I N F O Article history: Received 21 June 2010 Received in revised form 18	Adam Wasserstro Adam Wasserstro , Tel Aviv 69710, Israel netics, Department of Forensie November 2010	rensic tissue identification m ^{a,*} , Bruce Budowle ^b , Ariane Davidson ^a and Investigative Genetics, University of North Texas Health Science Center, Ft. Worth, TX 76107, USA Int J Legal Med (2012) 126:55–62 DOI 10.1007/s00414-011-0569-2	
Accepted 6 December 2010		ORIGINAL ARTICLE Potential forensic application of DNA profiling to body fluid identification Hwan Young Lee • Myung Jin Park • Ajin Choi • Ja Hyun An • Woo Ick Yang • Kyoung-Jin Shin Received: 1 October 2010/Accepted: 23 March 2011/Published online: 6 April 2	A methylation

Forensic Body Fluid Typing

Marker type	Effect	Feature	
mRNA/miRNA	-	Additional procedures: extraction of RNA, DNase treatment and cDNA preparation	
	+	DNA extract is not consumed	
	-	Cannot be applied when only DNA extract remains in an old case	
	+	Mixture analysis possible since markers are present/absent to certain extent	
	-	Variable expression: different per marker, influenced by physiological factors	
	-	Background signals from spurious transcription	
mRNA	+	Multiple candidates per tissue can be readily obtained	
miRNA	+	miRNA molecules are <i>in vivo</i> protected against RNases	
	+	miRNAs can have high expression levels	
DNA	-	Consumes DNA extract (and bisulphite-based assays use large quantities of DNA)	
methylation	+	Can be applied when only DNA extract remains in an old case	
	-	Often based on differential methylation level: not absence/presence but more/less	Dopondo
	-	Mixture analysis may be difficult as methylation level will dilute	
	-	Methylation show inter-person variation and is influenced by age and environment	J On Warkers:
Microbial	+	Both RNA and DNA-based assays can be used	
	+	Mixture analysis possible since markers are present/absent to certain extent	
	-	Microbiome various between and within persons, not always a core microbiome	
	-	Microflora may change with disease (e.g. vaginosis)	
	-	Microbes may not be human-specific and shared by other mammals	
	-	Microbiome may transfer to proximate body sites or locations/surfaces in contact	

What is an Epigenome?



DNA methylation

Histone modification



Gene Control by the Epigenome



DNA Methylation

DNA methylation is the addition of a methyl group to the DNA base cytosine followed by a guanine (5' CpG 3')



5 ' CAGCTCTTCAGGGGGCGAAGAGCAGGAACCGGAGCTACCTGAAGAGCGCG GCTTTCCCCCGGCTCTTCGGGGCTGTGGAGGCTGCGGGGCTCGCGCGCTTGTTCCG GGACAGGGGCGTGGCGCCTGCT 3 '

Analysis of DNA Methylation



Human Methylaion450 BeadChip Array



- 12 blood, 12 saliva, 12 semen, 3 vaginal fluid and 3 menstrual blood (GSE59505)
- Candidate marker selection
 - Body fluid-specific hyper or hypo-methylation with a low standard deviation in the same type of body fluid
 - Complete methylation or non-methylation in other types of body fluids

Comparison of Body Fluids

	Comparison ^a	Cut-off ^b	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

^aBL, SA, SE, MB and VF represent blood, saliva, semen, menstrual blood and vaginal fluid, respectively ^bAbs (delta_mean) represents the discrepancy in the mean values of average β-scores between a certain type of body fluid and the other type of body fluids

Selection of Body Fluid-Specific CpGs

			Mean beta values \pm 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	$\textbf{0.40} \pm \textbf{0.05}$	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	



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

Validation of Body Fluid-Specificity

Monoplex SBE reaction using bisulfite-converted DNA

Blood

Semen



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

Validation of Body Fluid-Specificity



Multiplex SBE for 7 CpG Markers



Jung SE et al., Electrophoresis, in press

Body Fluid Specificity of Selected CpGs







VF2

SA1



Lee HY et al., Forensic Sci Int Genet (2015), Lee HY et al., Forensic Sci Int Genet (2016)

A Collaborative Exercise by 7 Labs

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Electrophoresis 2016, 00, 1-8

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Research Article

A collaborative exercise on DNA methylation based body fluid typing

A collaborative exercise on DNA methylation based body fluid identification was conducted by seven laboratories. For this project, a multiplex methylation SNaPshot reaction composed of seven CpG markers was used for the identification of four body fluids, including blood, saliva, semen, and vaginal fluid. A total of 30 specimens were prepared and distributed to participating laboratories after thorough testing. The required experiments included four increasingly complex tasks: (1) CE of a purified single-base extension reaction product, (2) multiplex PCR of bisulfite-modified DNA, (3) bisulfite conversion of genomic DNA, and (4) extraction of genomic DNA from body fluid samples. In tasks 2, 3 and 4, one or more mixtures were analyzed, and specimens containing both known and unknown body fluid sources were used. Six of the laboratories generated consistent body fluid typing results for specimens of bisulfite-converted DNA and genomic DNA. One laboratory failed to set up appropriate conditions for capillary analysis of reference singlebase extension products. In general, variation in the values obtained for DNA methylation analysis between laboratories increased with the complexity of the required experiments. However, all laboratories concurred on the interpretation of the DNA methylation profiles produced. Although the establishment of interpretational guidelines on DNA methylation based body fluid identification has yet to be performed, this study supports the addition of DNA methylation profiling to forensic body fluid typing.

Keywords:

Body fluid identification / Collaborative exercise / DNA methylation / Forensic science / Methylation SNaPshot DOI 10.1002/elps.201600256

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Overview of the samples and experiments

Part	Samples	Required experiments
1	Purified SBE(single-base extension) reaction product 6 samples : for each cell type, single source samples, cell type indicated	Capillary electrophoresis
2	 Bisulfite converted DNA 5 samples : for each cell type, single source samples, cell type indicated 2 samples : single source samples, unspecified cell type 1 sample : mixture of two body fluids, unspecified cell type 	Multiplex PCR Multiplex SBE Capillary electrophoresis
3	 Genomic DNA 5 samples : for each cell type, single source samples, cell type indicated 2 samples : single source samples, unspecified cell type 1 sample : mixture of two body fluids, unspecified cell type 	Bisulfite conversion Multiplex PCR Multiplex SBE Capillary electrophoresis
4	 Body fluid swabs 4 samples : for each cell type, single source samples, cell type indicated 1 sample : mixture of two body fluids, cell type indicated 2 samples : single source samples, unspecified cell type 1 sample : mixture of body fluids, unspecified cell type 	DNA extraction DNA quantification Bisulfite conversion Multiplex PCR Multiplex SBE Capillary electrophoresis

CE of a Purified SBE Product



Multiplex on Bisulfite Converted DNA



*(Un) indicates samples provided with unspecified body fluid origin

Bisulfite Conversion of Genomic DNA



*(Un) indicates samples provided with unspecified body fluid origin

DNA Extraction from Body Fluid Samples



*(Un) indicates samples provided with unspecified body fluid origin

Body Fluid Swab Test (Lab 2)





*Data interpretation Swab 1: SE Swab 2: MB

Swab 3: SE + BL +SA



Comparison of VF and MB

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 ^b	No. of CpGs
3	MB day 1 vs. VF	Abs (delta_mean) ≥ 0.3, <i>P</i> < 0.05, sd < 0.1	165
	MB day 2 vs. VF	Abs (delta_mean) ≥ 0.3, <i>P</i> < 0.05, sd < 0.1	31
	MB day 3 vs. VF	Abs (delta_mean) ≥ 0.2, <i>P</i> < 0.05, sd < 0.1	15

				Mean beta	a values \pm SD ^a				Geno	ome 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	$\textbf{0.02} \pm \textbf{0.01}$	$\textbf{0.08} \pm \textbf{0.03}$	$\textbf{0.06} \pm \textbf{0.02}$	$\textbf{0.23} \pm \textbf{0.08}$	$\textbf{0.05} \pm \textbf{0.01}$	$\textbf{0.38} \pm \textbf{0.11}$	$\textbf{0.36} \pm \textbf{0.06}$	$\textbf{0.31} \pm \textbf{0.12}$	2	177029608	HOXD3
cg02009088	0.03 ± 0.01	$\textbf{0.02} \pm \textbf{0.01}$	$\textbf{0.40} \pm \textbf{0.21}$	$\textbf{0.70} \pm \textbf{0.03}$	0.02 ± 0.01	$\textbf{0.44} \pm \textbf{0.12}$	$\textbf{0.41} \pm \textbf{0.09}$	$\textbf{0.35} \pm \textbf{0.14}$	5	139228153	NRG2
cg14486338	0.02 ± 0.01	$\textbf{0.15} \pm \textbf{0.04}$	$\textbf{0.11} \pm \textbf{0.03}$	$\textbf{0.17} \pm \textbf{0.10}$	$\textbf{0.07} \pm \textbf{0.02}$	$\textbf{0.41} \pm \textbf{0.11}$	$\textbf{0.38} \pm \textbf{0.05}$	$\textbf{0.34} \pm \textbf{0.20}$	8	99440279	KCNS2
cg19893585	$\textbf{0.80} \pm \textbf{0.08}$	$\textbf{0.38} \pm \textbf{0.04}$	0.06 ± 0.04	$\textbf{0.05} \pm \textbf{0.03}$	0.09 ± 0.04	$\textbf{0.46} \pm \textbf{0.13}$	$\textbf{0.36} \pm \textbf{0.04}$	$\textbf{0.34} \pm \textbf{0.16}$	8	145025064	PLEC1
cg17124583	0.03 ± 0.01	$\textbf{0.09} \pm \textbf{0.02}$	$\textbf{0.37} \pm \textbf{0.13}$	$\textbf{0.05} \pm \textbf{0.02}$	0.03 ± 0.01	$\textbf{0.45} \pm \textbf{0.09}$	$\textbf{0.38} \pm \textbf{0.06}$	$\textbf{0.41} \pm \textbf{0.18}$	10	8097641	GATA3
cg04255276	0.02 ± 0.01	$\textbf{0.07} \pm \textbf{0.04}$	0.04 ± 0.03	$\textbf{0.05} \pm \textbf{0.02}$	$\textbf{0.09} \pm \textbf{0.07}$	$\textbf{0.41} \pm \textbf{0.11}$	$\textbf{0.36} \pm \textbf{0.07}$	$\textbf{0.28} \pm \textbf{0.13}$	11	65314021	LTBP3
cg18023065	$\textbf{0.15} \pm \textbf{0.10}$	$\textbf{0.39} \pm \textbf{0.03}$	$\textbf{0.10} \pm \textbf{0.06}$	$\textbf{0.20} \pm \textbf{0.05}$	0.06 ± 0.02	$\textbf{0.48} \pm \textbf{0.13}$	$\textbf{0.43} \pm \textbf{0.03}$	$\textbf{0.40} \pm \textbf{0.19}$	11	94278603	FUT4
cg09696411	0.01 ± 0.00	$\textbf{0.02} \pm \textbf{0.01}$	0.01 ± 0.00	$\textbf{0.05} \pm \textbf{0.02}$	0.01 ± 0.00	$\textbf{0.41} \pm \textbf{0.10}$	$\textbf{0.35} \pm \textbf{0.06}$	$\textbf{0.28} \pm \textbf{0.14}$	12	58013517	SLC26A10
cg18069290	$\textbf{0.01} \pm \textbf{0.01}$	$\textbf{0.02} \pm \textbf{0.01}$	$\textbf{0.02} \pm \textbf{0.01}$	$\textbf{0.03} \pm \textbf{0.01}$	0.02 ± 0.00	$\textbf{0.35} \pm \textbf{0.12}$	$\textbf{0.32} \pm \textbf{0.10}$	$\textbf{0.22} \pm \textbf{0.13}$	12	58013539	SLC26A10
cg16567290	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	$\textbf{0.13} \pm \textbf{0.05}$	0.04 ± 0.01	$\textbf{0.46} \pm \textbf{0.11}$	$\textbf{0.44} \pm \textbf{0.05}$	$\textbf{0.37} \pm \textbf{0.12}$	12	58013569	SLC26A10
cg20985399	$\textbf{0.13} \pm \textbf{0.12}$	$\textbf{0.15} \pm \textbf{0.03}$	0.07 ± 0.03	$\textbf{0.36} \pm \textbf{0.05}$	0.07 ± 0.02	$\textbf{0.50} \pm \textbf{0.12}$	$\textbf{0.50} \pm \textbf{0.04}$	$\textbf{0.41} \pm \textbf{0.18}$	15	65689263	IGDCC4
cg22320365	$\textbf{0.18} \pm \textbf{0.14}$	$\textbf{0.07} \pm \textbf{0.02}$	$\textbf{0.09} \pm \textbf{0.06}$	$\textbf{0.26} \pm \textbf{0.07}$	0.06 ± 0.02	$\textbf{0.37} \pm \textbf{0.12}$	$\textbf{0.36} \pm \textbf{0.04}$	$\textbf{0.33} \pm \textbf{0.17}$	17	36718198	SRCIN1
cg12798338	0.06 ± 0.02	$\textbf{0.02} \pm \textbf{0.00}$	0.03 ± 0.01	$\textbf{0.22} \pm \textbf{0.06}$	0.03 ± 0.01	$\textbf{0.37} \pm \textbf{0.11}$	$\textbf{0.34} \pm \textbf{0.08}$	$\textbf{0.27} \pm \textbf{0.14}$	17	76128683	TMC8
cg01032675	$\textbf{0.81} \pm \textbf{0.10}$	$\textbf{0.19} \pm \textbf{0.07}$	$\textbf{0.03} \pm \textbf{0.02}$	$\textbf{0.34} \pm \textbf{0.06}$	0.04 ± 0.01	$\textbf{0.45} \pm \textbf{0.12}$	$\textbf{0.43} \pm \textbf{0.07}$	$\textbf{0.39} \pm \textbf{0.21}$	19	3136430	GNA15
cg16606773	$\textbf{0.07} \pm \textbf{0.06}$	$\textbf{0.44} \pm \textbf{0.04}$	$\textbf{0.06} \pm \textbf{0.05}$	$\textbf{0.08} \pm \textbf{0.03}$	0.06 ± 0.01	$\textbf{0.38} \pm \textbf{0.06}$	$\textbf{0.36} \pm \textbf{0.06}$	$\textbf{0.23} \pm \textbf{0.12}$	20	19955806	RIN2

Multiplex SBE for 9 CpG Markers



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

Multiplex SBE for 9 CpG Markers





*Menstrual blood was collected from a sanitary pad (method 1) or from the inside of the vagina (method 2) using sterilized cotton swabs.

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

DNA Methylation in Menstrual Bloods

Methylation profiles vary with menstrual cycle and sample collection methods



*Menstrual blood was collected from a sanitary pad (method 1) or from the inside of the vagina (method 2) using sterilized cotton swabs. D1, D2, D3, D4 and D5 represent the first, second, third, fourth, and fifth day of menstrual bleeding, respectively.

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

Casework Samples: Mixture Analysis



Current Forensic Body Fluid Typing

Marker type	Effect	Feature	
mRNA/miRNA	_	Additional procedures: extraction of RNA, DNase treatment and cDNA preparation	
	+	DNA extract is not consumed	
	-	Cannot be applied when only DNA extract remains in an old case	
	+	Mixture analysis possible since markers are present/absent to certain extent	
	-	Variable expression: different per marker, influenced by physiological factors	
	-	Background signals from spurious transcription	
mRNA	+	Multiple candidates per tissue can be readily obtained	
miRNA	+	miRNA molecules are <i>in vivo</i> protected against RNases	
	+	miRNAs can have high expression levels	
DNA	-	Consumes DNA extract (and bisulphite-based assays use large quantities of DNA)	
methylation	+	Can be applied when only DNA extract remains in an old case	
	-	Often based on differential methylation level: not absence/presence but more/less	ו
	-	Mixture analysis may be difficult as methylation level will dilute	- Resolve
	-	Methylation show inter-person variation and is influenced by age and environment –	J
Microbial	+	Both RNA and DNA-based assays can be used	
	+	Mixture analysis possible since markers are present/absent to certain extent	
	-	Microbiome various between and within persons, not always a core microbiome	
	-	Microflora may change with disease (e.g. vaginosis)	
	-	Microbes may not be human-specific and shared by other mammals	
	-	Microbiome may transfer to proximate body sites or locations/surfaces in contact	

Age Prediction with DNA Methylation

Blood

Predicted age



DNA Methylation Profiling in Forensics

- DNA methylation has only recently come into focus in the forensic field, but its applicability is being highly estimated among an increasing number of forensic investigators
- □ Just like epigenetics to genetics, DNA methylation analyses are expected to add more informative layers to the forensic genetic analyses of the evidence materials

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