

Novel identification of biofluids using a multiplex methylation-specific PCR combined with single-base extension system

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Abstract

Purpose Knowledge of the composition of complex body fluid mixtures may aid forensic investigations greatly. However, many of the traditional tests are presumptive in nature and can lead to ambiguous results. The aim of this study is to establish a reliable method to identify various biofluids via analysis of their DNA methylation profiles.

Methods A total of eight biofluid-specific methylated markers for saliva, venous blood, vaginal fluids, and semen were isolated from the open database of Infinium HumanMethylation450 BeadChip. These biofluid-specific markers, a control marker to confirm bisulfite conversion,

and a gender marker, were combined into a 10-plex methylation-specific PCR single-base-extension (MSP-SBE) system.

Results Analysis of 65 DNA samples isolated from venous blood, semen, vaginal fluid, saliva, and menstrual blood that had been treated with bisulfite, resulted in all eight markers detecting the body fluid to which they were designed. Unambiguous body fluid identification occurred from both single sources of body fluids and complex mixtures. A threshold was devised for each marker to minimize the chance of a false inclusion. The efficacy of the assay and application to forensic practice was demonstrated using five non-probative samples from real alleged sexual assault cases. The system unambiguously determined the biofluid types for the non-probative forensic samples that previously resulted in inconclusive or conflicting results using traditional tests.

Conclusions The results demonstrated the 10-plex MSP-SBE system established in this study is both sensitive and specific when applied to body fluid identification and can be readily adopted into forensic practice.

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Keywords DNA methylation · Body fluids · Mixtures · Biofluid identification · Methylation-specific PCR (MSP) · Single-base-extension (SBE)

Introduction

Knowledge of the composition of complex body fluid mixtures may aid forensic investigations greatly. This is particularly the case in instances of alleged sexual assaults. Presumptive tests for the presence of a body fluid focus on identifying specific compounds, such as proteins, by using chemical and catalytic tests [1]. However, in comparison to

DNA, proteins and their associated activities are more prone to degradation and inactivity, affecting the potential for obtaining a positive result from old and compromised samples. Moreover, chemical and catalytic assays usually suffer from ambiguous results due to poor sensitivity or lack of specificity. Although immunological assays have been exploited as confirmatory tests with a few commercial immunochromatographic kits available, their applications are still limited in some situations [2, 3]. Examples include the semenogelin kit, which is highly specific to human semen but when it was compared to the p30 test, only exhibited a moderately high degree of sensitivity for fresh diluted semen and a very low sensitivity for frozen samples and long-term postcoital samples [2]. The p30 test, however, has been reported to suffer from false indications of semen in female urine as well as in male urine and blood samples. This is especially the case for males suffering from prostate cancer for which the p30 level was elevated [3]. Recently emerging techniques have been developed that examine gene expression through mRNA [4–8], miRNA [9–12], and also DNA methylation [13]. Issues with RNA degradation and the consumption of sample can remain when either mRNA or miRNA is analyzed, and this may affect the success of subsequent STR typing [14].

DNA methylation, occurring at the 5' position of cytosine within a CpG dinucleotide, is known to play a role in cell differentiation [15–17]. An increase in the methylation status of specific tissues has been observed previously [18–21], leading to a focus on identifying the cellular origins of biofluids for forensic purposes. There are two main approaches used in these studies, one is based on bisulfite conversion and the other is the use of a methylation-sensitive restriction enzyme.

Bisulfite conversion deaminates cytosines, changing a cytosine to uracil, while 5-methylcytosine resists this conversion [22]. Bases that are either converted or not converted can be detected using pyrosequencing [23, 24] or single-base-extension (SBE). SBE uses a primer that anneals exactly one base pair upstream or downstream of the cytosine within the targeted CpG site after amplification of bisulfite-converted DNA [25, 26]. The tissue type present in a sample is determined by evaluating methylation ratios using biofluid-specific methylated markers. The ratio could, however, be affected when the samples are mixtures of different body fluids. To overcome this limitation, it is essential that the target shows only a complete “on/off” methylation status, being almost completely methylated in only one biofluid (>90 %) and unmethylated in the others (<10 %) or vice versa [27].

Methylation-sensitive restriction enzyme (MSRE)-PCR is based on digestion of genomic DNA with a methylation-sensitive-restriction enzyme and then amplification of DNA fragments covering the enzyme recognition sites

[28]. An in-house MATLAB software was used to search for tissue-specific methylated markers with *HhaI* recognition sequences [29] and a multiplex PCR kit including the selected markers was developed to detect semen using MSRE-PCR [30]. However, these studies only focused on identifying semen and no other body fluid.

A consequence of genome-wide DNA methylation analysis is that extensive discovery of biofluid-specific methylated markers becomes feasible. The Infinium HumanMethylation450 BeadChip Kit (Illumina, San Diego, CA, USA) is array-based and allows assessment of the methylation status of more than 480,000 CpGs distributed over the whole genome [31]. It has been recently employed to analyze methylome data from different biofluids for forensic purposes [32]. A multiplex system containing biofluid-specific methylated markers, obtained from data generated by this analysis, was established to identify venous blood, saliva, semen, vaginal fluid, and menstrual blood in one reaction. Additionally DNA methylation profiles were produced for aged or mixed samples [33].

We report on the effective identification of multiple biofluids within both single source and mixtures of body fluids to aid in forensic investigations. The biofluid-specific methylated markers for saliva, vaginal fluid, venous blood, and semen used in this study were from the open database of the Infinium HumanMethylation450 BeadChip. We report on an effective identification system using these markers, based on a multiplex MSP-SBE combined test. Evaluation using non-probative forensic samples is reported as part of this comprehensive study.

Materials and methods

Sample collection

A total of 65 biofluid samples (10 semen, 15 saliva, 15 venous blood, 15 menstrual blood, and 10 vaginal fluids) were collected from 25 volunteers (10 males and 15 females) after informed consent using procedures approved by the Institutional Review Board (IRB) of Tao-Yuan General Hospital in Taiwan (IRB No. TYGH102011).

To evaluate the potential of the system established in this study for forensic applications, five non-probative forensic samples were collected from the Criminal Investigation Bureau in Taiwan including: 1 internal vaginal swab, 1 external anal swab, 2 sections of fabric, and 1 of tissue paper, all suspected to contain the semen of the alleged offenders. A part of each sample (approximately 5 mm²) was removed for the detection of blood using the Kastle–Meyer method, and the remainder was placed into a 1.5 mL tube containing 1000 µL PBS. This was followed

by agitation for 1 h at room temperature. The samples were centrifuged at 14,000 rpm for 3 min after which the supernatant was immunologically tested using SERATEC[®] PSA SEMIQUANT (SERATEC, Göttingen, Germany). The pellet was re-suspended in 100 μ L PBS, from which 10 μ L was used for Christmas tree staining [34] and the remaining 90 μ L of re-suspended pellet was used for DNA isolation.

DNA isolation and quantification

Genomic DNA was isolated from the collected samples using a Qiagen Mini kit (Qiagen, CA, USA) following the manufacturer's protocol for body fluids or tissues. Human total DNA and male DNA were quantified by using a Quantifiler[®] Human DNA quantification kit (Life Technologies, NY, USA) and a Quantifiler[®] Y Human Male DNA Quantification Kit (Life Technologies), respectively using a 7300 Real-Time PCR machine (Life Technologies).

Bisulfite conversion

The isolated genomic DNA was converted with bisulfite using a Zymo EZ DNA Methylation Kit (Zymo Research, CA, USA) according to the manufacturer's protocol. The bisulfite-converted DNA was quantified by using a Nanodrop[™] 2000 spectrophotometer (Thermo Scientific, Wilmington, NC, USA).

Marker selection

The markers used in this study were selected from the datasets of the Infinium HumanMethylation450 BeadChip Kit using the GPL13534 platform in the NCBI Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>). There were five methylation datasets including: 20 cervical tissues from GSE46306 [35]; 17 and 5 saliva samples from GSE39560 and GSE48472, respectively [36, 37]; 5 and 6 venous blood samples from GSE48472 and GSE35069, respectively [37, 38]; and 8 semen samples from GSE47627 [39], from which abnormal samples were excluded and only one of each monozygotic pair was selected. For each CpG locus in the Infinium HumanMethylation450 BeadChip Kit, mean and standard deviation of the beta-values (the calibration ratio of methylation) for each biofluid were calculated using Microsoft Office Excel 2007. The Student's *t* test was used to evaluate the significance of variation between the 4 different biofluids. Markers were selected with the criteria of a high beta-value for the target biofluid and a near zero value for any of the others, and a *p* value <0.05 was used between the target biofluid and the others.

A housekeeping gene, Beta-actin (ACTB), was used as an internal control to verify whether the bisulfite conversion was complete. A pair of primers was designed containing degenerative sequences, allowing them to complement to both thymine, which indicated bisulfite conversion from cytosine, and cytosine to indicate no conversion due to incomplete bisulfite treatment. No CpGs should be within the annealing site of the primer to prevent any such methylation influencing the results of bisulfite treatment. The status of whether the bisulfite conversion is complete can be determined by the presence or absence of a cytosine in the amplicon as this should be converted to thymine when bisulfite conversion is complete.

From the sequence alignment of AMEL (accession nos. NC_000023.11 and NC_000024.10 in GenBank) on chromosomes X and Y, a pair of primers was designed to amplify a fragment including a single nucleotide polymorphism (SNP) to differentiate between the chromosomes.

Sequences of the primers used in this study are shown in Online Resource 1 and 2.

Methylation-specific PCR (MSP)

Each of the selected candidate markers was preliminarily assessed by two independent methylation-specific PCR (MSP) amplifications, named M and U reactions, on the collected samples. A common forward primer was used in both reactions however, the reverse primer in the M reaction was the complement of the methylated cytosine of CpG after bisulfite conversion, and in the U reaction was the complement of thymine if converted from un-methylated cytosine of CpG. The specificity of the reverse primers in the M and U reactions allowed selective amplification of only the methylated and un-methylated CpG fragments, respectively. The primers used in MSP reactions are shown in Online Resource 1. The MSP reactions were performed in a total volume of 10 μ L containing approximately 2 ng of bisulfite-converted DNA, 0.5 unit of EpiTaq[™] HS (Takara Bio Inc., Shiga, Japan), 1 μ L of 10 \times PCR buffer (250 μ M Mg²⁺, 300 μ M each dNTP), and 300 μ M of each forward and reverse primers. The reaction was conducted in a MJ Mini[™] Thermal Cycler (Bio-Rad, CA, USA) at 95 °C for 10 min, then 35 cycles of 94 °C for 20 s, 61 °C for 2 min and 72 °C for 30 s, with a final extension at 72 °C for 5 min. The amplified products were separated on a 2 % agarose gel.

A 10-plex MSP including 8 biofluid-specific methylated markers, a bisulfite conversion control (ACTB), and a gender marker (Amelogenin) was performed in a total volume of 10 μ L. The MSP conditions were as above with exception of the primer concentrations. The optimal concentration for each primer of the 10-plex system is shown

in Online Resource 1. After the amplification, the excess dNTPs and primers were dephosphorylated and digested by using 3 units of Shrimp Alkaline Phosphatase and 1 unit of Exonuclease I (New England Biolabs, MA, USA) with incubation at 37 °C for 60 min. Samples were heat inactivated at 80 °C for 20 min.

Single base extension (SBE)

SBE was performed on the purified 10-plex MSP products by using the SNaPshot™ Kit (Life Technologies) in a total volume of 10 µL containing 5 µL of SNaPshot Multiplex Ready Reaction Mix, 4 µL of purified 10-plex MSP product and 300 µM of each the 10 SBE primers (Online Resource 2). The SBE reaction was conducted in a MJ Mini™ Thermal Cycler under the following conditions: 25 cycles at 96 °C for 10 s, 52 °C for 5 s, and 60 °C for 30 s. The unincorporated ddNTPs were dephosphorylated using 1 unit of Shrimp Alkaline Phosphatase by incubating at 37 °C for 60 min followed by heat inactivation at 65 °C for 5 min. The fluorescently labeled SBE products were separated on an ABI PRISM 310 Genetic Analyzer (Life Technologies) using LIZ 120 (Life Technologies) as the internal standard. Peak Scanner™ Software v1.0 (Life Technologies) was used to analyze the data.

The methylation status of the eight biofluid-specific methylated markers was determined by the addition of labeled ddCTP for “methylated” cytosine and labeled ddTTP for thymine converted from “un-methylated” cytosine on the forward strand, and labeled ddGTP and ddATP complementary to either the “methylated” cytosine or “un-methylated” thymine on the reverse strand. For ACTB, the complete conversion of cytosine was determined by the addition of ddATP, as this is complementary to thymine that is generated if a cytosine has been converted by bisulfite. Alternatively, it would be labeled with ddGTP, being the complement of the original cytosine, if the bisulfite conversion was not complete. Gender was determined by addition of labeled ddCTP and ddTTP for detection of the Amelogenin SNP on either the X or Y chromosome, respectively. To separate all the SBE products by capillary electrophoresis, different lengths of poly A were added to the 5' end of SBE primers (Online Resource 2).

Statistical analysis

To evaluate the methylation status, a value was termed “methylation indicative value” (abbreviated as MIV) and defined by the equation “ $M/(M + U)$,” in which M and U indicated the value of peak height of methylated (M) and un-methylated (U) signals on the electropherograms

obtained in this study. The mean and standard deviation of MIVs for each biofluid were calculated using Microsoft Office Excel 2007.

Mixture tests

To test the applicability of this system to the identification of biofluid mixtures encountered frequently in forensic investigations bisulfite-converted DNAs from vaginal fluid, blood, and saliva of one female were individually mixed with semen DNA from a male in ratios of 9:1, 1:1, and 1:9. Furthermore, DNA from the above four biofluids was mixed equally (1:1:1:1). To test the influence of incomplete bisulfite conversion, the bisulfite-converted DNA and original DNA from the same biofluid sample were also mixed in a ratio of 1:9.

Results

Marker selection

To select biofluid-specific methylated markers for vaginal fluid, saliva, venous blood, and semen, the public datasets of the Infinium HumanMethylation450 BeadChip from the GPL 13534 platform in GEO (Gene Expression Omnibus in NCBI) were searched. Following the marker selection criteria and taking into consideration the CpG density in the sequence and compatibility of primers, two markers for each biofluid were selected. This resulted in 8 markers with high beta-values in their respective target biofluid and near zero in the others selected for this study (Table 1).

Preliminary MSP amplification

Each of the eight selected markers was assessed by two independent MSP reactions, the methylated (M) and un-methylated (U) reactions. The M reaction was amplified using a forward primer (F) and a reverse primer complementary to cytosine from methylated cytosine after bisulfite conversion (RM). The U reaction was amplified using the same forward primer (F) and a reverse primer complementary to thymine bisulfite converted from un-methylated cytosine (RU). Bisulfite-converted DNA from one sample of each biofluid was used in the preliminary MSP reactions (Online Resource 3). The bands generated by the M reactions were only observed in the respective methylated specific biofluids, such as vaginal fluid (VG-A & VG-B), saliva (SA-A & SA-B), blood (BL-A & BL-B), and semen (SE-A & SE-B), except that faint bands were observed in M reactions of venous blood for the vaginal fluid markers.

Table 1 Mean beta-values for selected markers in different body fluids or tissues from open database

Marker name	CpG ID ^a	Gene ^b	Mean \pm SD (%) ^c			
			Cervical tissue (n = 20)	Saliva (n = 22)	Venous Blood (n = 11)	Sperm (n = 8)
VG-A	cg25416153	–	35.6 \pm 15.7	3.4 \pm 1.4	5.0 \pm 1.9	1.3 \pm 0.5
VG-B	cg09765089	–	39.6 \pm 13.8	5.1 \pm 2.5	5.7 \pm 2.3	1.3 \pm 0.4
SA-A	cg09107912	FNDC1	2.9 \pm 1.4	22.9 \pm 10.6	3.8 \pm 0.9	1.3 \pm 0.2
SA-B	cg16732616	DMRTA2	2.2 \pm 4.2	29.2 \pm 9.5	1.3 \pm 0.8	0.2 \pm 0.3
BL-A	cg24124443	BTBD11	4.2 \pm 2.7	2.1 \pm 1.6	18.9 \pm 4.3	0.3 \pm 0.3
BL-B	cg01607849	TPI1	4.1 \pm 1.7	3.6 \pm 1.2	13.8 \pm 1.8	1.6 \pm 0.3
SE-A	cg05261336	–	0.5 \pm 0.3	1.3 \pm 0.7	1.2 \pm 0.5	93.1 \pm 4.8
SE-B	cg17610929	ACCN4	0.6 \pm 0.8	1.0 \pm 1.1	1.2 \pm 0.7	97.9 \pm 1.7

VG vaginal, SA saliva, BL venous blood, SE semen

^a CpG ID indicates the ID in the Illumina HumanMethylation450 bead array

^b The gene where the CpG was observed; the symbol of “–” represents the CpG was not observed in any gene

^c Bold values showed the top two mean beta-values of the markers for the specific biofluids or tissues

Multiplex MSP-SBE

The eight biofluid-specific methylated markers, a control for bisulfite conversion (ACTB), and a gender marker (AMEL) were combined into a 10-plex MSP reaction. For each of the eight body fluid markers, the forward primer (F) and both reverse primers (RM and RU) were used in the assay. To emphasize the signals of methylated DNA, the reverse primers RM were adjusted to a concentration higher than the RU primers for some markers (Online Resource 1). Samples treated with bisulfite comprising 10 vaginal fluids, 15 saliva, 15 venous bloods, 10 semen, and 15 menstrual bloods, in addition to one untreated sample of semen DNA (no bisulfite conversion), were analyzed by this 10-plex MSP-SBE system. One example for each biofluid is shown in Fig. 1. A peak at the expected position for the ACTB product (P) was observed for the converted DNA from all biofluids confirming the complete conversion. Only the untreated semen sample generated an N peak as expected if no conversion had occurred. If partial bisulfite conversion of the DNA had occurred then both the P and N peaks would exist.

An obvious peak (M) was only generated at the expected position for each of the tested biofluids: vaginal fluid (VG-A and VG-B); saliva (SA-A and SA-B); venous blood (BL-A and BL-B); and semen (SE-A and SE-B). The peak (M) was observed in VG-A, VG-B, BL-A, and BL-B representing menstrual blood samples (Fig. 1e). It was therefore deduced that the menstrual blood was composed of both blood and vaginal fluid.

Statistical analysis

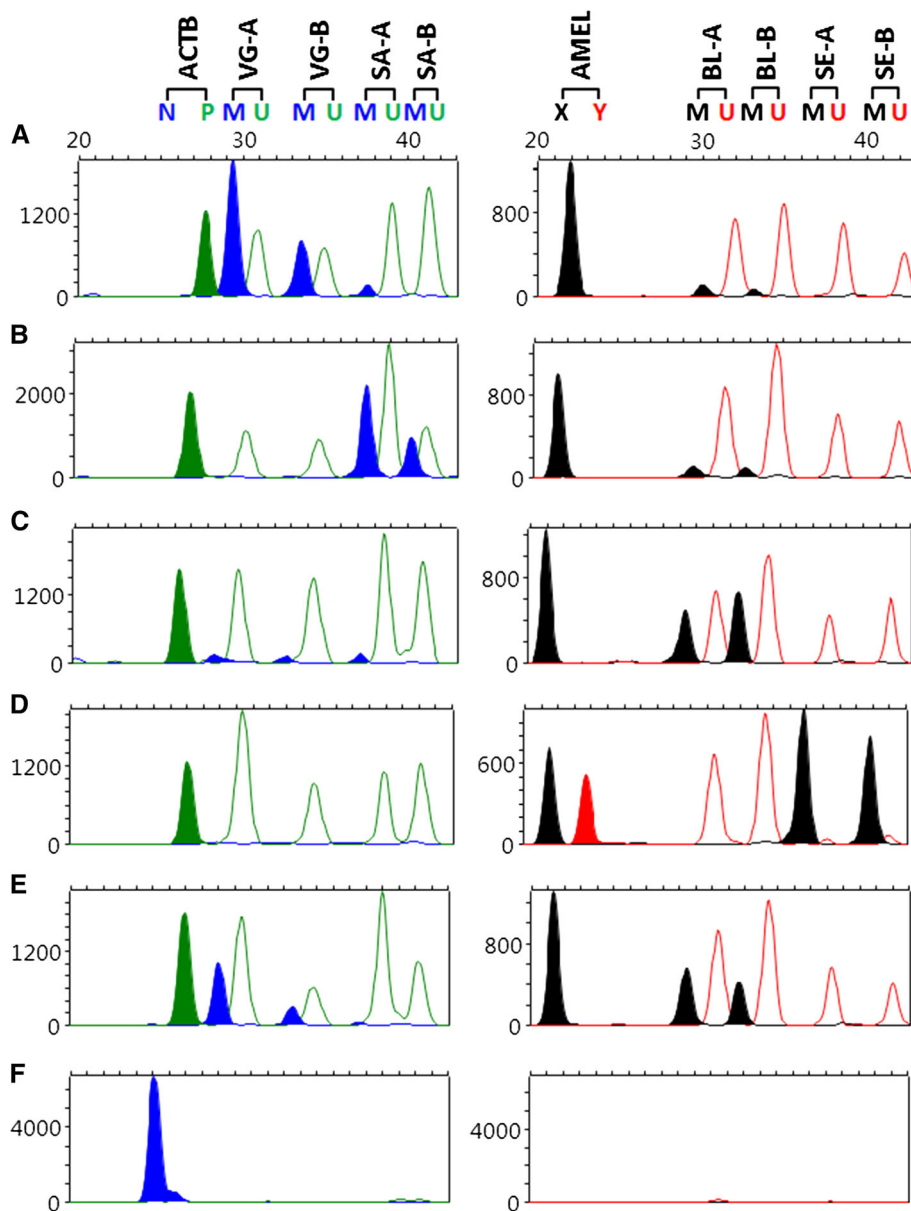
The methylation status of each biofluid for the eight selected markers was evaluated by MIV. The MIV was

markedly higher in the respective biofluid for each of the 8 selected markers (Fig. 2). A threshold to reduce the risk of any false positive interpretations for each marker was set at the highest “mean + 2 SD” of MIV for the non-specific biofluids. For example in respect to the VG-A marker, the highest “mean + 2 SD” of MIV for the non-specific biofluid was 16.74 % (“8.11 + 2 \times 4.32” %) (for venous blood, Online Resource 4). Only a MIV above the threshold was considered a positive result. All 65 collected biofluid samples were correctly identified without ambiguity by adopting the respective thresholds for these markers.

Mixture tests

Mixtures of semen and vaginal fluid (to simulate cases of sexual assault) were prepared by combining bisulfite-converted DNA in differing ratios for analysis by the 10-plex MSP-SBE system (Fig. 3). The M peaks of markers specific to vaginal fluid (VG-A and VG-B) and specific to semen (SE-A and SE-B) were observed when the bisulfite-converted DNAs of vaginal fluid and semen were mixed at ratios of 1:9, 1:1, or 9:1. The M peak of VG-B appeared as a low peak height in the ratio of 1:9 (Fig. 3a). When vaginal fluid and semen DNAs were mixed in the ratio of 1:1 and 9:1, the MIVs of the four specific markers were all above the respective thresholds (Online Resource 5). However, for the mixture of vaginal fluid and semen DNAs at a ratio of 1:9, although the MIV of VG-A was higher than the threshold, the MIV of VG-B was marginally lower than its threshold. Based on these data, the VG-B showed a false negative when vaginal fluid comprised only a small part (10 %) of the mixture. When the mixtures were prepared following the above ratios for combinations of saliva and semen, or venous blood and semen, the MIVs of the

Fig. 1 Electropherograms of an example for each biofluid identified by the 10-plex MSP-SBE system. The bisulfite-converted DNA used is from the vaginal fluids (a), saliva (b), venous blood (c), semen (d) and menstrual blood (e), and one control DNA sample from semen (not bisulfite-converted) (f). The symbol *N* represents the peak from the template including the cytosine from incomplete converted DNA and *P* represents thymine when converted from cytosine; *X* and *Y* for the X and Y chromosome, respectively; and *M* and *U* for the methylated and unmethylated CpG. The left blue/green signals represent guanine and adenine, and the right black/red signals represent cytosine and thymine, respectively



specific markers in all the mixtures were higher than the threshold for their respective biofluids. An exception was the BL-B marker as this was approximately equal to the threshold when venous blood and semen were mixed in a ratio of 1:9. When the DNA isolated from: vaginal fluid, saliva, venous blood, and semen was mixed at equal proportions, all the M peaks of the eight selected markers were clearly observed and all the MIVs of each marker were above their thresholds (Fig. 3d; Online Resource 5). In order to test if incomplete bisulfite conversion would affect the outcome, treated, and non-treated DNA that had been isolated from semen was mixed at a ratio of 1:9 (Fig. 3e; Online Resource 5) resulting in a strong N peak at the

correct position for ACTB and M peaks at SE-A and SE-B. This indicated that most of the DNA was non-treated as expected and the interpretation of the profiles for eight selected markers and AMEL was not affected.

Non-probative forensic sample identification

To test the applicability of this 10-plex MSP-SBE system in real cases, five non-probative forensic samples were collected from sexual assault cases (Table 2). Previous testing of a stain for the presence of blood using the KM test resulted in only one sample (Sample 2) giving a positive result. An immunochromatographic test for PSA using

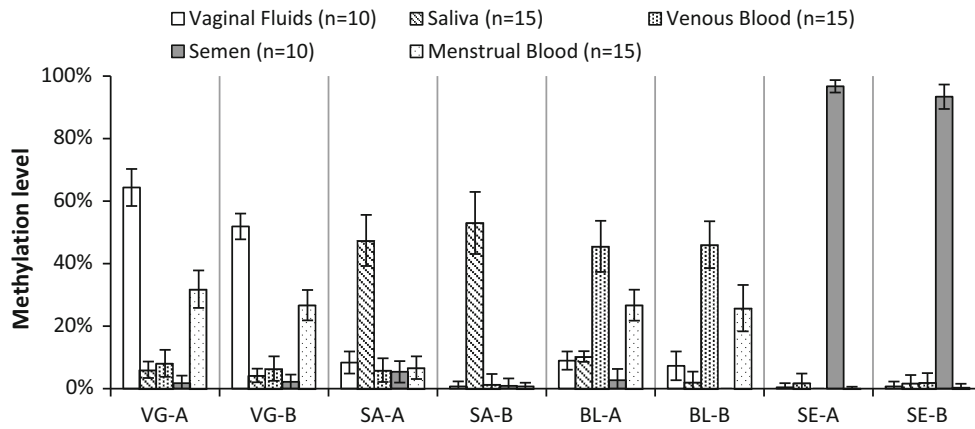


Fig. 2 Methylation indicative value (MIV) of 8 biofluid-specific methylated markers in vaginal fluids, saliva, venous blood, semen, and menstrual blood determined by the 10-plex MSP-SBE system

Fig. 3 Electropherograms of the simulated biofluid mixtures identified using the 10-plex MSP-SBE system. Bisulfite-converted DNA of vaginal fluid and semen was mixed at a ratio of 1:9 (a), 1:1 (b), and 9:1 (c). Bisulfite-converted DNA of vaginal fluid, saliva, venous blood, and semen was mixed at equal amounts (d). In (e), DNA isolated from semen that was both treated and not treated with bisulfite was mixed at a ratio of 1:9. The 10-plex MSP-SBE system was performed using 2 ng of the mixed DNA

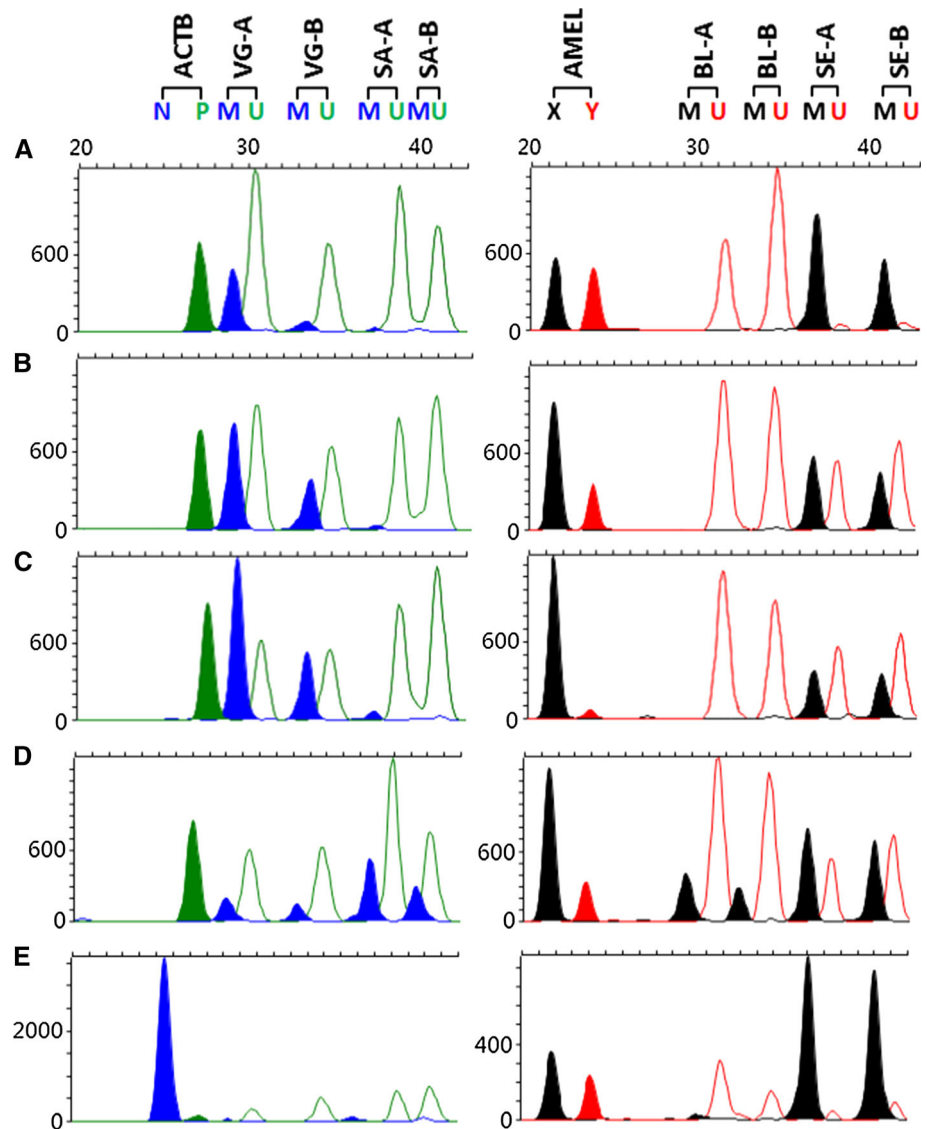


Table 2 Results from forensic investigation of the 5 non-probative forensic samples

Forensic sample	Sample type	KM test ^a	PSA test ^a	Microscopic examination ^a	HQ (ng/ μ L) ^b	YQ (ng/ μ L) ^b	Y ratio (%) ^{b,c}	Determination of 10-plex MSP-SBE identification
1	Internal vaginal swab	–	INC	–	15.6	NR	NR	Vaginal fluid
2	External anal swab	+	INC	–	7.11	NR	NR	Venous blood
3	Tissue paper	–	+	+	0.77	0.36	31.86	Vaginal fluid, Saliva, Semen
4	Fabric cut from panty	–	+	–	18.55	1.50	8.07	Vaginal fluid, Semen
5	Fabric cut from panty	–	+	–	11.64	0.22	1.89	Vaginal fluid, Semen

^a The symbols “+”, “–”, and INC represent the positive, negative, and inconclusive result, respectively

^b HQ and YQ represent the quantification of total human DNA and human male DNA using Quantifiler[®] Human DNA quantification kit and Quantifiler[®] Y Human Male DNA Quantification Kit, respectively. NR indicates the quantity was under the limitation of the quantification kit

^c Proportion of Y Male DNA in total DNA (Y ratio) was calculated with the equation of YQ/HQ

the SERATEC[®] PSA SEMIQUANT resulted in three samples (Sample 3, 4, and 5) being positive and two samples inconclusive (INC) (Sample 1 and 2) due to the very faint color of the test line. Spermatozoa had only been observed in Sample 3 of the three PSA positive samples based on microscopic examination. From these tests, semen was identified confidently only in Sample 3, and the remaining four samples were inconclusive due to the ambiguous PSA results for Sample 1 and 2 and the inconsistent PSA result and microscopic examination for Sample 4 and 5. Using real-time quantification of the human total DNA and male DNA, Y DNA was not detected in the two samples that generated an inconclusive PSA result (Sample 1 and 2) but detected in the three PSA positive samples (Sample 3, 4, and 5). For the two samples that were positive for PSA but negative for microscopic examination (Sample 4 and 5), the majority of DNA was from a female since the Y ratios were very low (8.07 and 1.89 % for Sample 4 and 5, respectively). Although the male Y DNA was observed in these two samples, the tissue from which the Y DNA had originated was still in question.

In this study, DNA from these 5 forensic samples was initially bisulfite converted and then analyzed by the 10-plex MSP-SBE system. Interpretation of the results was based on the MIVs of the 2 markers specific to each biofluid (Online Resource 6). The results showed that the MIVs of the blood-specific markers (BL-A and BL-B) were higher than their respective thresholds in Sample 2 and thus the presence of venous blood was determined confirming the positive result for KM test (Table 2). For the two PSA inconclusive samples (Sample 1 and 2), no male Y DNA was recorded and the MIVs of the semen specific markers (SE-A and SE-B) were zero. However, for the three PSA positive samples (Sample 3, 4 and 5), the MIVs of semen-specific methylated markers (SE-A and SE-B) were all above their threshold values and thus the presence of semen was determined.

Discussion

To establish a DNA-methylation-based method for identification of biofluid types, tissue-specific methylated markers were selected from the public datasets of the Infinium HumanMethylation450 BeadChip from the GPL 13534 platform in GEO. Vaginal fluid and semen samples were not available from this platform therefore relevant datasets for cervical tissues and spermatozoa were used to search for candidate markers. From results of the preliminary MSP amplification, the methylation patterns of the eight selected markers for vaginal fluid and semen were as expected and similar to those of the cervical tissues and spermatozoa respectively. Due to the selective amplification of the M reactions for the eight biofluid-specific methylated markers, an approximate “on/off” effect was in accordance with a previous recommendation [25].

In the preliminary test of this study, initial PCR was performed using primers with degenerate sequences that flanked the CpG islands. The methylation status of the amplicon was checked by direct sequencing but it was noted that the dominant signals were from the un-methylated products and the signals for methylated products were often absent or very low (data not shown). An alternative approach was therefore adopted using 2 reverse primers (RM and RU) to mimic the methodology of the type I assay design in Infinium HumanMethylation450 BeadChip [29] and to emphasize the signals of methylated CpGs by increasing the concentration of RM primer in the MSP amplification.

An attempt was also made to label the forward primer of each marker with a fluorescent dye in the 10-plex MSP reaction and directly detect the fluorescent signals with capillary electrophoresis. However, this resulted in the generation of extra peaks that made interpretation problematic (data not shown); this may be due to non-specific amplification products generated from the 28 primers in

such a complex MSP reaction. It was decided to use the alternative detection method of SBE by annealing the SBE primers to the purified MSP products.

To prevent false interpretations, the “methylation indicative value” (MIV) was used to describe the methylation status and a threshold was set for each marker. The reproducibility of the MIVs and an indication of the limit of detection were tested using serial dilution of bisulfite-converted DNA from 2 ng to 0.0625 ng. The values obtained were reproducible down to an input of 0.25 ng DNA (data not shown). It should be noted that MIV was not comparable with the beta-values [40] in the public datasets (Table 1). “MIV” does not therefore reflect the real ratio of methylation due to factors such as PCR bias, uneven concentrations of RM and RU primers, and uneven fluorescence intensity in this system. With the thresholds of MIVs, all the collected biofluids were unambiguously identified.

To simulate mixtures as typically encountered in a forensic investigation, the vaginal swab was dipped into liquid semen before DNA extraction as part of this preliminary study. Both of the biofluids were identified subsequently as expected (data not shown) although the actual ratios could not be determined accurately due to the initial unknown cell number within each biofluid. In sexual assault cases, accurate quantification of human DNA and male DNA can be performed to obtain the DNA ratio of male offenders and female victims. The bisulfite-converted DNA of different biofluids was therefore mixed instead of mixing the biofluids in this study. As a result, even though DNA from semen comprised only 10 % of the total DNA, it was still unambiguously identified. The results from mixtures of original DNA and bisulfite treated DNA indicated that incomplete bisulfite conversion would not affect the accurate determination of the presence of a body fluid. Primers were designed to be specific to bisulfite-converted DNA and therefore should not anneal and amplify non-treated DNA as this is similar to incompletely converted DNA.

For identification of the non-probative forensic samples from real sexual assault cases, the applicability of semen detection was demonstrated even if only 1.89 % male DNA was present in the mixtures. At this ratio, assuming the male DNA was predominately from spermatozoa and the remaining DNA was from vaginal cells, the cell numbers of spermatozoa and vaginal cells would be calculated as 1:26 (given that a spermatozoon is haploid). The very few numbers of spermatozoa would have been very difficult to identify confidently from the mass of vaginal cells under microscopic examination. This 10-plex system unambiguously determined the biofluid types for the non-probative forensic samples that gave ambiguous results from tests that had a catalytic, immunological, and microscopic basis. Furthermore, the system has advantages over current and traditional assays due to the potential that samples can be

massively processed in a batch and four different biofluids can be identified in a single reaction.

This study adds to that of others [31] and demonstrates that the biofluid-specific methylated CpGs from the Infinium HumanMethylation450 BeadChip are highly informative for selecting markers for the identification of biofluids. In this study, we further established a novel 10-plex MSP-SBE system containing one bisulfite conversion control marker, one gender marker, and eight biofluid-specific methylated CpG markers for identifying vaginal fluid, saliva, venous blood, and semen.

Conclusions

We report on the establishment of a 10-plex MSP-SBE assay that includes a bisulfite conversion control marker, a gender marker, and eight biofluid-specific methylated markers for the body fluids of vaginal fluid, venous blood, saliva, and semen. In total 65 samples were tested and in every case an unambiguous identification of the body fluid present was recorded. This included not only single source samples but also complex mixtures even when the body fluid comprised only 10 % of the total DNA. The presence of semen was determined even when the DNA from semen was only 1.89 % of the total DNA within a non-probative forensic sample. We have demonstrated that this system can be used as a valuable method in the identification of biofluids.

Key points

1. A 10-plex methylation-specific PCR single-base-extension (MSP-SBE) system was established to unambiguously identify the presence of specific biofluids encountered frequently in forensic investigations. These included saliva, venous blood, vaginal fluids, and semen.
2. An unambiguous identification of the biofluid was recorded not only for samples from a single source but also for complex mixtures even if one of the body fluids contributed only 10 % of the total DNA.
3. The system accurately determined the biofluid types for five non-probative forensic samples that previously gave ambiguous results based on results from presumptive and microscopic tests.
4. Based on the results provided, this assay can be used as a valuable method in the identification of biofluids.

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Compliance with ethical standards

Ethical standard The body fluids used in this study were collected from volunteers using procedures approved by the Institutional Review Board (IRB) of Tao-Yuan General Hospital in Taiwan (IRB No. TYGH102011).

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