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High-resolution melt analysis of DNA methylation to discriminate semen in biological stains

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ABSTRACT

The goal of this study was to develop a method for the detection of semen in biological stains using high-resolution melt (HRM) analysis and DNA methylation. To perform this task, we used an epigenetic locus that targets a tissue-specific differentially methylated region for semen. This specific locus, ZC3H12D, contains methylated CpG sites that are hypomethylated in semen and hypermethylated in blood and saliva. Using this procedure, DNA from forensic stains can be isolated, processed using bisulfite-modified polymerase chain reaction (PCR), and detected by real-time PCR with HRM capability. The method described in this article is robust; we were able to obtain results from samples with as little as 1 ng of genomic DNA. Samples inhibited by humic acid still produced reliable results. Furthermore, the procedure is specific and will not amplify non-bisulfite-modified DNA. Because this process can be performed using real-time PCR and is quantitative, it fits nicely within the workflow of current forensic DNA laboratories. As a result, it should prove to be a useful technique for processing trace evidence samples for serological analysis.

DNA typing technologies using short tandem repeats allow comparisons to be made between body fluid stains and individuals. In cases where the presence of a suspect is expected, the type of body fluid present can make the distinction between innocent and criminal contact. In previous work, we have shown that analysis of DNA methylation patterns can identify the tissue source of a DNA sample [1,2]. DNA methylation is a natural process in the mammalian genome that involves the addition of a methyl group to the 5′ carbon of cytosines in a dinucleotide CpG pair. DNA methylation is involved in gene expression by regulating transcription factors that lead to gene activation or gene silencing [3]. Several reports demonstrate that specific regions of the genome have different DNA methylation patterns depending on the cell type studied [4–6]. Those regions are called tissue-specific differentially methylated regions and can be used as a powerful tool for body fluid identification [7–9]. Common methodologies for DNA methylation analysis include DNA digestion by methylation-sensitive endonucleases followed by polymerase chain reaction (PCR) amplification [9] and bisulfite conversion of genomic DNA that causes the unmethylated cytosines to be chemically converted to uracil while the methylated cytosines are protected. After bisulfite modification, the DNA is amplified by PCR using specific primers [10] and the amplicons can be analyzed by pyrosequencing, which provides quantitative methylation values for each CpG site present in the target sequence [11].

High-resolution melt (HRM) analysis can detect DNA sequence variants based on their different melting temperatures [12–14]. PCR products can be differentiated even if they differ from each other at only a single nucleotide [15], making this an optimal method to analyze single nucleotide polymorphisms [12,16,17]. HRM involves the amplification of a DNA template by real-time PCR in the presence of a double-stranded DNA (dsDNA) intercalating dye such as EvaGreen. The fluorescence is maximized at the end of amplification when the largest quantity of dsDNA is present. Once the PCR is complete, the melting step begins, whereby PCR

Abbreviations used: PCR, polymerase chain reaction; HRM, high-resolution melt; dsDNA, double-stranded DNA; T_M, melting temperature; gDNA, DNA before bisulfite conversion; bDNA, DNA after bisulfite conversion.

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products are heated in increments of 0.1 °C and a graph illustrating the change in fluorescence with respect to changes in temperature ($-dF/dT$) is obtained [15]. HRM is an in-tube method, meaning that analysis occurs in the same tube as amplification, thereby saving time and avoiding sample transfer steps. The procedure is a quick and nondestructive method to characterize PCR products [12]. HRM can also be used to explore the melting differences between unmethylated and methylated DNA after bisulfite conversion. During bisulfite conversion, the unmethylated cytosines, but not the methylated ones, are converted into uracils and subsequently thymine during PCR. Thus, amplicons resulting from the PCR of unmethylated DNA have a lower GC content and concomitant lower melting temperature when compared with amplicons resulting from the PCR of methylated DNA. If the bisulfite conversion fails, the GC content of the amplicon is similar to methylated DNA and an overestimation of methylation occurs [18,19]. Even though the available commercial kits for bisulfite conversion modify 99% of the DNA, appropriate controls should be in place to confirm that there is no amplification of unmodified DNA. One way of guaranteeing this step is to design primers that anneal only to bisulfite-modified DNA.

Several reports [14,19–22] have exploited the application of HRM for detecting differentially methylated DNA. Even though DNA methylation analysis by HRM cannot determine the methylation status of individual CpGs, it does provide a robust and inexpensive method to differentiate DNA based on the overall methylation of a specific amplicon.

One challenge faced in PCR-based analysis is the potential decrease of amplification efficiency due to inhibition. DNA is often coextracted with substances that can hinder PCR. There are two main mechanisms of PCR inhibition. One mechanism occurs when the inhibitor binds the DNA, and the other mechanism occurs when the inhibitor hinders the catalytic activity of *Taq* polymerase. When the inhibitor binds to the DNA, it can diminish the processivity of the DNA polymerase or prevent primers from annealing to the template DNA, thereby decreasing PCR efficiency. Moreover, for inhibitors binding to the DNA, a simple cleanup step might not be sufficient to remove the decrease in PCR efficiency. Humic acid is one substance known to inhibit PCR because it binds to the template DNA [23,24].

In this study, we aimed to investigate whether the methylation differences among blood, saliva, and semen for the locus ZC3H12D can be identified using HRM. When pyrosequencing analysis is performed in the locus ZC3H12D, blood and saliva show hypermethylation in comparison with semen [1]. The successful analysis of this locus using HRM would be extremely useful in the identification of the source of DNA samples in forensic investigations. To our knowledge, this is the first time that HRM has been used to discriminate body fluids by exploring differences in DNA methylation.

Materials and methods

DNA collection, extraction, and bisulfite conversion

In total, 10 blood samples, 9 buccal swabs, and 7 semen samples were collected from volunteers according to the approved IRB-13-0555 from Florida International University. Swabs were air-dried, and DNA extraction was performed using the EZ1 DNA Investigator Kit (Qiagen, Valencia, CA, USA) and the BioRobot EZ1 automated purification workstation (Qiagen) according to the manufacturer's specifications. Quantification was performed using the PicoGreen method (Life Technologies, Carlsbad, CA, USA). DNA (1 and 50 ng) was bisulfite modified using the EpiTect Fast DNA Bisulfite Kit (Qiagen) according to the manufacturer's instructions. For the sensitivity studies, samples were serial diluted to obtain 0.5 and 0.25 ng of input DNA to bisulfite conversion. In parallel, the DNA samples that were modified using 1 ng of input to bisulfite modification were serially diluted after modification to determine the lowest amount amplifiable by HRM. Samples that were not bisulfite modified were diluted to 0.5 ng/μl in order to obtain a 1 ng input to the HRM reaction.

DNA amplification

Amplification reactions were performed using the EpiTect HRM Kit (Qiagen) on a Rotor Gene 6000 real-time machine (Qiagen). The kit is composed of an HRM buffer that contains EvaGreen, HotStarTaq Plus, and dNTP mix. DNA (2 μl) was added to the master mix composed of HRM buffer and 0.75 μM of each forward and reverse primer. Primers were designed using the online tool MethPrimer [25]. The primer sequences are given on Table 1. Amplification was made by initially holding the temperature at 95 °C for 5 min, followed by 45–50 cycles of 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 10 s. Melt analysis was performed by increasing the temperature from 65 to 85 °C in 0.3 °C increments and detecting fluorescence in the HRM channel. Melt curve analysis was made using the Rotor-Gene 6000 series software (version 1.7).

Humic acid inhibition

Humic acid was added to each DNA sample at a final concentration of 1.92×10^{-3} ng/μl prior to bisulfite conversion. Both samples without humic acid (control samples) and with humic acid were bisulfite converted and amplified as described above. A master mix containing humic acid with a final concentration of 1.92×10^{-3} ng/μl was made, and control samples were amplified as described above.

Statistical analysis

One-way analysis of variance (ANOVA) with Tukey's post hoc test was performed using SPSS (version 22, IBM) to compare the melting temperatures among the 7 semen samples, 10 blood samples, and 9 saliva samples and to determine whether the difference observed is statistically significant. For *P*-values inferior to 0.05, the melting temperature was considered to be significantly different.

Results and discussion

High-resolution melt can be used to discriminate among fragments of DNA that present varying degrees of methylation. HRM has the advantage of being cost-effective, besides being a useful alternative/additional method to pyrosequencing protocols, because it permits the analysis of amplicons with sizes larger than 70 bp. From the work of Madi and coworkers [1], we note that for locus ZC3H12D semen presents low levels of methylation (~10%), whereas other body fluids have levels of methylation around 100%. The hypomethylation of semen is expected to result in a melt curve with a lower melting temperature when compared with blood and saliva cells. Figs. 1 and 2 illustrate that DNA from semen presents an

Table 1
Sequences of primers used in this study.

Primer	Sequence
ZC3H12D forward	GGG TGA GGG TTT AAG GGT
ZC3H12D reverse	CTC CCC TCA AAA CCT CAT