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.

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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

products are heated in increments of 0.1 °C and a graph illustrating the change in fluorescence with respect to changes in temperature ($-\mathrm{d}F/\mathrm{d}T$) 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, Hot-StarTaq 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

average melt temperature of 75.5 °C with a standard deviation of 0.2, which is lower than the values for other body fluids (78.2 and 78.1 °C for blood and saliva, respectively). Our primers amplify a specific genomic region that is hypermethylated in saliva and blood, resulting in amplicons with high GC content. Methylated cytosines are protected from bisulfite conversion and remain as cytosines. Amplification will result in amplicons with a high GC content, which results in higher melting temperature (T_M) because higher temperatures need to be reached to break the triple hydrogen bonds that bind guanine to cytosine. In semen, the same region amplified by our primers is hypomethylated. The lack of methyl groups in the cytosines from semen DNA causes them to convert to uracil through the bisulfite reaction. Those uracils are amplified as thymines and result in an amplicon with lower GC content, resulting in melt curves with lower T_M values. The differences in T_M are visible in Figs. 1 and 2. To determine the sensitivity of this method, we performed serial dilutions of DNA samples from blood, saliva, and semen to obtain inputs of DNA to bisulfite conversion of 1, 0.5, and 0.25 ng. Those samples were amplified, and the T_M value was quantified by HRM. Fig. 3 shows that for inputs of DNA lower than 1 ng, some samples fail to amplify even when the amplification is extended for 50 cycles. It seems that 1 ng of genomic DNA is the minimum amount to input to bisulfite conversion to guarantee amplification using this protocol for HRM analysis.

To further explore whether it is the bisulfite conversion or the HRM amplification that limited the efficiency of amplification of DNA of DNA levels below 1 ng, we performed serial dilutions of bisulfite-modified samples prepared with 1 ng of input genomic DNA. Four different quantities of DNA were amplified based on dilutions of the original 1 ng level of input DNA. Fig. 4 shows that a dilution of 1:2 is sufficient to impair amplification for two of nine samples. Because the bisulfite-modified samples are further reduced in quantity, fewer samples amplify successfully, as can be seen for the 1:16 dilution having only five successful amplifications out of a total of nine samples. Fig. 4 also shows that for the dilutions of 1:2 and 1:4, when amplification is successful the expected T_M value is observed for the body fluid tested. The saliva samples at 1:8 dilution show a higher standard deviation (77.50 ± 0.72) when compared with 1:2 dilution (78.50 ± 0.15) and 1:4 dilution (78.25 ± 0.00), possibly due to stochastic effects on amplification and melt analysis when low amounts of DNA are used. The results displayed in Figs. 3 and 4 demonstrate that samples containing less than 1 ng of input DNA prior to bisulfite conversion may result in impaired amplification due to the presence of low levels of DNA. However, in situations where amplification occurs below 1 ng, distinctions among semen, blood, and
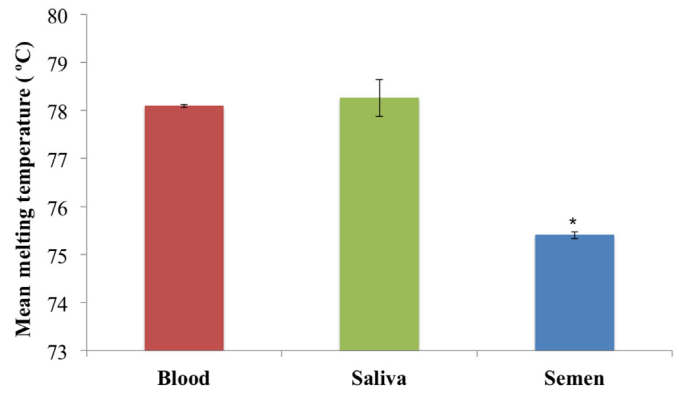
saliva may still be possible using HRM analysis. The fact that the melting temperatures do not change when low amounts of DNA are successfully amplified demonstrates that this method is unlikely to give false results that would otherwise lead to a misclassification of a body fluid.

Fig.2. Chart showing the mean values for melting temperatures (°C) determined by HRM for 10 blood samples, 9 saliva samples, and 9 semen samples. Analysis of variance was performed showing that the melting temperature is significantly different for semen when compared with blood and saliva (*$P < 0.05$).
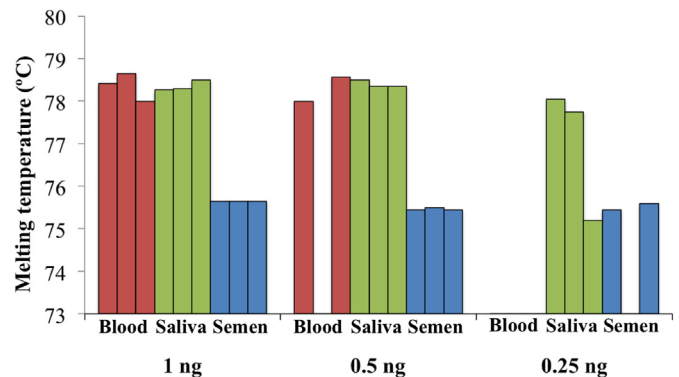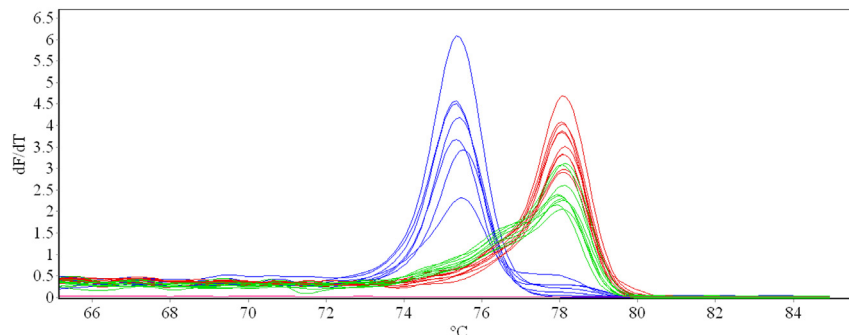
Fig.3. Chart showing the melting temperatures (°C) for three individual samples from three different volunteers of each body fluid: blood (red), saliva (green), and semen (blue). The x-axis shows the different amounts of input DNA added to bisulfite modification. Each body fluid should have three individual bars for individual amplifications. The absence of a bar indicates that amplification failed, presumably due to insufficient levels of input DNA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig.1. First derivative of the change of fluorescence with temperature (dF/dT) for high-resolution melt curves showing 7 semen samples (blue, T_M = 75.5 °C), 10 blood samples (red, T_M = 78.2 °C), and 9 saliva samples (green, T_M = 78.1 °C). A no-template control (no DNA) was also included and is seen at the baseline in pink color. Body fluid samples were donated from individual volunteers. Samples were amplified from 50 ng of genomic DNA added to bisulfite conversion. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
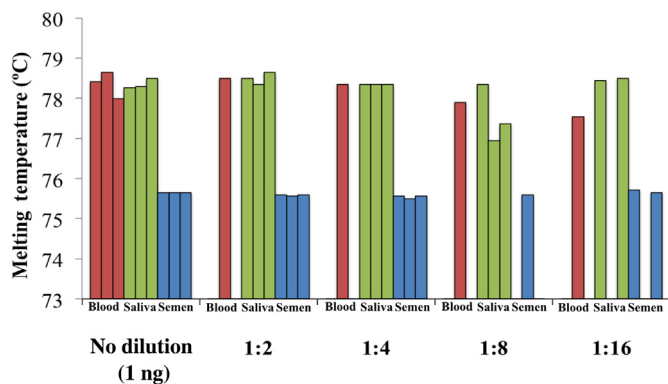
Fig.4. Chart showing the melting temperatures (°C) for three individual samples from three different volunteers of each body fluid: blood (red), saliva (green), and semen (blue). The *x*-axis shows the dilution factor from samples that were bisulfite modified with 1 ng of DNA. For comparison, the nondiluted samples (1 ng) are displayed. Each body fluid should have three individual bars for individual amplifications. The absence of a bar indicates that amplification failed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

To further test the robustness of the method, we explored whether the primers can amplify non-bisulfite-modified DNA. Differences in methylation can be explored accurately only if the DNA is bisulfite modified prior to amplification and HRM. Incomplete bisulfite modification could result in an overestimation of methylation in DNA because an amplicon containing unmodified cytosines, regardless of their methylation status, will have a melting curve similar to a fully methylated amplicon [18]. To determine whether our primers are specific for bisulfite-converted DNA, we performed HRM for the same extracted samples of semen, blood, and saliva before bisulfite conversion (gDNA) and after bisulfite conversion (bDNA). Fig. 2 demonstrates that gDNA samples did not amplify (pink lines) even after 50 cycles. The bDNA amplified properly (blue, orange, and green lines in Fig. 5A) and presented the expected melt curves, with a lower T_M for semen when compared with blood and saliva (Fig. 5B).

Because some samples contain substances that, when coextracted with DNA, can cause inhibition of the amplification, we determined whether the cleanup steps in the bisulfite conversion reaction are sufficient to remove inhibition. Because humic acid is an inhibitor known to impair amplification by binding to the template DNA [23], we decided to examine whether the cleanup step can release DNA from that substance. Moreover, to determine whether the PCR efficiency is altered in the presence of humic acid, we also added that inhibitor following bisulfite conversion. PCR efficiency is evaluated by the number of copies of DNA synthesized per cycle (or unit of time). On a graph that displays the amplification curve, such as in Fig. 6A, a steeper slope on the exponential curve means that the PCR was more efficient. Efficiency is 100% when $E = 2$ ($E = 10^{\text{slope}}$), and it means that the PCR product doubles at each cycle.

In the first experiment, humic acid was added before bisulfite conversion to simulate coextraction with DNA. Fig. 6A shows that samples without humic acid (control, thin lines) and samples where the humic acid was added before bisulfite conversion (dashed lines) amplify with similar efficiency. The fact that the steepness of the curves for the control samples and those where humic acid was added before bisulfite treatment are similar allows us to conclude that the cleanup steps performed as part of the bisulfite kit are sufficient to remove humic acid coextracted with DNA.
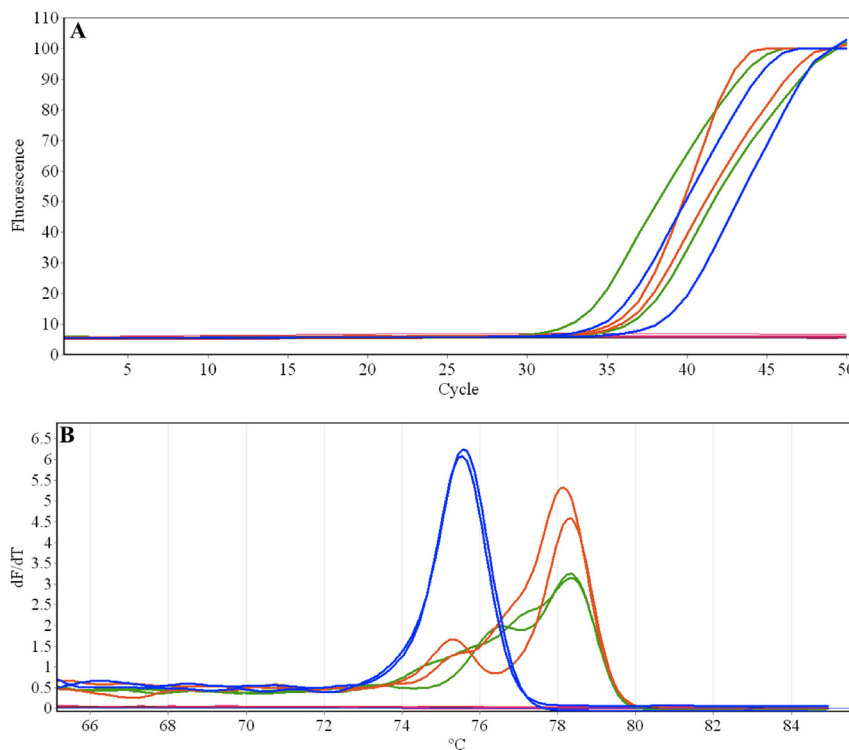

Fig.5. Graphs showing samples before and after bisulfite modification. bDNA from semen samples is in blue, from blood samples is in orange, and from saliva samples is in green. gDNA samples are shown in pink. (A) This panel shows the increase in fluorescence as a result of incorporation of the green fluorescent dye EvaGreen as the cycle number increases. DNA samples that were not bisulfite modified (gDNA) do not amplify and are in pink color at the baseline. The bDNA samples amplify, showing an increase in fluorescence. (B) Melt curve analysis shows that the bDNA samples amplified show a melt profile consistent with Figs. 1 and 2. Semen samples are in blue, blood samples are in orange, and saliva samples are in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
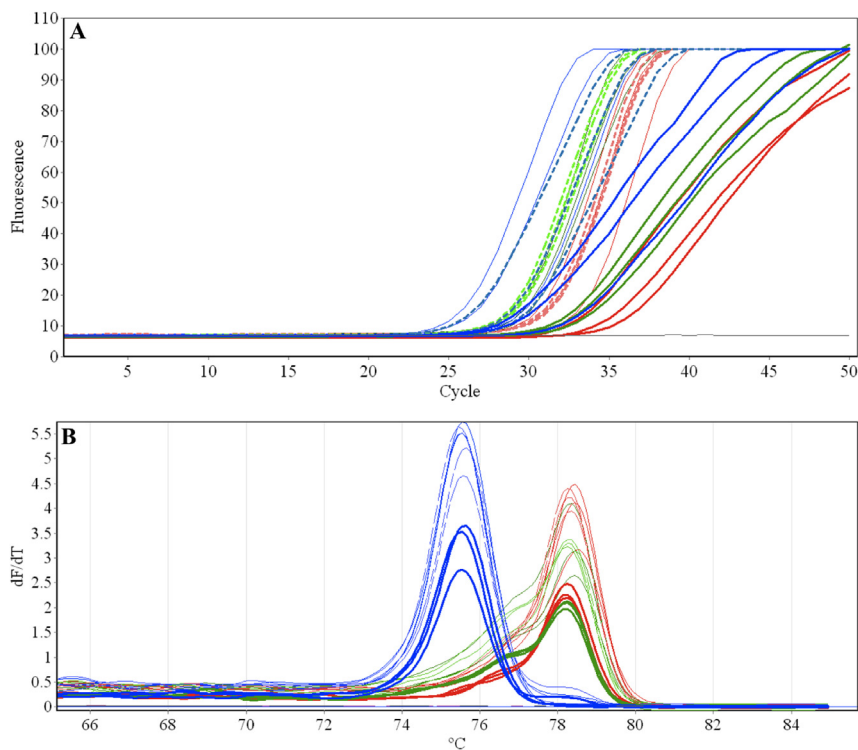
Fig.6. Graphs showing that the addition of humic acid to extracted genomic DNA from blood, saliva cells, and semen prior to bisulfite modification does not cause a change in PCR efficiency. DNA from blood is in red, from saliva cells is in green, and from semen is in blue. Thin lines are control samples without humic acid, dashed lines are samples where humic acid was added prior to bisulfite conversion, and thick lines are samples where humic acid was added during amplification. (A) The presence of the inhibitor during amplification (thick lines) causes a decrease in the steepness of the slope of the curve when compared with samples without humic acid (thin lines) or when humic acid was added prior to bisulfite conversion (dashed lines). (B) The presence of humic acid does not affect the melt curve because all samples have similar T_M values to those demonstrated in Fig. 1, even when amplified in the presence of humic acid. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

To determine whether the same concentration of humic acid causes changes in PCR efficiency, we performed another experiment in which the humic acid was added to the PCR mix after bisulfite conversion. All samples that were amplified in the presence of humic acid (thick lines) show a decrease in PCR efficiency, as indicated by a reduction in the slope of the amplification curve (Fig. 6A). Even though the presence of inhibitor in the amplification decreased PCR efficiency as seen by a reduction in the slope in Fig. 6A, it did not cause a shift in the melting curve (Fig. 6B). The T_M of DNA extracted from semen samples (blue in Fig. 6B) is still lower when compared with the T_M values of DNA extracted from blood (red in Fig. 6B) and saliva (green in Fig. 6B) regardless of the presence of humic acid (thick lines). Thompson and coworkers [23] speculated that changes in the melt curve can depend on the size and GC content of the amplicon. Our amplicon has a size of 91 bp and a GC content of 21% for complete methylated DNA strands (blood and saliva cells) and 14% for unmethylated DNA strands (semen). Pionzio and McCord [24] concluded that long amplicons with low GC content are more prone to display effects of inhibition. Our amplicon is considered to be short and with low GC content. Therefore, it demonstrates robustness for melt curve analysis even when the PCR efficiency is reduced by the presence of inhibitors.

Conclusion

This HRM analysis method is able to amplify and distinguish DNA from semen even when only 1 ng of input DNA is bisulfite converted. Lower levels of input DNA can also provide useful results. When tested with DNA that is not bisulfite converted, our primers failed to amplify the DNA, which proves that incomplete conversions will not result in false results possibly leading to misidentification of a body fluid. The results also show that when inhibitors like humic acid are coextracted with the DNA, the cleanup step performed as part of the bisulfite conversion step is capable of eliminating detrimental effects given that no decrease in the amplification efficiency was observed. Furthermore, even when humic acid was added to the amplification step following bisulfite conversion, the HRM method provided reliable results, with T_M values similar to those obtained in the absence of inhibitor. These results demonstrate that HRM analysis can be a promising technology to identify the tissue source of a given DNA sample.

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