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# Differentiating between monozygotic twins through DNA methylation-specific high-resolution melt curve analysis

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ABSTRACT

Although short tandem repeat profiling is extremely powerful in identifying individuals from crime scene stains, it is unable to differentiate between monozygotic (MZ) twins. Efforts to address this include mutation analysis through whole genome sequencing and through DNA methylation studies. Methylation of DNA is affected by environmental factors; thus, as MZ twins age, their DNA methylation patterns change. This can be characterized by bisulfite treatment followed by pyrosequencing. However, this can be time-consuming and expensive; thus, it is unlikely to be widely used by investigators. If the sequences are different, then in theory the melting temperature should be different. Thus, the aim of this study was to assess whether high-resolution melt curve analysis can be used to differentiate between MZ twins. Five sets of MZ twins provided buccal swabs that underwent extraction, quantification, bisulfite treatment, polymerase chain reaction amplification and high-resolution melting curve analysis targeting two markers, *Alu*-E2F3 and *Alu*-SP. Significant differences were observed between all MZ twins targeting *Alu*-E2F3 and in four of five MZ twins targeting *Alu*-SP ($P < 0.05$). Thus, it has been demonstrated that bisulfite treatment followed by high-resolution melting curve analysis could be used to differentiate between MZ twins.



Although standard DNA profiling targeting short tandem repeats is successful in identifying individuals from stains, there have been a number of incidences where the suspects were monozygotic (MZ)[1] twins. In such cases, it has not been possible to determine from which of the twins the stain could have originated.

Efforts in this area have included whole genome sequencing, which looks at potential single nucleotide polymorphisms through mutation analysis [1]. Such specialized techniques might not be readily available in general forensic laboratories. One particular area showing promise is DNA methylation, an epigenetic feature that changes in response to environmental exposure [2–4]. The principle behind DNA methylation analysis on samples from twins is that as the MZ twins mature, they will be exposed to different environmental stimuli; for example, if one twin were to take up smoking, this person may present a different DNA methylation pattern. This could be characterized following bisulfite treatment and sequencing to identify the differences [5,6].

This study explored the use of high-resolution melting curve analysis (HRMA) [6,7] to identify differences in DNA methylation patterns between MZ twins. The principle behind this is that as the DNA methylation patterns change through exposure to different stimuli, the use of the bisulfite conversion step will change the sequences. Different sequences can lead to different melting temperatures. Consequently, the aim of this study was to evaluate whether HRMA will allow for the differentiation between MZ twins.

## Materials and methods

### *Sample collection and extraction*

Five sets of MZ twins provided buccal swabs after informed consent was obtained, providing 10 samples in total. An additional buccal swab was obtained from a full sibling of one pair of twins. DNA extraction was conducted using the buccal swab protocol of the QIAamp DNA Mini Kit (Qiagen, UK).

### *DNA quantification*

All extracted samples then underwent quantitative polymerase chain reaction (qPCR) DNA quantification using an Investigator

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[1] *Abbreviations used:* MZ, monozygotic; HRMA, high-resolution melting curve analysis; qPCR, quantitative polymerase chain reaction.

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Quantiplex Kit (Qiagen, UK) on the RotorGene Q Real-Time PCR Machine (Qiagen, UK). This allows for the normalization of the DNA prior to bisulfite treatment.

*Bisulfite treatment*

Extracted DNA then underwent bisulfite treatment using the EpiTech Bisulfite Kit (Qiagen, UK) as per the manufacturer's recommendations. This step converts all nonmethylated cytosines into uracil, leaving any 5-methylcytosines unchanged. Thus, differences in methylation patterns result in differences in sequences following bisulfite treatment.

*Amplification*

Both treated and untreated DNA extracts then underwent amplification targeting the *Alu*-E2F3 (2 CpGs) promoter and fragments of the *Alu*-SP (17 CpGs) regions using the Applied Biosystems 7500 Fast Real-Time PCR Machine and SYBR Green chemistry using the following primers: *Alu*-SP Forward 5′-tttggtgattaggaaggtgggta-3′, Reverse 5′-aaactaatctcaaactccctacctc-3′, *Alu*-E2F3 Forward 5′-ggtaataattttaaaatttgggggt-3′, and Reverse 5′-attaaaaaaaccaatcaacccataa-3′ [8]. Fraga and coworkers' study [8] provides further information regarding the positions of DNA methylation in both regions.

*High-resolution melting curve analysis*

HRMA was conducted immediately after amplification on the Fast 7500 Real-Time PCR Machine (Life Technologies, UK), with an initial phase of 95 °C for 15 s, then at 60 °C for 60 s, before a 1% increase to 95 °C. Data collection took place during the 1% increase. The data were analyzed using SDS 7500 software version 2.0.6.

## Results

Following bisulfite treatment and subsequent amplification of *Alu*-SP, as can be seen in Fig. 1, one set of MZ twins had different melting temperatures. This is the largest difference observed and was obtained from 53-year-old MZ male twins.

As can be seen in Fig. 2, the extent of the differences in the melting temperatures between different sets of MZ twins varies considerably. However, there are significant differences in the melting temperatures of products amplified by targeting *Alu*-E2F3 across all MZ twins ($P < 0.05$), with two sets of MZ twins showing a particularly higher significant difference ($P < 0.001$).

Fig. 3 shows the same set of samples but when targeting a different set of markers, namely *Alu*-SP. In this case, only four of the five sets of MZ twins showed a significant difference in melting temperature with the *Alu*-SP amplicon.

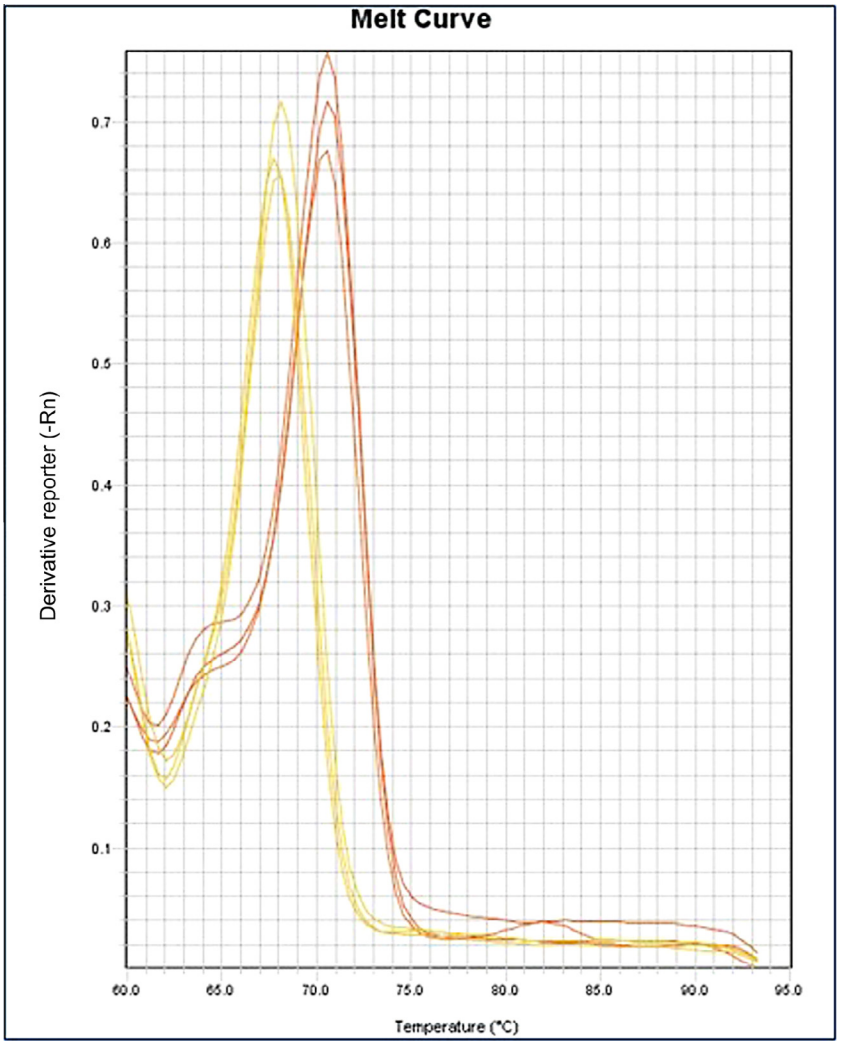


**Fig.1.** Representative melt curve of one set of twins (53-year-old men) exhibiting a difference in melting temperature targeting *Alu*-SP. Each group of peaks represents one individual.