Application Note

Tissue source attribution using the PyroMark® Q48 Autoprep System: Sperm identification in forensic casework

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Introduction

In recent years, DNA profiling has become the mainstay of forensic science and is the primary method of choice for human identification in criminal cases. The association of a highly discriminating DNA profile from a crime scene with a suspect linked to that crime can provide powerful evidence in the construction of a criminal case. However, the strength of evidence with which a DNA profile is linked to a suspect varies significantly depending on multiple factors such as quantification result, results of presumptive or confirmatory tests for biological fluids, or visual stain information [1]. In particular, attribution of a DNA profile to a specific cell or tissue type can enable scientists to address source-level questions often posed by the judiciary (e.g., how likely is it that the DNA profile was obtained from evidentiary bloodstains?) [1]. Furthermore, even where presumptive tests have identified a body fluid, the chemical test used may show more or less sensitivity than DNA profiling, and also lack specificity to the identified cell type. Therefore, the test may not provide an accurate answer that can be used with confidence in attributing a DNA profile to a given cell type. Here we describe a method for tissue source identification by examining DNA methylation patterns, and propose a novel workflow for sperm cell identification in casework samples.

Forensic tissue source identification by DNA methylation pattern analysis

Recent research efforts have led to the development of new assays for identification of body fluids based on epigenetic markers such as DNA methylation. DNA methylation represents a promising methodology since it is tissue specific, provides quantitative results, and has greater long-term stability in comparison to protein targets. Testing is performed on the same DNA extract used for profiling, and can be easily incorporated into a laboratory's current workflow and validated for forensic casework [2]. DNA methylation is an epigenetic modification that is involved in transcriptional regulation and affects gene expression. Although the mechanism is not fully ▷

understood, methylation patterns can differ between tissue types and these differences in methylation, which have been found to be both stable and reproducible, form the basis for a body fluid identification assay.

We have previously described a method for determining the tissue type of body fluids typically encountered at crime scenes using DNA methylation analysis of tissue-specific regions of the genome, followed by Pyrosequencing (2–7). The basis of the approach is that different tissues may express different methylation patterns at certain loci. For example, at a specific locus, methylation levels may be very low in sperm cells, but high in blood, saliva and other tissues. In this way, a Pyrosequencing assay can be designed for each locus to measure the relative level of methylation in the tested DNA sample.

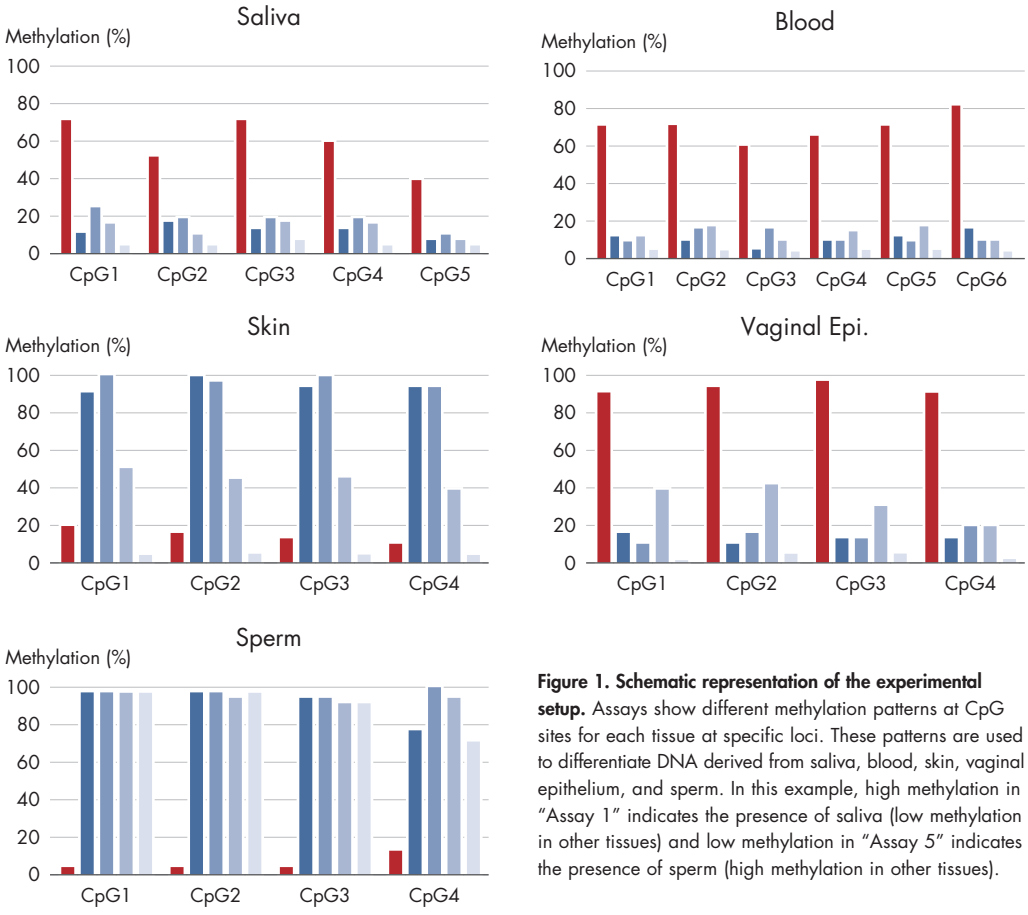

Figure 1. Schematic representation of the experimental setup. Assays show different methylation patterns at CpG sites for each tissue at specific loci. These patterns are used to differentiate DNA derived from saliva, blood, skin, vaginal epithelium, and sperm. In this example, high methylation in "Assay 1" indicates the presence of saliva (low methylation in other tissues) and low methylation in "Assay 5" indicates the presence of sperm (high methylation in other tissues).

We now asked whether we can positively identify sperm cell DNA with the sensitivity that correlates with STR sensitivity. If so, we can establish a workflow whereby the presence of sperm can be confirmed by testing DNA after a STR profile has been developed. The adoption of such a workflow would streamline sexual assault sample processing by removing the need for presumptive serological and laborious microscopic slide screening methods.

DNA methylation and Pyrosequencing®

Methylated cytosines typically occur at CpG dinucleotides with the presence of a methyl group at the C-5 position of the molecule. The methylation status of each cytosine can be identified by bisulfite treatment of the extracted DNA, followed by Pyrosequencing. The bisulfite conversion treatment is the basis of the assay. Extracted DNA is treated with a bisulfite buffer and incubated. During this incubation, non-methylated cytosines present at CpG sites are converted to uracils, which are replaced as thymines during PCR. Methylated cytosines present at these same positions are protected from the conversion and remain unchanged. Thus, the ratio of cytosines to thymines at these CpG sites determines the methylation values. A high ratio of cytosines to thymines would indicate a high level of methylation at that locus.

The Pyrosequencing method is based on the sequencing-by-synthesis principle, utilizing an enzymatic cascade and bioluminescence. In brief, a sequencing primer is hybridized to a single-stranded, PCR-amplified DNA template, which is incubated with enzymes and substrates. With each dispensation, one of the four nucleotides is added to the reaction. If the nucleotide is complementary to the template strand, it will be incorporated into the DNA strand (DNA polymerase) accompanied by the release of pyrophosphate, which is converted into ATP (ATP sulfurylase) and in turn is used to generate a light signal (luciferase). Before adding the next nucleotide into the reaction, unincorporated nucleotides are degraded (apyrase). The generated light signal is proportional to the number of incorporated nucleotides and can therefore be used for sequence calling and variant detection such as DNA methylation (8).

Materials and methods

Samples

Aspermic semen and sperm-positive semen swab samples were purchased from the Serological Research Institute. In addition, samples were obtained from a known semen donor with informed consent and blood samples used in previous CTS tests as well as saliva samples were provided by a lab personnel.

Sample preparation and quantification

Samples were extracted on the EZ1® Advanced XL using the EZ1 DNA Investigator® Kit, following manufacturer's instructions. DNA extracts were quantified using the Applied Biosystems® Quantifiler® Trio Kit, following manufacturer's instructions.

Bisulfite conversion

This protocol uses the EpiTect® Fast DNA Bisulfite Kit which is designed for bisulfite conversion of 1–500 ng DNA in a volume of up to 40 µl. A mix of 85 µl of bisulfite solution and 15 µl of DNA protect buffer was added to wells in a 96-well PCR plate. Purified DNA was then added to this mix, up to a maximum volume of 40 µl per well. Where less than 40 µl of the DNA extract was ▷

added, samples were made up to this volume with Molecular Biology Grade water. The samples are vortexed and spun briefly before being placed onto a thermal cycler. The bisulfite conversion PCR program used is described in Table 1.

Table 1. Cycling parameters for bisulfite conversion using the EpiTect Fast DNA Bisulfite Kit.

Step	Time	Temperature
Denaturation	5 min	95°C
Incubation	20 min	60°C
Denaturation	5 min	95°C
Incubation	20 min	60°C
Hold	∞	20°C

Bisulfite cleanup

The QIAcube® was used to automate the bisulfite cleanup. However, this cleanup process can also be performed manually by following the manufacturer's protocols.

The QIAcube has two protocols for cleanup: the "Standard," or Cleanup of 100 ng or more bisulfite-converted DNA, and "Up to 100 ng DNA," or Cleanup of up to 100 ng bisulfite-converted DNA. The Up to 100 ng DNA protocol was selected. An aliquot of 310 µl of reconstituted (with Molecular Biology Grade water) cRNA was added to Buffer BL, 30 ml ethanol (96–100%) was added to Buffer BW, and 27 ml ethanol (96–100%) was added to Buffer BD. Reagent bottles were filled with the appropriate buffers and placed into their proper positions on the QIAcube:

a. Position 1: Buffer BL
b. Position 2: Buffer BW
c. Position 3: Buffer BD
d. Position 4: Buffer EB
e. Position 5: Ethanol (96–100%)

A 1.5 ml tube of reconstituted cRNA was inserted into Position A. Rotor adapters containing 1.5 ml elution tubes and MinElute® columns as well as 1000 µl and 200 µl tips were loaded onto the QIAcube.

Samples were then removed from the thermal cycler, transferred to 2 ml tubes, and loaded into appropriate positions on the shaker rack. Manufacturer's recommended tube type was used to ensure proper fitting when placed in the QIAcube.

PyroMark PCR setup

A master mix of PyroMark PCR Master Mix and sperm-specific tissue ID primer ZC3H12D was prepared (see Table 2 for other tissue markers available from QIAGEN®).

Table 2. Primer sets available for tissue identification (only the ZC3H12D (sperm) marker was used in this study).

Tissue type	Primer set
Sperm	ZC3H12D
Saliva	BCAS4
Blood	Cg06379435
Vaginal epithelial cells	PFN3A

A total volume of 15 µl of master mix per sample was prepared using 12.5 µl of the PyroMark PCR Master Mix and 2.5 µl of the sperm primer ZC3H12D. When reconstituted, the sequencing primer is at a final concentration of 4 µM and the PCR primers are at a final concentration of 2.55 µM each. The master mix and 10 µl of bisulfite-converted DNA were added to appropriate wells on a 96-well PCR plate, which was then placed on the thermal cycler. The PyroMark Q48 Pyrosequencing PCR program used is outlined in Table 3.

Table 3. PyroMark Q48 PCR cycling parameters.

Step	Time	Temperature
Initial	15 min	95°C
Denaturing	30 s	94°C
Annealing	45 cycles 30 s	55°C
Extension	30 s	72°C
Final extension	10 min	72°C
Hold	∞	4°C

Pyrosequencing on the PyroMark Q48 Autoprep System

With the PyroMark Q48 Autoprep Software, an assay setup for each marker was created once, and this specifies the sequence to be analyzed in the tissue-specific region of the DNA and automatically generates a nucleotide dispensation order. For each tissue ID run, a run file was created specifying the samples to be analyzed and the specific control wells. The PyroMark Q48 Autoprep System (Figure 2) was used according to the manufacturer's instructions.

Figure 2. The PyroMark Q48 Autoprep System.

Results

Verification of sperm marker ZC3H12D specificity

The efficacy of the ZC3H12D sperm marker was verified by comparing results for this marker when used with sperm and saliva samples. Figure 3 shows the pyrograms for this study. The five ZC3H12D CpG positions, highlighted in the grey bars, show the different methylation results for the C nucleotide and the corresponding percentage methylation above the bars. The saliva sample exhibits significantly higher methylation, as the methylated C nucleotide inhibits expression of the sperm-specific ZC3H12D marker in the saliva sample.

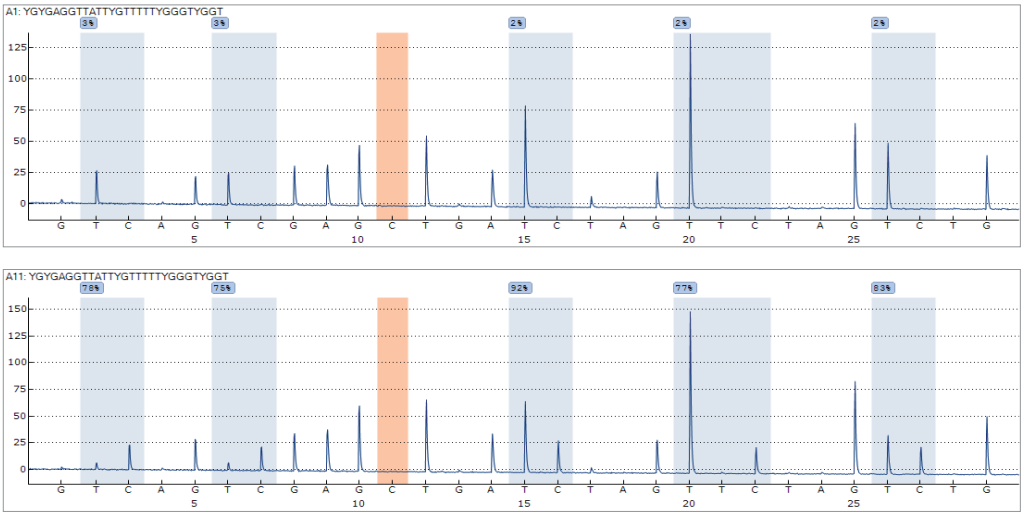

Figure 3. Pyrogram result for sperm and saliva samples amplified with primers for the sperm-specific ZC3H12D marker.

The expression of ZC3H12D was then compared between an aspermic semen sample and the sperm and non-sperm fractions of a semen sample. These results are shown in Figure 4. Aspermic semen showed a comparable level of methylation to the saliva sample in Figure 1 (as expected for a sample with no sperm), while the non-sperm fraction of a semen sample exhibited an intermediate level of methylation, most likely as a result of residual levels of sperm DNA in the fraction.

These results correspond well with those previously published by Silva et al. [2] for ZC3H12D.

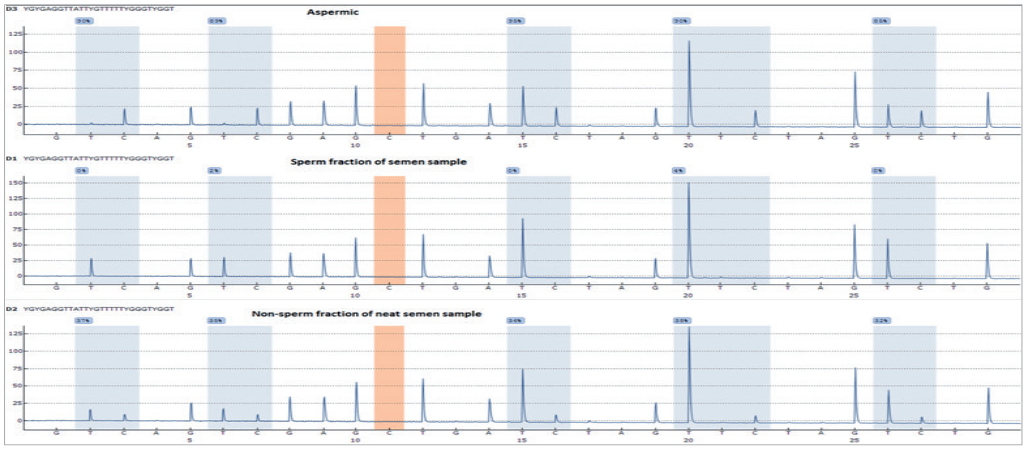

Figure 4. Pyrogram result for sperm and non-sperm fractions of a semen sample compared with an aspermic sample, when analyzed with the sperm-specific ZC3H12D marker.

Optimization of cycling parameters

Pyrosequencing is routinely used in various fields of life sciences other than human identity testing, where levels of input DNA for PCR are not limited. Because forensic samples typically have a finite amount of DNA, it is important to optimize the PCR enrichment step in the Pyrosequencing workflow when used on forensic casework samples to maximize the chances of success. To this end, we varied PCR cycling ramp rates for the tissue-specific marker PCR enrichment step, comparing 1°C/second with 2°C/second and 3°C/second, all of which are slower than the standard 6°C/second. The slowest ramp rate of 1°C/second was found to produce more optimum and reliable results for low level DNA samples (below 1 ng) (Figure 5) compared with the higher ramp rates and was used for further studies.

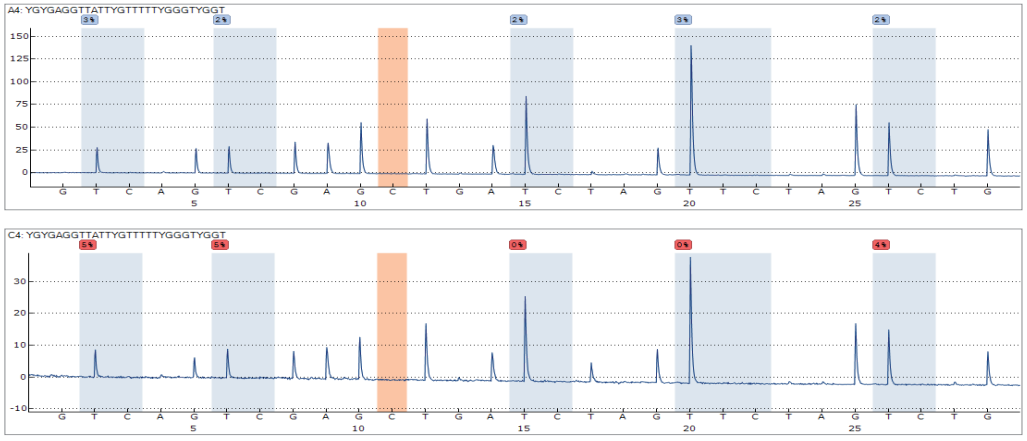

Figure 5. Pyrograms showing the effect of PCR ramp rate on Pyrosequencing. The slower ramp rate (1°C/second) produced more reliable results while the higher ramp rate (3°C/second) produced either no result or significantly elevated methylation percentages for the relevant ZC3H12D marker.

Sensitivity studies

Using the optimized cycling parameters, the sensitivity of the Pyrosequencing workflow was assessed using a dilution series of DNA extracted from semen samples. Results are shown in Figure 6 A–C. Results were obtained down to 0.05 ng but with slightly higher methylation values, indicative of a slight decrease in specificity and therefore accuracy. Silva et al. [2] identified 0.1 ng input DNA as providing good quality methylation profiles and the results shown here support this level of input DNA for optimal results. However, further studies are needed to confirm the accuracy of the methylation results for ZC3H12D in identifying sperm at 0.05 ng input DNA level.

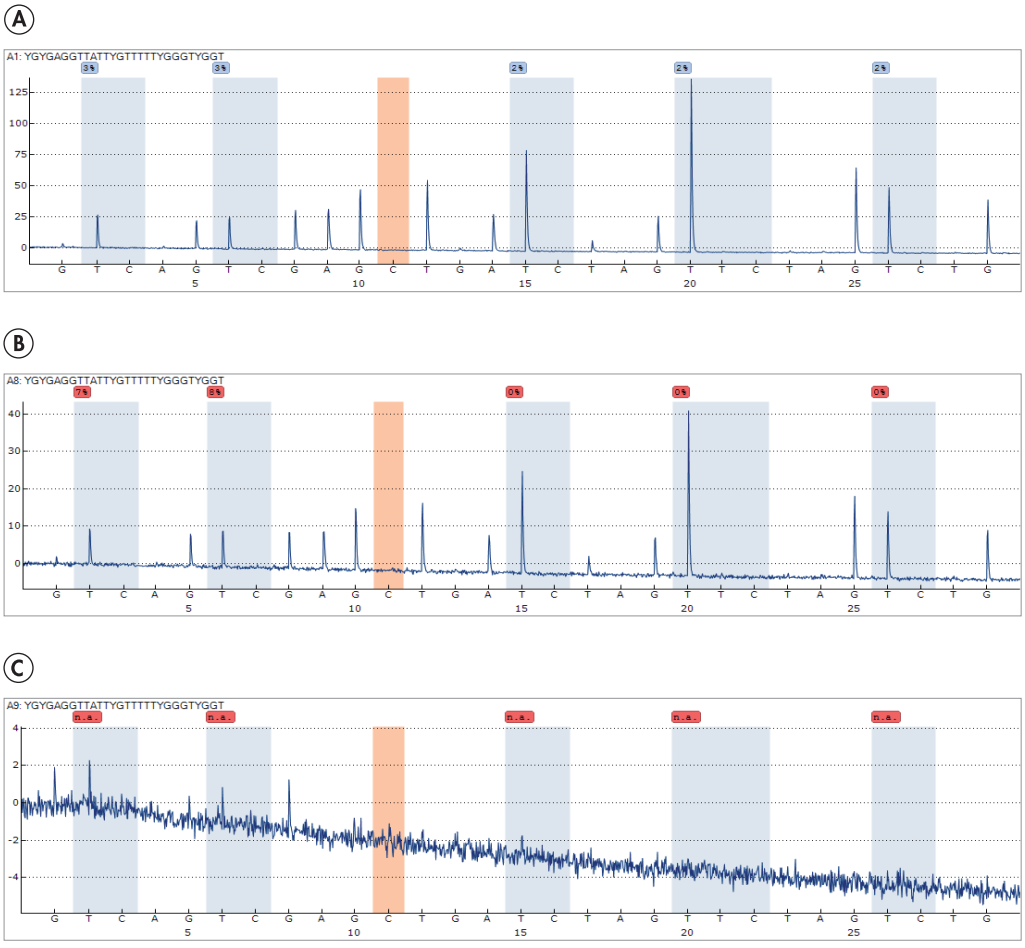

Figure 6. Effect of decreasing input DNA on Pyrosequencing results for semen samples using the ZC3H12D marker A **0.1 ng input DNA,** B **0.05 ng input DNA, and** C **0.01 ng input DNA).**

Conclusion

DNA profiles generated from crime scene samples are indispensable in the identification of criminal perpetrators. However, the strength of evidence associated with these DNA profiles can be significantly diminished if the profile is not accurately attributed to a body fluid or tissue type. DNA methylation and Pyrosequencing represent an accurate and reliable method for using the same DNA sample for both tissue type identification and subsequent DNA profiling, thereby enabling an association of the two results and increasing the strength of the evidence. Here we have described a DNA methylation and Pyrosequencing workflow that can be easily implemented into casework laboratories using the PyroMark Q48 Autoprep System and associated QIAGEN automation solutions. Furthermore, we have demonstrated the successful utilization of this workflow to characterize the sperm-specific marker ZC3H12D. The next steps will be to conduct a more detailed evaluation of accuracy at low DNA input levels (0.05 ng) and expansion of the study to include other markers for saliva, blood and vaginal epithelial cells.

Summary

- DNA methylation and Pyrosequencing using the PyroMark Q48 Autoprep System enable accurate and reliable sperm identification for forensic samples.
- A QIAGEN workflow comprising EZ1 Advanced XL, QIAcube and the PyroMark Q48 Autoprep System enable automation of this workflow, from DNA purification and bisulfite conversion through to data analysis, seamlessly integrating into the laboratory's current workflow.
- Utilizing DNA for tissue identification, as opposed to chemical presumptive tests, removes any uncertainty over the alignment of the tissue ID with the DNA profile and represents a paradigm shift in body fluid analysis for DNA profiling.
- Identification of sperm-derived DNA using the proposed method could enhance laboratory workflow efficiency during sexual assault sample processing.

Acknowledgment

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References

[1] Taylor, D., Abarno, D., Hicks, T., Champod, C. Evaluating forensic biology results given source level propositions. Forensic Science International: Genetics **21** (2016) 54–67

[2] Silva, D.S.B.S. et al. Developmental validation studies of epigenetic DNA methylation markers for the detection of blood, semen and saliva samples. Forensic Science International: Genetics **23** (2016) 55–63

[3] Antunes, J., Silva, D.S.B.S., Balamurugan, K., Duncan, G., Alho, C.S., McCord, B. Forensic discrimination of vaginal epithelia by DNA methylation analysis through pyrosequencing. Electrophoresis (2016) **37**(21):2751–2758

[4] Balamurugan, K., Bombardi, R., Duncan, G. and McCord, B. Identification of spermatozoa by tissue-specific differential DNA methylation using bisulfite modification and pyrosequencing. Electrophoresis (2014) **35**:1079–86

[5] Jung, S.E., Cho, S., Antunes, J., Gomes, I., Uchimoto, M.L., Oh, Y.N., Giacomo, L.D., Schneider, P.M., Park, M.S., van der Meer, D., Williams, G., McCord, B., Ahn, H.J., Choi, D.H., Lee, Y.H., Lee, S.D. and Lee, H.Y. A collaborative exercise on DNA methylation based body fluid typing. Electrophoresis (2016) **37**(21):2759–2766

[6] Madi, T., Balamurugan, K., Bombardi, R., Duncan, G., McCord, B. The determination of tissue-specific DNA methylation patterns in forensic biofluids using bisulfite modification and pyrosequencing. Electrophoresis (2012) **33**, 1736–1745

[7] Antunes, J., Balamurugan, K., Duncan, G., McCord, B. Tissue-specific DNA methylation patterns in forensic samples detected by Pyrosequencing. Methods in Molecular Biology (2015) **1315**, 397–409

[8] Ronaghi, M., Uhlén, M., Nyrén, P. A Sequencing Method Based on Real-Time Pyrophosphate. Science (1998) **281**:363–365

Ordering Information

Product	Contents	Cat. no.
QIAcube	Robotic workstation for automated purification of DNA, RNA or proteins using QIAGEN spin-column kits: includes 1-year warranty on parts and labor	Inquire
PyroMark Q48 Autoprep System	PyroMark Q48 Instrument, multistep pipet, software, documentation and installation	9002470
EZ1 Advanced XL, System	Robotic workstation for automated purification of nucleic acids from up to 14 samples using EZ1 Kits: includes installation, training, 1-year warranty on parts and labor	9001874
EZ1 DNA Investigator Kit	For 48 preps: Reagent Cartridge (DNA Investigator), Disposable Filter-Tips, Disposable Tip-Holders, Sample Tubes (2 ml), Elution Tubes (1.5 ml), Buffer G2, Proteinase K, Carrier RNA	47016
EpiTect Fast DNA Bisulfite Kit (200)	For 200 preps: Bisulfite Solution, DNA Protect Buffer, MinElute DNA Spin Columns, Carrier RNA, and Buffers	59826
PyroMark PCR Kit (200)	For 200 reactions: 2x PyroMark PCR Master Mix (includes HotStarTaq DNA Polymerase and optimized PyroMark Reaction Buffer containing 3 mM $MgCl_2$ and dNTPs), 10x CoralLoad Concentrate, 5x Q-Solution, 25 mM $MgCl_2$, and RNase-Free Water	47016

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