Use of FTA® classic cards for epigenetic analysis of sperm DNA

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FTA® technologies provide the most reliable method for DNA extraction. Although FTA technologies have been widely used for genetic analysis, there is no literature on their use for epigenetic analysis yet. We present for the first time, a simple method for quantitative methylation assessment based on sperm cells stored on Whatman FTA classic cards. Specifically, elution of seminal DNA from FTA classic cards was successfully tested with an elution buffer and an incubation step in a thermocycler. The eluted DNA was bisulfite converted, amplified by PCR, and a region of interest was pyrosequenced.

Our research developed in order to address the need to perform high-throughput screening of seminal samples. For convenience given the vast number of samples required, it was deemed suitable to use Flinders Technology Associates (Whatman FTA®) classic cards. This not only provides the most reliable method for DNA storage and extraction, but also allows researchers to reanalyze the samples at a later stage since samples can be stored for up to 20 years. Samples may also be stored and transported at room temperature in areas where cold storage and transport are not available, or when the transport of biohazardous agents is hindered by international regulations.

FTA classic cards are impregnated with a patented chemical formula that lyses cell membranes and denatures proteins on contact. Nucleic acids are physically entrapped, immobilized, and stabilized for storage at room temperature. FTA classic cards also protect nucleic acids from nucleases, oxidation, UV damage, and microbial and fungal attacks. Infectious pathogens, including blood-borne pathogens and seminal pathogens are inactivated on contact with the card matrix. With FTA classic cards, DNA remains tightly bound while proteins and inhibitors are washed away from the matrix, enabling them to be used directly for PCR amplification. The same card pieces may later be reused for additional PCR reactions. It is also possible to successfully extract sufficient DNA from previously processed FTA cards stored at 4°C for up to 1 year (1).

Because of all these features, FTA classic cards have been widely used in forensic laboratories that need to process a great number and wide variety of crime scene samples for DNA analysis (2). FTA classic card-based technologies are also desirable for common use in assisted reproduction laboratories where demand for genetic and epigenetic analysis of gametes and embryos is increasing.

In the existing literature, FTA classic cards are used for blood, saliva, and sperm DNA extraction (1,3,4). The mammalian spermatozoa is one of the most specialized haploid cells, basically consisting of a condensed nucleus, very little cytoplasm, and an extremely long flagellum; overall, it has a hydrodynamic shape. Sperm chromatin is mostly condensed by protamine, with the remaining part condensed by histones(5). Spermatozoa possess germ cell-specific epigenetic patterns involving DNA methylation and histone to protamine transitions during spermatogenesis (6).

One important aspect of germ cell-specific epigenetic patterns is genomic imprinting. It was recently demonstrated in spermatozoa that hypermethylation of the maternally imprinted gene *MEST* and hypomethylation of the paternally imprinted gene *H19* were significantly associated with decreased sperm counts. Specifically for *MEST*, hypermethylation was also

METHOD SUMMARY

Sperm cell DNA can be successfully eluted from FTA® Classic Cards and analyzed for DNA methylation. The DNA elution step takes only 55 minutes using an elution buffer containing proteinase K, after which DNA can be purified by phenol chloroform extraction before bisulfite conversion and pyrosequencing.

associated with decreased sperm motility and abnormal morphology (7).

Since epigenetic alterations do not induce modifications in the gene sequence or copy number, they could potentially account for idiopathic infertility cases in which no genetic abnormality is detected using conventional genetic analysis techniques (8). DNA bisulfite conversion represents a viable method for detecting CpG methylation since, in bisulfite-treated DNA samples, cytosine turns to uracil while 5' methyl-cytosine remains unchanged.

Until now, FTA classic card-based technologies have been used just for genetic analysis (4,9), but no evidence of their suitability for epigenetic analysis is known. Previously, successful epigenetic analysis (methylome profiling) has been performed from samples collected on Guthrie cards (10,11). Unlike FTA technology, these methods do not directly extract DNA from cells, but actually require a prior lysis step to release the DNA into solution.

We present here for the first time, a simple method for methylation assessment based on sperm cells stored on FTA classic cards. Using this approach, we successfully reduced the time for analysis since the cell lysis starts when the cells are placed on the FTA classic card; the protocol we set up involves eluting DNA from the FTA card as bisulfite conversion does not happen on the DNA stored on the card, and eliminating cellular and protein debris through a subsequent purification. We then analyzed DNA methylation status of the *MEST* gene since it is a putative epigenetic biomarker of infertility.

Materials and methods

Semen samples

All subjects provided written informed consent and agreed to the analysis of genetic material as approved by the University of Parma Ethics Committee (reference number of 29284, 05/09/2015). 50 semen samples were collected from different male subjects who donated them to the Medically Assisted Procreation Center (University Hospital of Parma). Semen samples were obtained after a sexual abstinence lasting between 2 and 5 days. After liquefaction at 37°C for 30 min, semen parameters were measured as specified by the WHO laboratory manual directives (12).

Sample collection

Swim-up purification of all semen samples was performed to avoid contamination by somatic cells. Semen was diluted 1:1 in Sperm Preparation Medium (Origio, Målov, Denmark), centrifuged (390 x g, 10 min), then the supernatant was removed. The pellet was then washed in 2 ml Sperm Preparation Medium (390 x g, 10 min). After washing, 1 ml Sperm Preparation Medium was slowly added to the pellet and incubated for 1 h at 37°C with 5% CO_2. In order to ensure that the swim-up purified sperm samples were not contaminated by somatic cells, samples were microscopically examined (Nikon Eclipse 50i, 400x). 500 µL of the supernatant containing motile spermatozoa was collected on FTA classic cards; semen samples were then distributed onto the card in a concentric circular motion within the spotted circle. The samples were air dried for about 1 h then stored for 1 year at room temperature.

Evaluation of DNA quality and its availability on FTA® classic cards

Following the protocol provided by Whatman for FTA® classic cards, polymerase chain reaction (PCR) was performed by inserting three 3mm disks directly into the PCR tubes without treatment of FTA® classic cards with DTT as suggested by Fujita and Kubo 2006 (9).

DNA elution methods

Three different methods were used to elute DNA from FTA classic cards. For all methods, eight 3 mm diameter circle punches were obtained from each sample using a Whatman™ Harris Micro Punch. The paper punch was cleaned between samples with 70% ethanol.

Method I. Sample spots were fixed by overlaying them with methanol—four drops delivered via Pasteur pipette three times—and allowed to air-dry in between (the first time at room temperature for 20 min and the next two times incubated at 37°C for 40 min each) (13). Using flat forceps, dry sample spots were transferred to a thin-walled 200 µl PCR tube. Elution was performed by adding either an elution buffer containing or not containing proteinase K (50 µg/ml). The two elution solutions contained, respectively: a) 5 µL 10X PCR buffer without $MgCl_2$* (Thermo Fisher Scientific), proteinase K at 50 µg/ml and water up to 50 µL final volume; or b) 5 µL 10X PCR buffer without $MgCl_2$* (Thermo Fisher Scientific) and 45 µL water. *Buffer Composition (10X): 200 mM Tris-HCl (pH 8.4), 500 mM KCl without $MgCl_2$.

The tubes were then subjected to one cycle of 60°C for 30 min, 99.9°C for 10 min, and then cooling to 4°C. The high temperature inactivates proteinase K. The FTA classic card was left in the tube with the eluent and stored at 4°C.

Method II. Sample spots were processed according to the Whatman FTA classic card protocol, which involved first washing with FTA Purification Reagent and then rinsing with TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8). Using this procedure, DNA should remain tightly bound while proteins and inhibitors are washed from the matrix. As previously described, the spot was subsequently fixed with methanol and genomic DNA eluted from the paper through a heat incubation step run on a thermal cycler.

Method III. This method consisted of just the elution step in the thermal cycler in the presence of the elution solution. Neither methanol fixation nor the Whatman FTA classic cards cleaning protocol were performed.

DNA quantity and quality

To evaluate the efficiency of the procedure in terms of DNA yield, the DNA concentration in all eluted DNA samples was measured by optical density (OD) at 260 nm (NanoDrop2000, Thermo Scientific). To evaluate the DNA purity the OD 260/280 ratio was also measured.

DNA amplification before bisulfite conversion

The DNA quality was tested by PCR amplification with two pairs of primers (metabion international AG, Planegg/Steinkirchen, Germany): one for the *XRCC1* gene and the other for paternally imprinted *MEST*. Both cycling conditions were performed with 10–100 ng per sample. Primer sequences and PCR conditions are reported in Table 1.

DNA purification

Since the FTA classic cards lyse cells, the step for cellular lysis was skipped and the eluted sample moved directly to the purification step. Regardless of the method used to elute DNA, we performed purification using the following protocol: DNA eluted from FTA classic cards in a final volume of 50 µl was transferred into new 1.5 ml tubes.

Table 1. Primers and PCR conditions.

Name	Primer sequence (5' to 3')	PCR conditions
XRCC1 rev	TGTCCCGCTCCTCTCAGTAG	95°C, 3 min, 25x (95° 30 s; 59°, 30 s; 72° C 30 s) 72°, 10 min
MEST rev	AGGAGTGACACCCCCTCCTCAAGT	95°C, 3 min, 40x (95° 30 s; 72°, 30 s; 72° C 30 s) 72°, 10 min
MEST rev bisulfite	Biotin-AAAAATAACACCCCCTCCTCAAAT	95°C, 3 min, 40x (95° 30 s; 60°, 30 s; 72° C 30 s) 72°, 10 min

The paper dishes were briefly re-washed with 50 µl of water, which was subsequently added to the previously eluted DNA.

Wizard® Genomic DNA Purification

DNA samples were subjected to the purification steps suggested by the Promega kit protocol. Given that the Wizard® Genomic DNA Purification–Promega kit does not contain a suitable protocol for sperm cells, the procedure was performed in accordance with two protocols suggested by the instruction manual, one specified for blood cells, and the other for cells and tissues.

Protein precipitation solution and DNA ethanol precipitation

A modified Wizard Genomic DNA Purification method for DNA purification was performed, using just Protein Precipitation Solution from the Promega kit. Specifically, in order to elute DNA from FTA classic cards, 200 µl of protein precipitation solution was added and the samples placed on ice for 5 min, after which, they were vortexed and centrifuged for 30 min at 4°C (13000 x g). Following this procedure, the proteins remain as pellets in the tube and the supernatant contains the DNA, which can be precipitated with ethanol.

Phenol-chloroform and ethanol DNA precipitation

Protein precipitation was performed with Phenol:Chloroform:Isoamyl Alcohol 25:24:1 (Sigma). DNA precipitation was carried out with 2x volume of ice cold 96% ethanol, 1/10 of sodium acetate (3M; pH 5.2) and 1 µl of glycogen (20 mg/ml). Finally, DNA was resuspended in 40 µl of Tris-HCL (10 mM; pH 8).

To evaluate the efficiency of the procedure, the DNA concentration was measured by measuring the optical density (OD) at 260 nm (NanoDrop2000, Thermo Scientific) in all the eluted samples. DNA purity was also evaluated by calculating the 260/280 OD ratio.

DNA bisulfite conversion

Sperm DNA eluted from FTA classic cards was bisulfite converted with the EpiTect Fast DNA Bisulfite kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The low concentration samples protocol (1–500 ng) was followed for all samples. Because of the low DNA yield (lower than 100 ng), clean-up of converted DNA was performed using carrier RNA.

DNA amplification after bisulfite conversion

PCR amplification of bisulfite converted-DNA was performed using a biotinylated reverse primer and a forward primer (respectively MEST rev bisulfite and MEST fwd bisulfite, GenBank accession no. Y10620, nucleotides 609–898, see Table 1). The PCR mixture (25 µL) contained 1X reaction buffer (Invitrogen™), 1.5 mm $MgCl_2$, 100 µm of each dNTP (Promega), primers (metabion international AG), 1.5 U Invitrogen™ Platinum™ Taq DNA Polymerase, and 4 µL bisulfite converted DNA (about 5ng/µl). PCR conditions were 3 min at 95°C; 40 cycles of denaturation (95°C; 30 sec), primer annealing (60°C; 30 sec), elongation (72°C, 30 sec), and a final extension at 72°C (10 min). Methylated and unmethylated DNA was used as controls (EpiTect PCR control DNA set, Qiagen Srl, Milan, Italy).

Pyrosequencing

Quantitative methylation analysis of a portion of the *MEST* gene was performed on DNA prepared as described above and sequenced on a PyroMark Q96 ID platform (Qiagen Srl, Milan, Italy). Briefly, the biotinylated PCR products were captured and annealed to a sequencing primer. The pyrosequencing reaction was performed with Pyromark gold Q96 reagents (Qiagen Srl, Milan, Italy) encompassing a region containing 6 CpG sites.

Global DNA methylation

The global DNA methylation status of 5 semen samples, freshly collected or stored on FTA classic cards, was detected using MethylFlash™ (Epi-gentek Group, Farmingdale, NY, USA), following the instructions of the manufacturer. The OD of DNA was normalized to 200 ng.

Results and discussion

The use of FTA classic cards allows the transport of semen samples at room temperature as well as storage of the samples for as long as 20 years. Using our approach, once DNA has been collected on FTA classic cards, it is possible to analyze the methylation status of the gene of interest at a later date.

Methylation analysis of sperm DNA could be used as diagnostic and prognostic biomarker of fertility for assisted fertilization techniques. Our study provides a distinctive contribution by offering a convenient and simple analytical method that is easy to perform. This approach can be implemented in geographically distant laboratories, eliminating the problems associated with biological sample transportation. Our procedure allows repeated testing over an extended time-span of many years following sample collection, especially because it has been demonstrated that methylation of sperm DNA is not subject to change over time (14). Since assisted fertilization techniques have increasingly shown problems arising after a significant time-span (15), it becomes increasingly important to monitor the methylation status of germ cell genes.

Taking into account the variability of sperm production in humans, we evaluated different samples collected following the

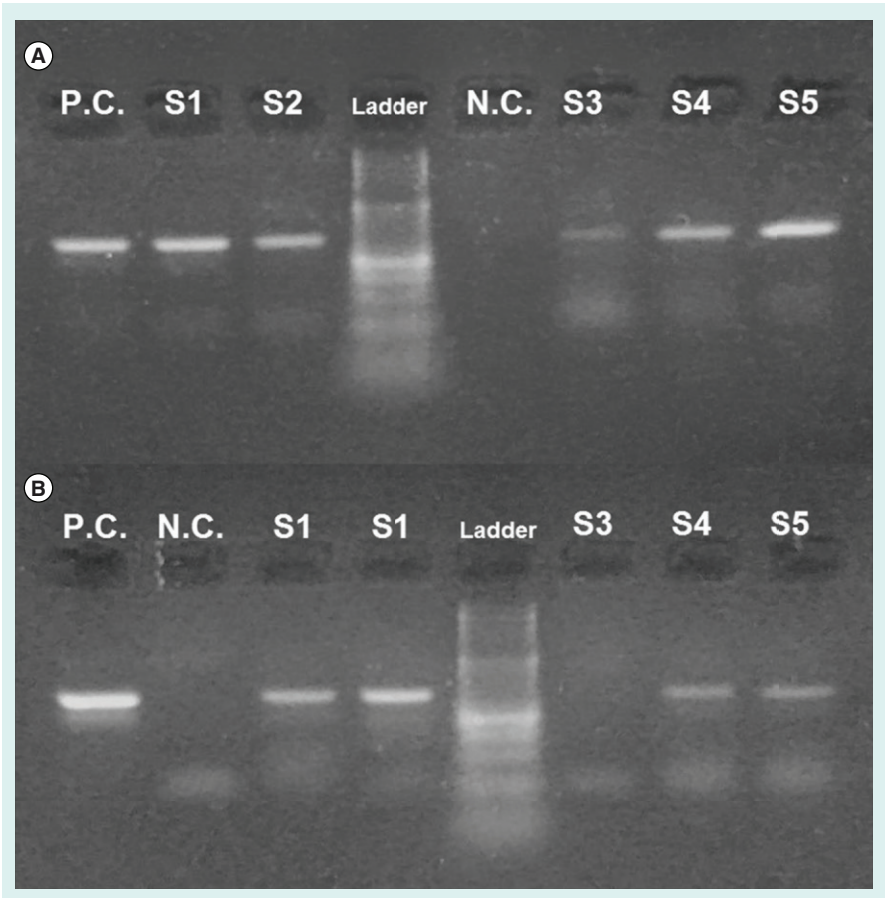

Figure 1. Elution solutions prepared with (A) and without (B) proteinase K. The same numbers represent the same sample under the two different conditions. N.C.: Negative control; P.C.: Positive control.

Table 2. Comparison between the three different DNA elution methods. DNA concentrations shown are an average of 50 independent samples.

	Method I	Method II	Method III
OD 260/280 nm SD	0.40 0.06	0.55 0.34	0.40 0.04

WHO directives (12). We analyzed 50 semen samples from different subjects that were stored on FTA classic cards. DNA quality was tested by PCR amplification of DNA from three disks directly placed in the PCR tubes using primers for *XRCC1* and *MEST* genes (data not shown). To develop a suitable DNA bisulfite conversion protocol, three different methods of DNA elution from FTA classic cards were assessed. Evaluating the amount of sperm DNA derived from FTA cards is not reliable since the number of spermatozoa varied between samples. Moreover, the distribution of seminal fluid on the card surface is unlikely to be homogeneous, generating variability when cutting the disks. The DNA yields of methods I and III were comparable, while method II performed worse; given the initial inadequacy of method II, only six samples were tested with this method (Table 2). Removing the methanol treatment resulted in faster elution without DNA loss (Method III). All methods were performed in the presence of proteinase K , which was shown to help DNA elution (Figure 1). Nevertheless, evaluation of the 260/280 nm ratio evidenced a possible protein contamination.

DNA was of sufficient quality for amplification, meaning that for common amplifications, it is not necessary to purify DNA eluted from Whatman® FTA cards. It is possible to directly convert the eluted DNA with sodium bisulphite (data not shown), but the yield is not always sufficient due to the aforementioned high intra- and inter-individual variability. To convert DNA with sodium bisulfite, it is necessary to know the concentration of the template DNA; if it is contaminated with proteins, no reliable result will be obtained. The EpiTect Fast DNA Bisulfite kit states that the starting DNA has to have a 260/280 OD ratio higher than or equal to 1.7. To allow the conversion of sperm DNA with sodium bisulfite, it was therefore necessary to purify the DNA in order to improve the 260/280 OD ratio. Treatment with phenol–chloroform showed the most effective protein removal, as evidenced by the OD 260/280 ratio (Table 3). Standard deviation is again high because of the starting material (seminal fluid) and, consequently, the DNA yield is varies between different subjects.

According to previous research, *MEST* gene primers (see Table 1) were chosen in an area rich in CpG sites in the 5' UTR (7). Amplification using primers for bisulfite-converted DNA was efficient for almost all samples with only a few showing inefficient amplification. Figure 2 shows some of the samples we amplified; S stands for sperm, while the numbers correspond to different subjects. Samples S2-3-4-7-8 show a consistent amplification whereas S1 and S5 show low and no amplification, respectively. Using a higher concentration of eluted DNA while performing the PCR was sufficient to solve this problem.

In order to demonstrate the advantage of the procedures described above for epigenetic analysis, quantitative methylation of the imprinted gene *MEST* was performed. As shown in Supplementary Table S1, all the samples showed little to no methylation in the considered region. Sample names refer to the amplicons shown in Figure 2. Reproducibility of the technique was established by analyzing five samples in duplicate; each replicate was the result of an independent elution (Supplementary Tables S2). Furthermore, five samples were analyzed both when fresh and after collection from FTA classic cards to verify that there was no variation in methylation (Supplementary Table S3).

Table 3. Comparison between the three DNA purification methods.

	Wizard® Genomic DNA Purification kit	Protein precipitation	Phenol–chloroform
OD 260/280 nm	0.97	0.85	1.73
SD	0.05	0.54	0.23

Analysis of the concentration (ng/µl) of sperm DNA eluted with Method III and purified through three different protocols (OD 260nm). DNA concentration was evaluated by NanoDrop2000, Thermo Scientific. Values are representative of the average of three different measurements; DNA purity was also calculated by analyzing the 260/280 OD ratio. Low values indicate protein contamination.

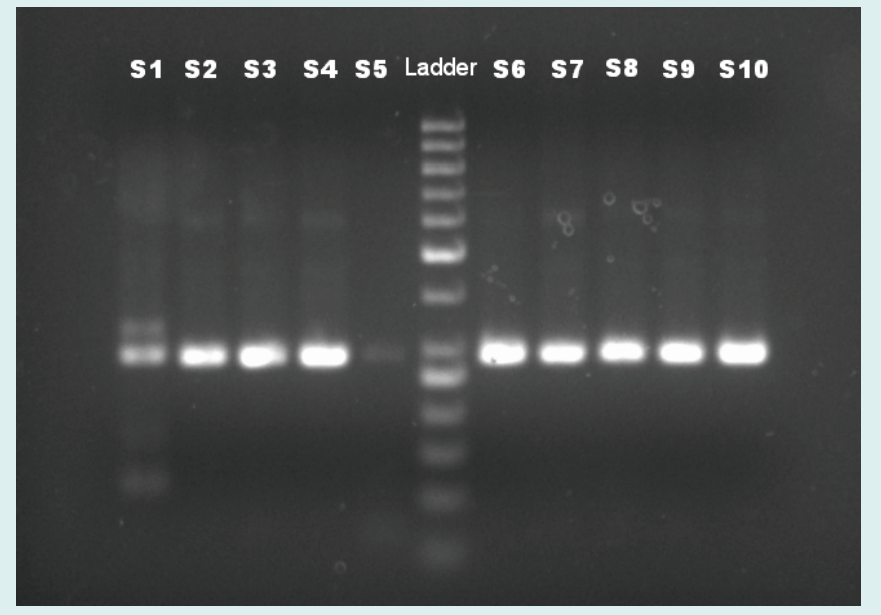

Figure 2. PCR amplification efficiency for the MEST gene after bisulfite conversion.

Methylation analysis of the MEST gene through bisulfite conversion and pyrosequencing was carried out successfully, demonstrating that it is possible to collect semen samples on FTA classic cards and then use them to perform epigenetic analysis (specifically methylation analysis of CpG sites).

Also, the global methylation level was comparable between the fresh samples and those stored on FTA Cards (Supplementary Table S4), showing an interesting correspondence between the level of methylation of the promoter of the *MEST* gene and the level of global methylation.

Finally, we think that this method could work well even for other cell types, as we have also tested it on buccal mucosa samples, with positive results (data not shown).

Author contributions

O.S. and A.B. designed the study. O.S., R.F., and A.P. carried out the experiments. O.S. and R.F. carried out data analysis. L.B. provided sperm samples. O.S. drafted the manuscript. All authors contributed to the critical revision and final version of the paper. A.B. supervised the project.

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Competing interests

The authors declare no competing interests.

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