



The author(s) shown below used Federal funding provided by the U.S. Department of Justice to prepare the following resource:

Document Title: Development of a Real-time PCR Assay for the Detection of Blood, Saliva, and Semen

Author(s): Cynthia B. Zeller, Ph.D.

Document Number: 251633

Date Received: April 2018

Award Number: 2008-DN-BX-K137

This resource has not been published by the U.S. Department of Justice. This resource is being made publically available through the Office of Justice Programs' National Criminal Justice Reference Service.

Opinions or points of view expressed are those of the author(s) and do not necessarily reflect the official position or policies of the U.S. Department of Justice.

NATIONAL INSTITUTE OF JUSTICE

2008-DN-BX-K137

Development of a Real-time PCR Assay for the Detection of
Blood, Saliva and Semen

Cynthia Zeller, Ph.D.
czeller@towson.edu
410-704-2170

EIN: 526002033

DUNS: 143372741

Towson University
College of Graduate Studies and Research
and
Fisher College of Science and Mathematics
Department of Chemistry
Forensic Science Program
8000 York Rd.
Towson, MD 21252

PROJECT START DATE: November 1, 2008

FINAL REPORT

February 28, 2013

Cynthia B. Zeller, Ph.D
Principal Investigator

ABSTRACT

During the process of forensic DNA genotyping, evidence containing potential DNA evidence undergoes serological screening to determine which body fluid, if any, it contains. The majority of the DNA genotyping process has undergone automation, leaving a bottleneck at the serological analysis stage. This is due in large part to the methods employed to identify body fluids. Since most of the commonly used assays test for the presence or function of a particular protein prevalent in a specific body fluid, these tests cannot be multiplexed and therefore can use a fairly large portion of a stain if multiple body fluids need to be identified. The purpose of this study was to attempt to develop an assay for the detection of sperm, blood and saliva that could be multiplexed, save time and sample and could be dovetailed into the current workflow of a forensic DNA laboratory.

The method of choice for this study was the development of a methylation specific PCR assay base on a dual labeled TaqMan® probe. Two assays for the identification of semen were generated, each with appropriate tissue specificity, single cell sensitivity in single source samples and in mixtures. The thought was that individual assays for the detection of sperm, blood and saliva could be multiplexed by utilized different 5'dyes. Unfortunately, this did not come to fruition since none of the primer sets tested for the detection of blood and saliva were specific enough to be used for identification. Although the ultimate goal of this project was not reached, the two assays for the detection of sperm provide a proof of concept for this type of assay. With the advent of more information about the epigenome of normal tissues, this type of testing could provide a quick, sensitive detection system for multiple body fluids in the forensic laboratory.

Table of Contents

ABSTRACT	2
Table of Contents	3
Executive Summary:	4
Synopsis of the problem and purpose of this research:.....	4
Research design	5
Findings and conclusions	5
Implications for policy and practice.....	6
Technical Report:	7
Goals of the Project:.....	7
Literature Review:.....	9
Why perform serology testing?	9
Methods for Body fluid Identification:.....	9
Role of Methylation of DNA in Gene Expression:	11
Materials and Methods	14
Bioinformatics	14
Body Fluid Samples	16
Bisulfite treatment of DNA	16
Quantitative PCR.....	16
Results:.....	18
Developmental Pluripotency Antigen 5 (DPPA5):.....	18
Figure 1: Tissue specificity of DPPA5 assay for the detection of semen	19
Figure 2: Species specificity of DPPA5 Assay for the detection of sperm.....	20
Figure 3: Sensitivity of the DPPA5 assay for the detection of sperm	21
Figure 4: Detection of sperm DNA in the presence of DNA from other body fluids	22
Figure 5: Detection of sperm cells in samples of mixed body fluids.....	24
Adenosine Deaminase Domain Containing 1 (ADAD1):.....	24
Figure 6: Tissue specificity of ADAD1 assay for the detection of sperm	25
Figure 7: Species specificity of ADAD1 for the detection of sperm	26
Figure 8: Sensitivity of the ADAD1 assay for the detection of sperm cell	27
Figure 9: Detection of Sperm DNA in the presence of Blood or Saliva DNA	28
Microseminoprotein-Beta (MSMB).....	28
Figure 10: Tissue Specificity of the MSMB assay for the detection of semen	29
Figure 11: Sensitivity of the MSMB assay for the detection of semen.....	30
Other gene assays:.....	31
Group I Primers: MSMB-EK2, WFDC, SKP2, UPK3A #1-2 and MMP9	31
Group II Primers: UPK3A #3-4, CD52, SPINT2 #1-3.....	32
Group III Primers: MSMB-EK and MSMB-SC.....	33
Discussion.....	35
Table 1: Primers and Probes Used in this Study	36
Bibliography.....	42

Executive Summary:

Synopsis of the problem and purpose of this research:

When dealing with forensic evidence, time is often of the essence either because a suspect has been identified and a court date is eminent or because the perpetrator is not known and is roaming free in the population to perhaps strike again (1). Forensic DNA analysis has become a victim of its success. Once reserved for only the most violent crimes, genotyping has found utility in all types of crimes from property crimes to heinous crimes leading to an increasing backlog of evidence (1). A typical piece of biological evidence from a crime scene must undergo several types of analyses and procedures prior to the generation of a DNA profile that can be used to identify a match, including serological analyses for the identification the body fluids, DNA extraction, quantitation, amplification, separation, visualization, interpretation and statistical analysis. Initial steps taken to attempt to alleviate this backlog have focused on increased personnel and automation in the stages of the analysis from DNA extraction to interpretation and statistical analysis. (2). The modifications to this part of the process have resulted in eliminating the backlog that existed at the end of the procedure, but have resulted in the formation of a new bottleneck. Currently, one of the biggest detractors to the timely analysis of DNA based evidence is not the DNA genotyping itself, but the serological identification of body fluids that precedes it. (2)

The purpose of this research was to develop a fully automatable, species specific, quantitative method for the identification of body fluids in forensic samples in order to provide the forensic community with a method that could help to diminish the bottleneck that has been demonstrated at the stage of serological testing.

Research design

In order to develop an assay that incorporated all of the characteristics necessary in a newly developed procedure, including high sensitivity, high specificity, ease of use and relatively low cost; the method of choice was the use of a quantitative methylation specific TaqMan® assay. This type of assay utilizes procedures and equipment currently in use in forensic laboratories across the nation. By taking a portion of a DNA extract, treating it with sodium bisulfite to distinguish between methylated and non-methylated cytosine, then using DNA primers and labeled DNA probes which preferentially anneal to the treated DNA in a polymerase chain reaction, it is possible to quantitatively detect very small quantities of tissue specific DNA, thereby identifying tissues which typify blood fluids of interest in a forensic case.

As the three most commonly identified body fluids are semen, blood and saliva, the study was restricted to these body fluids. It is anticipated that once the proof of concept has been demonstrated that other body fluids could and should be add to this group of body fluids. Additional testing and validation will be required prior to this technique being implemented in forensic casework.

Findings and conclusions

Several genes proved to be sufficient for the identification of sperm cells under conditions that mimicked conditions that might be seen in forensic casework. DPPA5 and ADAD1 proved to be as sensitive as the gold standard for the identification of sperm in that they were able to detect single sperm cells even in the presence of an overwhelming excess of other cell types. This type of assay allows for the quantitation of sperm numbers over at least a

5000 fold range, easily and quickly and in a format which is easily automated, reducing hands-on analyst time, while maintaining the same high standards for sperm identification. The assays for the identification of saliva and blood, proved more problematic in that it was quite difficult to find genes that would allow for specific identification of these body fluids. The methylation specific TaqMan® assay proved to be a sensitive assay for the identification of sperm cells, however identification of blood and saliva were not possible utilizing the primers and probes generated in this study.

Implications for policy and practice

This procedure could be one way in which the backlog of samples in the queue for forensic DNA testing is reduced. By providing an automatable method that can identify sperm in even very low quantities, this technique could provide a method to reduce the number of analyst hours spent on the identification of sperm and allow the analyst to use that time on additional tasks that can result in an increase in productivity without an increase in staff or costly equipment.

Technical Report:

Goals of the Project:

The purpose of this project was to gather data for the development of a set of genomic DNA-based Real-Time PCR assays for the identification of forensically relevant biological fluids, including blood, semen and saliva from evidentiary samples. By performing serological testing using a genomic DNA based molecular biological technique as opposed to using traditional protein based techniques, it is anticipated that a forensic DNA laboratory could perform a single preparatory step, DNA extraction, and use this extracted DNA as a substrate for all downstream steps in the identification and individualization of the samples. This approach should allow for (1) identification of body fluids included in the sample, (2) human and/or human male quantitation, as well as (3) genotyping from the same extracted DNA. It was the goal of this project to develop individual assays for the detection of human sperm, blood or saliva, which could function even in the presence of other cell types from other body fluids. These assays should match or exceed the sensitivity and specificity of current serological procedures commonly used in the forensic community.

The final objective of the proposal was to multiplex the individual methylation specific quantitative PCR assays developed allowing for the simultaneous detection of the presence of blood, sperm and saliva in up to 96 samples at a time, including positive and negative controls and reagent blanks, utilizing equipment currently in place in forensic laboratories in order to dovetail into the current quantitation and genotyping technologies which could significantly reduce the amount of time and sample necessary for the identification of body fluids in evidentiary stains.

Statement of Problem:

As DNA genotyping has demonstrated its utility, the success of the technique has created a burden in many crime laboratories. Once reserved for only the most violent crimes, many different types of cases including property crimes have been subjected to DNA genotyping, resulting in an inability for many crime laboratories to keep up with the workload. As the backlog of cases increases, the time from collection of sample and submission to the laboratory to production of the final DNA report increases, which may have a significant impact on public safety (1). Evidence may sit waiting for analysis allowing perpetrators to remain free to commit other, perhaps increasingly violent crimes (3). In most laboratories the process of biological evidence analysis involves a workflow that starts with visualizing stains followed by serological testing of stains to identify body fluids then DNA genotyping analysis for individualization of the sample. To attempt to alleviate the backlog, initial efforts have been targeted to automating the process from the point of DNA extraction to final analysis (1, 4). These efforts have allowed for a significant decrease in the time it takes to perform DNA genotyping on a sample, but has revealed a bottleneck in the process at the stage of serological testing. Since most of this process requires hands-on attention of the analyst, it can be time- and sample- consuming (2). The most commonly used testing practices for blood, semen and saliva are based on identification of proteins, either through structure or function. These tests require fairly large amounts of sample, particularly when samples must be tested for multiple body fluids (5)

Literature Review:

Why perform serology testing?

With the advent of DNA testing procedures that can accurately and efficiently determine the genotype of the donor, why even bother with serological testing? Serological testing answers questions that genotyping does not. While genotyping allows for the identification of WHO had contact, it does not give any information about the type of contact. Serological analysis does this. From a criminal justice point of view it can mean the difference between the types of charges that are justified. It is the serological results that can corroborate a witness statement about the TYPE of interaction; genotyping results can be used to identify the parties involved.

Methods for Body fluid Identification:

The most commonly used methods for the identification of body fluids in crime laboratories rely on the identification of proteins that are specifically found in the body fluid in question. Since proteins perform most of the critical functions in these body fluids they make reasonable targets for identification of these fluids. Common targets are hemoglobin for blood (6), alpha amylase for saliva (7), and acid phosphatase (8, 9) or P-30 (prostate specific antigen-PSA) for semen (10). Testing for body fluids by detection of these proteins can use a fairly large portion of sample in addition to the amount of sample that is necessary for genotyping. Typical assays use a sample size 9 mm² to 25mm²(11). In order to test for multiple body fluids, multiple

tests must be performed; thus using additional sample and increasing the time of analysis.

If one recalls that the central dogma of biology describes that genomic DNA is transcribed to mRNA, which is then translated to protein (12), it can be easily understood that instead of using proteins to identify body fluids it is possible to identify them by looking to nucleic acids, both RNA and DNA. By looking at the mRNAs and other small RNAs that are unique to cell types it is possible to reduce the amount of analysis time and sample required for testing have utilized a shift in approach, by using nucleic acids for testing instead of proteins (12). Most of the published methods use either mRNAs or panels of miRNAs in order to identify body fluids. (13-19).

These RNA-based approaches allow for automation of the process, reducing the hands-on time of the analyst, however the use of RNA as a template requires special handling and techniques that may not be easily integrated into the workflow of a typical crime laboratory. The mRNA based approaches use panels of genes to infer the origin of the body fluid present. Since some of the targets appear in multiple fluids, the identity of a particular body fluid is predicted by the presence of groups of mRNAs or miRNAs and performing multivariate statistics in order to place the body fluid in a predetermined group.

We chose to develop an assay that that could be simpler to perform from a technical as well as a statistical standpoint. By using genomic DNA, which can be more stable than RNA under the unfavorable conditions that many forensic samples exist (20), we attempted to use signals such as methylation patterns that are used by cells to determine which genes are transcribed to mRNA and ultimately translated to proteins. Methylation patterns have been long recognized as one method used by cells to control gene expression in a tissue-specific or

developmental stage-specific manner (21-24). Recent studies have used methylation specific PCR for the identification of forensically relevant body fluids, although these studies did not combine this approach with quantitative PCR (5, 25-27).

Role of Methylation of DNA in Gene Expression:

While methylation of genomic DNA does not alter the sequence of the nucleotides, it results in the modification of cytosine residues 5' to guanosine residues that results in the stable alteration of gene expression in many genes (24). Genomic DNA of many vertebrates, including humans, have specific regions of highly methylated DNA interspersed with regions of non-methylated DNA localized to the 5' untranslated region of housekeeping genes, developmentally- and tissue-specific genes. These unmethylated regions are characterized as having a high GC content with clusters of the dinucleotide CpG, and thus have been named CpG islands (CGI) (24, 28, 29).

Methylation of cytosines in this region can act to silence gene expression, while demethylation of 5mC in these regions has been shown to induce expression of previously silenced genes (29-31). Methylation acts as an epigenetic switch that can turn off the transcription of genes without altering the sequence of the genomic DNA. This can be particularly useful for genes that are expressed in a limited number of cell types or in genes that are only expressed during different developmental stages of cells. Genes that are under methylation control are methylated in non-expressing cells and unmethylated in expressing cells.

The production of 5 methyl cytosine (5mC) appears to be mediated by a family of enzymes, known as DNA methyltransferases (DNMTs), including DNMT1, DNMT3a, and

DNMT3b in mammals that catalyze the methylation of DNA through an S-adenosyl-methionine methyl donor(32). De novo methylation, catalyzed by DNMT3a and DNMT3b, is an important feature in the imprinting of methylation pattern during development (33, 34), differentiation {Illingworth {Aranyi (22, 23, 29, 35-41). DNMT1 supports the other methyltransferases in the maintenance of the methylation patterns once established (32). Production of 5mC has been associated with inhibition of DNA transcription by binding of transcription factors, inhibition of the formation of active chromatin structures, or by altering the locations of nucleosome binding. (21, 42). Although the mechanism for this inhibition is still being elucidated, it is clear that the methylation of cytosines in CGI causes a stable inhibition of gene expression through the inhibition of transcription. (29, 37)

DNA sequence analysis predicts that the percentage of CpG dinucleotides found in the genome is approximately 75% less than would be expected relying on the base composition of the genome, except in the CGI, where this motif is enriched. It has been proposed that the diminution of the CpG dinucleotides in the genome is due to the ease at which a 5mC is converted to thymine by deamination (24). There is a concomitant increase of TpGs and CpAs that bolsters 5mC to T conversion supposition.

The genomic DNA of pluripotent stem cells has been demonstrated to be primarily unmethylated, but upon cell differentiation, the overall level of methylation is increased through the activity of DNMTs that use CpG pairs as its target (32, 41, 43). Of interest to our project, this has been demonstrated in the differentiation of spermogenic germ cells to mature spermatozoa (44) and in the differentiation of erythroid progenitor cells to reticulocyte, the nucleated immediate precursor to the red blood cell(38, 45).

Genomic DNA may be a more useful molecule to identify a specific body fluid. Genomic DNA is found in every nucleated cell and possesses a number of epigenetic modifications that allow for tissue specificity, such as DNA methylation, histone modification, non-coding RNAs and higher order chromatin structure (34, 43). DNA methylation in particular can easily be exploited to link a genomic DNA sequence to a single body fluid type. In animals methylcytosine is mainly found in cytosine-guanine (CpG) dinucleotides. About 80% of all 5'...CpG...3' dinucleotides in mammalian genomes are found to be methylated, whereas the majority of the twenty percent that remain non-methylated are within promoters or in the first exons of genes (24). DNA methylation of cytosine residues plays a key role in the regulation of gene expression and generally leads to transcriptional silencing in non-expressing cells (23, 34, 46).

Two different types of methods are currently used to study the degree of methylation in DNA sequences: non-bisulfite methods and bisulfite modification methods (47). Non-bisulfite methods generally rely on methylation-sensitive restriction endonucleases combined with Southern blot or PCR analysis; however, results with this method are limited by the presence of corresponding cleavage sites in the gene and area of interest and generally require larger sample sizes than are typically used in forensic casework (48). Bisulfite modification methods overcome some of the issues associated with non-bisulfite methods. Bisulfite methods are also more conducive to forensic applications because they are rapid and require very small amounts of DNA sample (30, 49). Bisulfite treatment causes deamination of unmethylated cytosine to uracil, while leaving methylated cytosine unchanged. Following treatment, methylation-specific (MS)-PCR amplification is performed by engineering primers that anneal to either the

methylated or unmethylated form of the sequence to detect specific sequences and amplifying only the template with the appropriate methylation state. Standard PCR does not replicate the modifications that are found on DNA, including the methylation of cytosines since the dCTPs added to the amplification mixture are not methylated and Taq polymerase will not distinguish between the addition of methylated or unmethylated dCTP in the amplified product (50). MS-PCR exploits bisulfite-induced changes and utilizes primers designed to a specific methylation status and can thus distinguish between expressing and non-expressing cells. Specifically, MS-PCR primers are designed to discriminate between methylated and unmethylated alleles following bisulfite treatment and to discriminate between DNA modified by bisulfite and that which had not been modified (51). By extending this approach to include a dual labeled probe, a quantitative TaqMan® assay has been developed to simultaneously detect and quantitate the amount of the body fluid present in a sample. Additionally, this method utilizes instrumentation and techniques already in use in a forensic DNA lab, providing an excellent basis for a new type of serological assay.

Materials and Methods

The method of choice in this study was a methylation-specific TaqMan® assay. This type of assay combines the ability to distinguish between methylated and non-methylated cytosines due to pretreatment of the DNA samples with sodium bisulfite and the specificity, sensitivity and ease of use of a quantitative PCR assay.

Bioinformatics

Genes were chosen for study by one of three methods. Firstly, genes were identified through literature searches as being specifically localized to a specific cell type or body fluid. In the absence of clear candidates, the TiGER- Tissue-Specific Gene Expression and Regulation Database (<http://bioinfo.wilmer.jhu.edu/tiger/>) was utilized as a tool to identify genes, which have been shown by extensive EST data to be expressed in restricted tissues (52). Lastly, the reduced representation bisulfite sequencing data from Epigenome Browser at Washington University (<http://epigenomegateway.wustl.edu/browser/>) was utilized in conjunction with the KEGG pathway feature to compare methylation patterns for genes in the phagocytosis and hematopoietic pathways between blood cells and other types of tissues for which this data was available (53). Additional literature searches were performed on these genes to obtain information about the control of gene regulation. In the absence of any definitive proof of methylation control of gene expression, the genomic sequences of these genes were obtained and scanned for CpG islands using Methyl Primer Express (Applied Biosystems). If CpG islands were identified, primers were designed based on the unmethylated versions of the gene to have an annealing temperature of approximately 60C and probes were designed to have an annealing temperature of approximately 70C and contain as many CpG sites as possible. This was performed either by inspection or with the aid of Meth Primer (54) (<http://www.urogene.org/methprimer/index1.html>). Using the ePCR feature of BiSearch, a tool designed to design primers against bisulfite treated genomes, the designed primers and probes were amplified in silico to determine the number of PCR products that would be expected to be produced in bisulfite treated human and other bisulfite treated genomes, including dog, dogfish, chicken, mouse, chimpanzee, rat and yeast. Primer sets that produced

only one PCR product in human and higher primate genomes, but no products in other organisms were produced and tested in the laboratory. Primers and probes tested are listed in Table 1 at the end of this report. Primers and probes were ordered from IDT DNA Technologies (www.idtdna.com). All Probes were dual labeled with 56FAM and Black Hole Quencher.

Body Fluid Samples

Blood and saliva were obtained from healthy volunteers under protocols approved by the Towson University Institutional Review board. Semen samples were purchased from Lee BioSolutions (St Louis, MO). Genomic DNA was extracted by either organic extraction (55) or using DNA IQ (Promega) following the manufacturers instructions. Genomic DNAs from baboon, rhesus monkey, chicken, turkey, pig, cow, salmon, sheep, mouse, rat, cat, dog, mouse, rat, ferret, horse, and rabbit were purchased from Zyagen (San Diego, CA). Human DNA was quantitated using Quantifiler Human (Life Technologies) following manufacturers instructions on the ABI Prism 7000.

Bisulfite treatment of DNA

DNA was bisulfite treated using either the Epitect kit (Qiagen) or the EZ Gold DNA methylation kit (Zymo) following manufacturer's recommended protocols. As quantitation of the low quantities (ng range) of treated DNA was not possible following treatment, it was assumed that 100% yield was obtained following treatment for the purposes of reporting DNA concentrations used in the following assays. This probably results in an over reporting of the quantity of the DNA, therefore underestimating the sensitivity of the assays.

Quantitative PCR

Quantitative PCR assays were performed using the following basic approach. 100-300nM

forward and reverse primers and 100-300nM probe, 0-10 ng bisulfite treated template DNA and 12.5 μ L 2x master mix (Fermentas) in a 25 μ l reaction. All reactions were performed with a minimum of 3 replicates. Amplification was performed with an Applied Biosystems ABI Prism 7000 using the FAM detection filter. Cycling parameters included a 10 minute pretreatment at 95C followed by 40-50 cycles of 15 second incubation at 95C and 1 minute at and annealing temperature of 50-60C. The threshold was set at 0.2 and background determined between 6 and 15 cycles. Data is reported as the cycle at which the reporter dye crossed the threshold (C_t). Each experiment included no template controls.

Results:

A total of 57 sets of primers and probes were generated and screened for this study with the goal of developing assays for the detection of sperm, semen, blood and saliva. Many of these results were negative and will not be covered in detail. The failed primer sets could be categorized into two groups: those that did not amplify for any of the body fluids tested, and those that amplified for every body fluid tested. This suggests that the methylation status of the template sequences tested was similar for all of the body fluids, either fully methylated and therefore not amplifiable or unmethylated or having an unmethylated subset of cells and therefore amplifiable. However, three of the assays produced interesting results that will be discussed in detail in this report.

Developmental Pluripotency Antigen 5 (DPPA5):

The utility for methylation specific Real time PCR in the detection and identification of sperm in forensic samples has been demonstrated as a method for identification of sperm cells. This approach has led to the development of an assay that rivals the current gold standard of microscopic examination of evidence in that it has been shown to specifically detect single sperm cells even in the presence of DNA from other tissues. This assay was developed using the DPPA5 gene, which has been shown to be methylated in all tissues except sperm and testes (31). Briefly, DNA is bisulfite treated to convert non-methylated cytosines to uracils, thereby allowing for the detection of genes that contain no methylated cytosines by using primers and probes designed to preferentially amplify non-methylated forms of tissue specific genes, i.e. the expressed versions of the genes. The methylation specific quantitative PCR assay for DPPA5 has been shown to be specific for semen DNA; blood and saliva DNAs

were not detected.

Figure 1: Tissue specificity of DPPA5 assay for the detection of semen

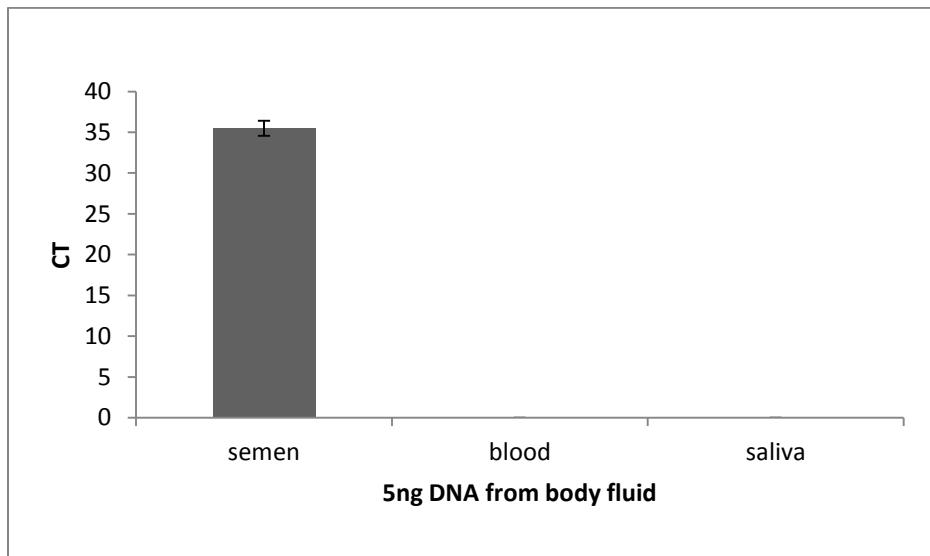


Figure 1: 5 ng of bisulfite treated DNA from semen, blood and saliva were used as a template for the Real-Time PCR assay using the DPPA5 primers and probes shown in Table 1. Signal was only generated from bisulfite treated seminal DNA; no signal was generated when bisulfite treated blood or saliva was used as a template. Signal was also absent in no template control and in non-bisulfite treated DNA (data not shown). Data shown is average C_t for triplicate assays \pm standard deviation and representative of multiple trials.

The species specificity of this assay was determined by performing the assay using 15 different, forensically relevant animals; none of the DNAs were detectable in this assay except for the higher primate, Rhesus monkey. All other animals including: Baboon, cow, chicken, ferret, rat, mouse, salmon, turkey, pig, cat, dog, horse, rabbit and guinea pig showed no reactivity in this assay thus demonstrating higher primate specificity for the DPPA5 set of

primers for the detection of sperm.

Figure 2: Species specificity of DPPA5 Assay for the detection of sperm

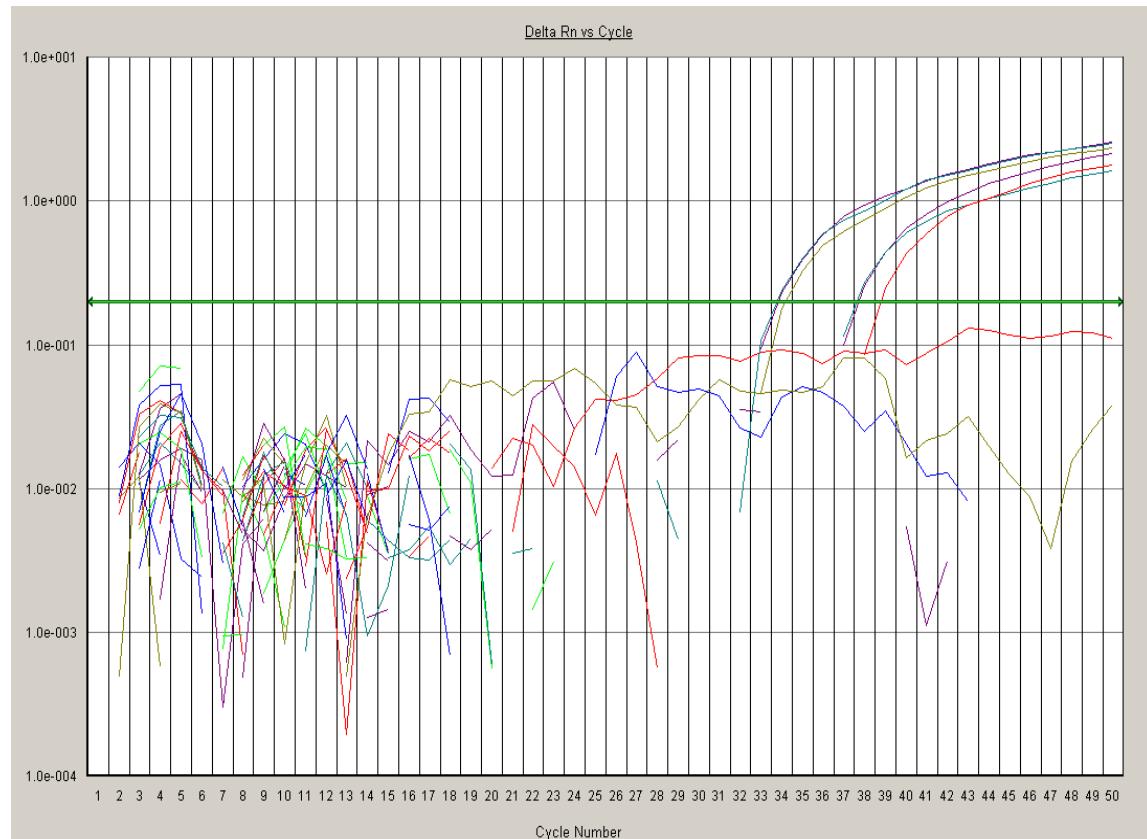


Figure 2: Species specificity using 15 forensically relevant non-human species

2 ng of bisulfite treated human semen DNA and 10 ng of bisulfite treated non-human DNAs was tested for DPPA5 detection. The human semen signal is indicated. Except for Rhesus monkey DNA, all other DNAs are below threshold and therefore not detected.

In order to determine the limit of detection for the DPPA5 assay, varying amounts of human semen DNA from 2pg (the DNA contained in a single sperm) to 5ng (the DNA contained in 2000 sperm cells) were tested. These quantities were obtained by serial dilution of a stock and are presented as a good faith estimate of the DNA quantity tested. The assay produced a signal when the equivalent of the DNA from a single sperm cell was used as a

template and produced a linear signal throughout the entire range tested. The sensitivity of the assay is unprecedented and matches the sensitivity of microscopic examination of evidence in that DNA from a single cell is detectable.

Figure 3: Sensitivity of the DPPA5 assay for the detection of sperm

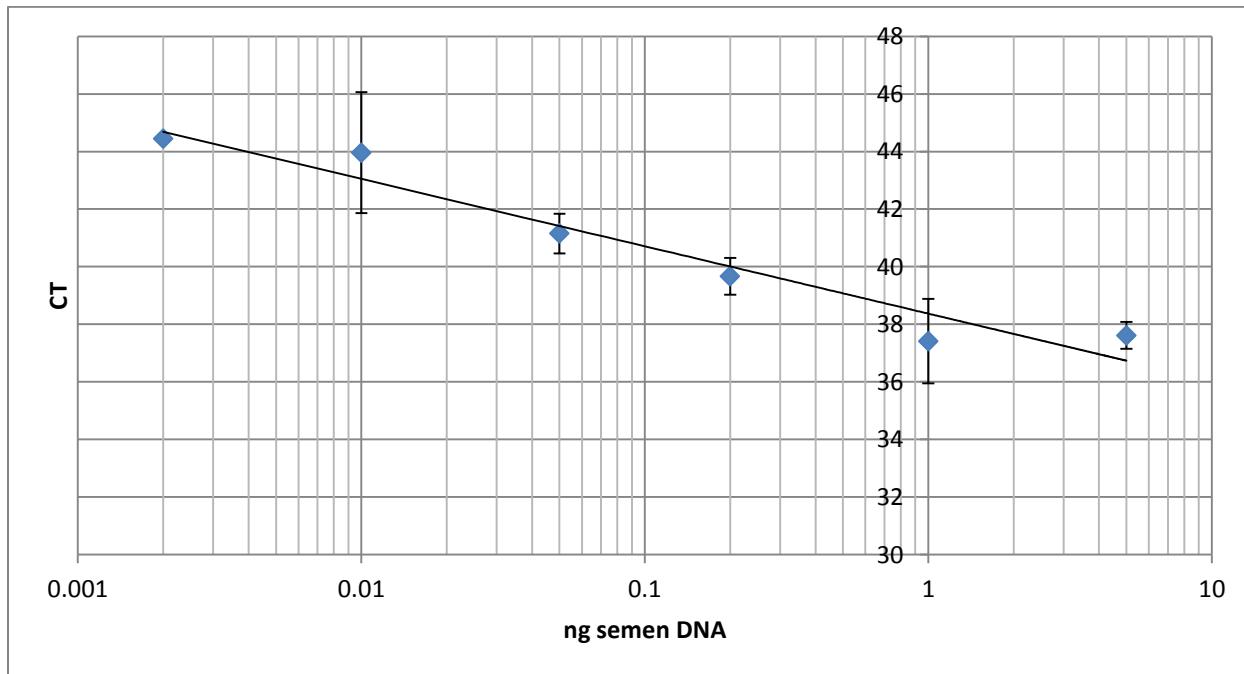


Figure 3: Sensitivity of DPPA5 detection of bisulfite treated sperm DNA

Serial dilutions of bisulfite treated semen DNA from 2 pg to 5ng of DNA were tested using the DPPA5 assay. Data represents average Ct values \pm standard deviation of triplicates of a representative assay. The signal at this low level of DNA concentration was subject to sampling issues and routinely amplified in only one or two of the triplicates and therefore does not represent a mean \pm standard deviation, only the mean. No signal was obtained with non-bisulfite treated DNA or in the absence of template (not shown).

It is particularly important in the evaluation of forensic evidence that the assay be tested for the detection of sperm DNA in the presence of other body fluids, since mixed source body fluids are frequently seen in forensic casework. Mixture studies were performed

in which serial dilutions of bisulfite treated sperm DNA (2 pg to 5ng) were mixed with 5ng of bisulfite treated DNA from either blood or saliva. As little as 2 pg of sperm DNA was detectable when diluted 1:2500 fold in either blood or saliva DNA with no significant difference in the Cycle threshold (C_t) between the three groups. This indicates that the sperm DNA was detectable without interference from the competing DNAs. In this set of experiments, the mixture studies have shown that mixtures of previously extracted and bisulfite treated DNAs allowed for the detection of sperm DNA at single cell levels even in the overwhelming presence of other DNAs.

Figure 4: Detection of sperm DNA in the presence of DNA from other body fluids

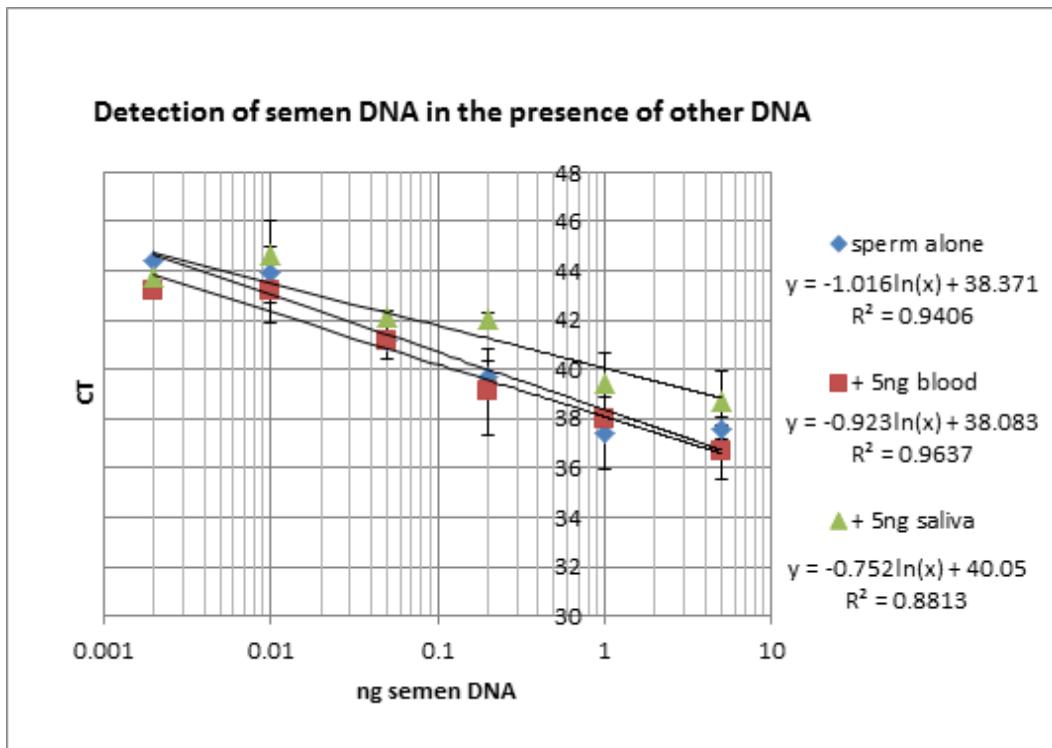


Figure 5: Mixture Studies: Detection of semen DNA in the presence of other DNA

Serial dilutions of bisulfite treated semen DNA was mixed with 5ng of bisulfite treated DNA obtained from either blood or saliva. Each data point represents the average of triplicates

\pm standard deviation for a representative assay.

It is important to note that in forensic casework, mixed samples are not combinations of DNAs, but are mixtures of body fluids. Therefore to test the ability of this assay to detect sperm in the presence of other body fluids was tested by mixing semen, which had been diluted 10,000 fold with either blood stains or buccal swabs and then testing for the presence of sperm, in order to mimic the mixture of body fluids which might be seen in a forensic sample. This specifically mimics situations in which sperm are found at very low concentrations in the presence of high concentrations of other fluids. Calculations estimate that approximately 6 sperm were likely to be present in the portion of the stains used for testing. When these diluted semen samples were mixed with full strength concentrations of other body fluids, then extracted and bisulfite treated, even these small numbers of sperm cells were detectable indicating that this assay might be useful in the identification of sperm in forensic casework samples.

Figure 5: Detection of sperm cells in samples of mixed body fluids

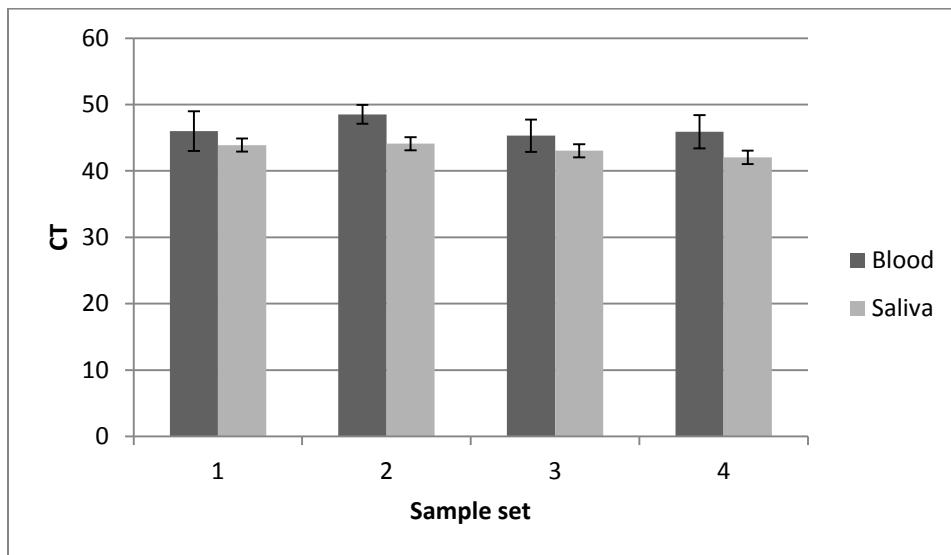


Figure 5: Detection of DPPA5 in casework like samples

Whole semen was diluted 10,000 fold and 10 µl of the dilution were added to either a dried saturated buccal swab or a dried blood saturated filter paper. Approximately 10% of the sample was taken for DNA extraction and bisulfite treatment prior to DPPA5 detection. Total DNA concentration tested was 5 ng. Data represents the average of triplicates ± standard deviation for 4 separate sample sets.

Adenosine Deaminase Domain Containing 1 (ADAD1):

In order expand the utility of this technique; additional assays for the identification of sperm were developed and tested. A myriad of genes were evaluated and 8 genes were found to have CpG islands in which primers and probes could be generated. Of the 8 assays, only one, ADAD1, demonstrated the appropriate specificity for semen, but not blood or saliva.

Figure 6: Tissue specificity of ADAD1 assay for the detection of sperm

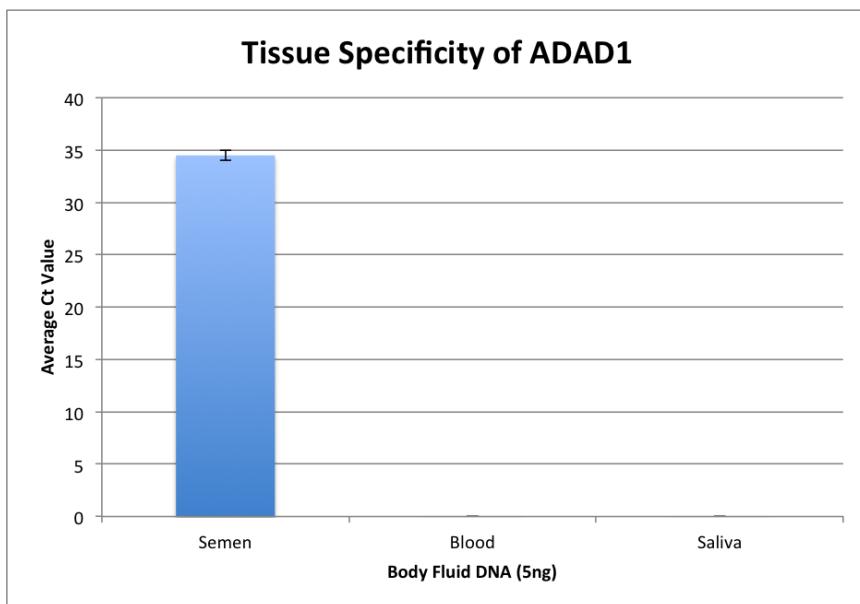


Figure 6: The assay demonstrated tissue specificity for semen, as blood and saliva DNA were not detected using ADAD1 primers. Data shown is the average of triplicates consolidated from 14 different tissue-specificity assays \pm standard deviation for a representative study.

Once the tissue specificity of the ADAD1 assay had been established, the species specificity of this assay was evaluated. 5ng of bisulfite treated human semen DNA was tested along with 10ng of bisulfite treated DNA from 15 different forensically relevant species. In this assay the human DNA was the only DNA that reacted.

Figure 7: Species specificity of ADAD1 for the detection of sperm

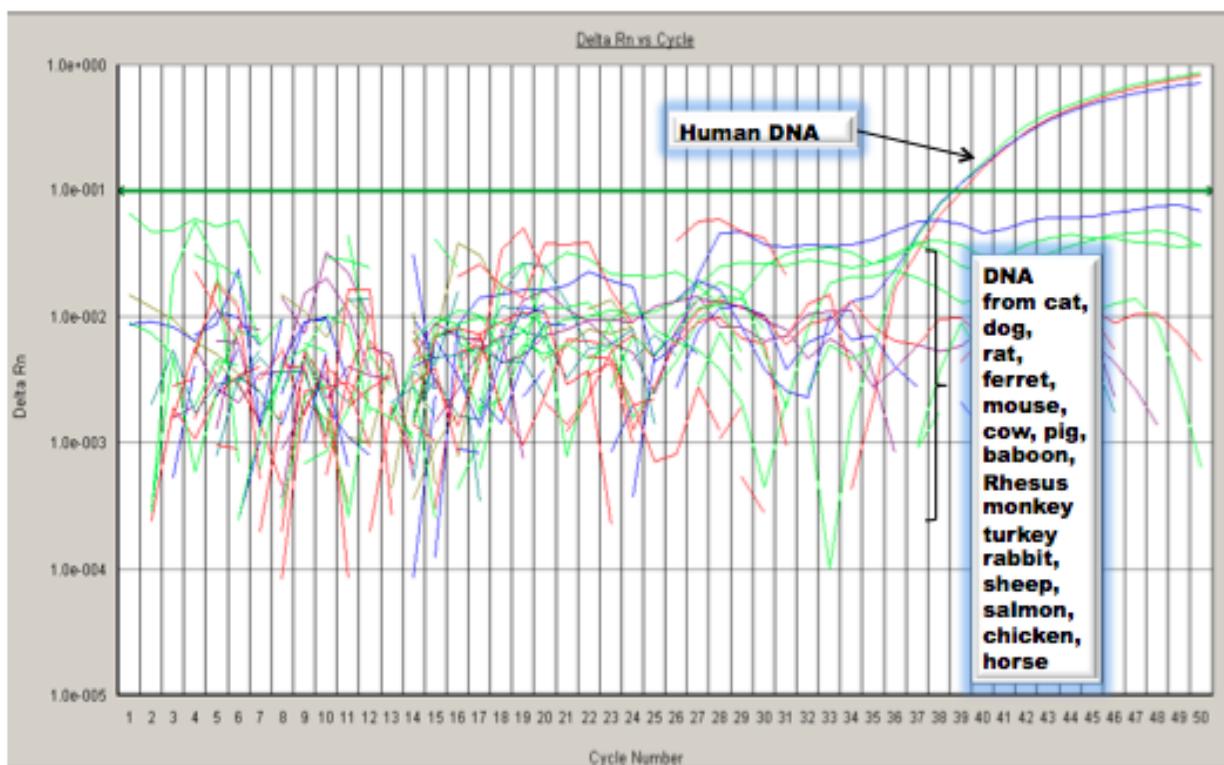


Figure 7: 5ng of human DNA and 10ng of 15 forensically relevant animals were tested in the ADAD1 assay. Only the human semen DNA crossed the threshold. The DNA from the non human animals was not detected.

In order to determine the sensitivity of the ADAD1 assay for the detection of semen, serial dilutions of bisulfite treated semen from 3pg to 5ng were tested. This assay was capable of detecting 3pg of semen DNA, the equivalent of a single sperm cell, as evidenced by the reproducible detection of a signal for this dilution, indicating that this assay, like the DPPA5 assay matches the sensitivity of microscopic visualization of sperm, the current gold standard of sperm detection.

Figure 8: Sensitivity of the ADAD1 assay for the detection of sperm cell

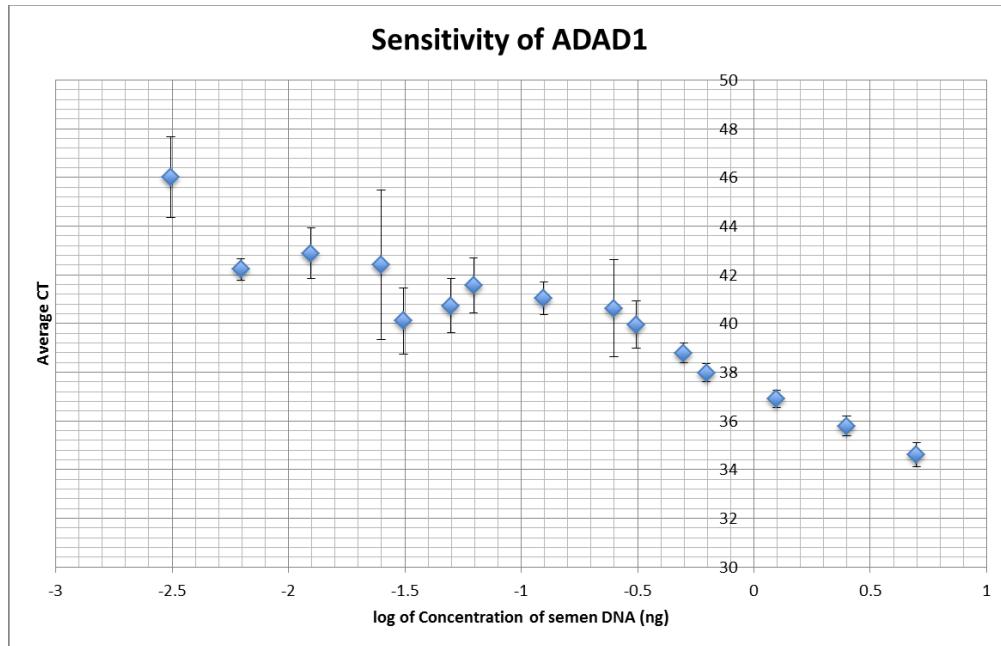


Figure 8: A concentration dependent signal is detected down to 3 pg of semen DNA. Data shown is the average of quadruplicates \pm standard error for a representative study.

In order to determine the ability of this assay to detect sperm in the presence other body fluids, various amounts of bisulfite treated sperm DNA was mixed with 5ng of either bisulfite treated DNA from human blood or human saliva and tested in the ADAD1 assay. Even in the presence of overwhelming quantities of competing DNA, 8pg of human sperm DNA, the equivalent of 2 to 3 sperm cells was detectable. Although this assay was less sensitive than the DPPA5 assay, it remains a sensitive assay for the detection of sperm cells.

Figure 9: Detection of Sperm DNA in the presence of Blood or Saliva DNA

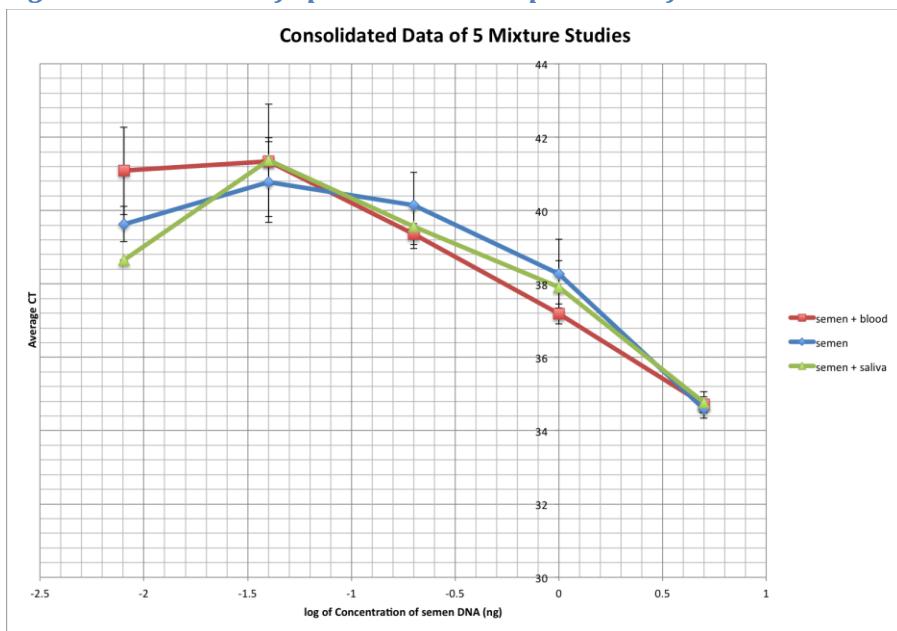


Figure 9: 8pg to 5ng of bisulfite treated sperm DNA was mixed with 5 ng of bisulfite treated DNA from blood or saliva. Data shown is the average of triplicates \pm standard error for a representative study.

Microseminoprotein-Beta (MSMB)

As sperm are not always present in all semen samples, for example in semen from post-vasectomized males, a similar assay was designed for the detection of prostatic cells that are also present in semen. We have demonstrated that these cells can be detected in very low numbers using a methylation specific PCR assay. In experiments similar to those previously explained, microseminoprotein beta (MSMB) was used as a target for prostate cells. The MSMB gene is unmethylated in normal prostate and methylated in prostate cancer. MSMB is expressed only in prostate cells and is therefore a good candidate for this type of assay. The methylation specific quantitative PCR assay for the detection of MSMB demonstrated semen specificity, with no signal generated in when bisulfite-treated blood or saliva DNA was used as

a substrate.

In order to determine if the assay was specific to the unmethylated MSMB gene and thus semen, real-time PCR was performed using 5ng of extracted and treated seminal fluid, blood and saliva/buccal swabs. For all tests, seminal fluid was amplified with an average Ct value of 22. Neither blood nor saliva produced a signal; thus, these fluids were undetected by the MSMB RT-PCR assay. When non-treated DNA samples as well as non-template controls were tested with the MSMB assay, no signal was detected. Therefore, the MSMB assay is specific only to bisulfite-treated seminal fluid DNA.

Figure 10: Tissue Specificity of the MSMB assay for the detection of semen

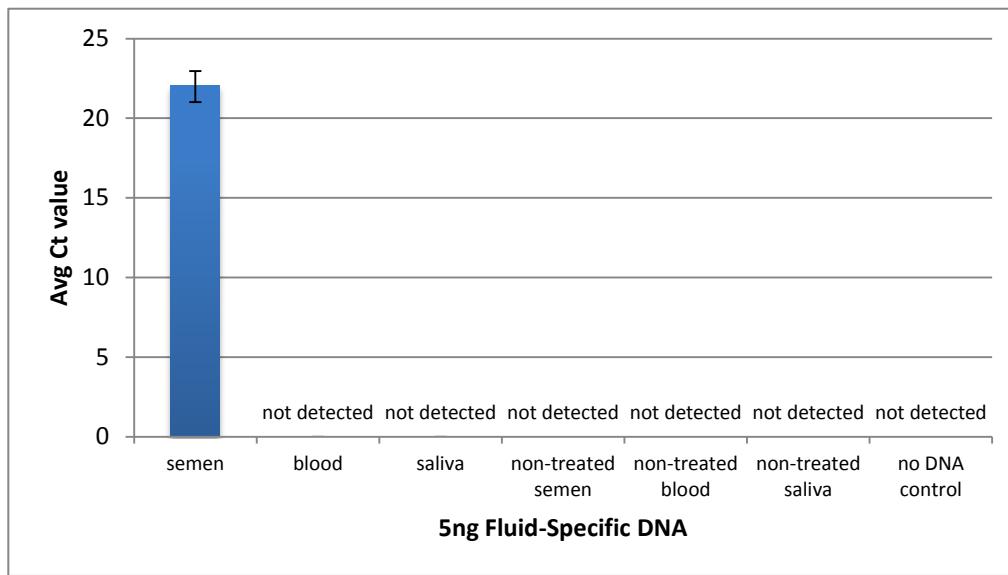


Figure 10: 5 ng of semen, blood and saliva DNA were tested with the MSMB assay for the detection of semen. Data shown is the mean +/- standard deviation of triplicate assays.

In order to determine the sensitivity of this assay, serial dilutions of seminal fluid were constructed (dilution concentrations ranged from 5ng to 0.005ng) and amplified in triplicate.

An average Ct value was calculated for each concentration. As expected, the Ct value for each consecutive dilution increased with the lowest concentration crossing the threshold well before the final amplification cycle (average Ct value is ~34). This concentration is equal to about 1 cell's worth of DNA and was consistently detected in repeated trials. Any variation in Ct value at this concentration can be accounted for by the fact that one cannot be certain that 1 cell's worth of solely prostatic DNA was amplified. Despite that, this assay appears to be sensitive enough to detect the DNA of at least one prostate cell present in seminal fluid.

Figure 11: Sensitivity of the MSMB assay for the detection of semen

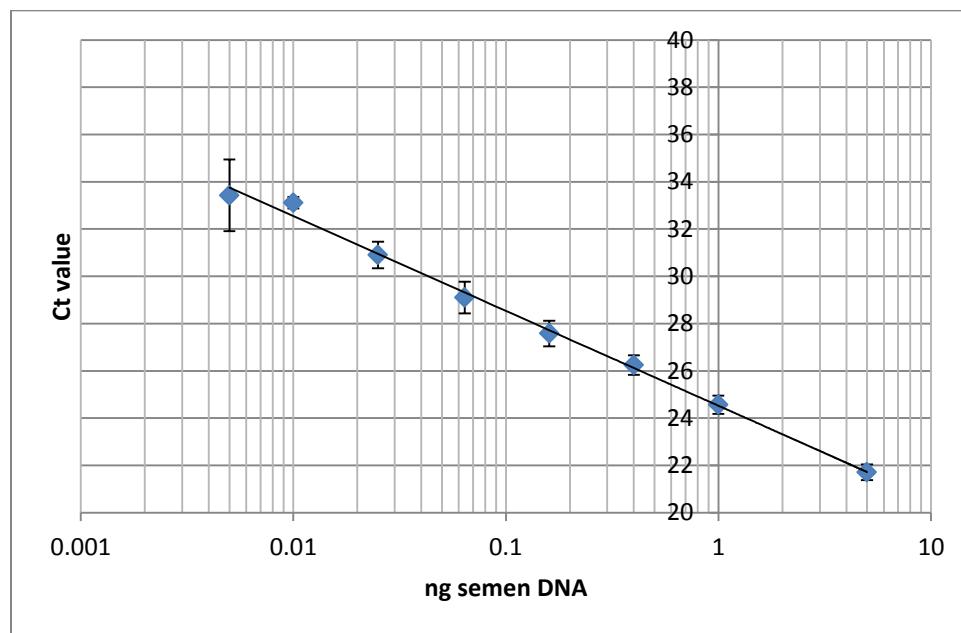


Figure 11: 5pg to 5ng of bisulfite treated semen DNA were tested with the MSMB assay for the detection of semen. Each data point is the mean +/- standard deviation of the triplicate trials.

When mixture studies were performed however, the detection of semen became much less sensitive when blood or saliva DNA was introduced. In the presence of other DNAs, the sensitivity was lost, at concentrations less than 50 pg of semen DNA, no amplification of

MSMB was detected. This was problematic since it suggests that low concentrations of semen when mixed with other body fluids may not be detected in this assay.

Other gene assays:

We have screened dozens of genes and primers sets for utility in the detection of blood and saliva. We have unfortunately been hampered in our progress by a series of failed experiments. In one subset of these experiments, all of the body fluids amplified. We hypothesized that this may be due to populations of hemimethylated DNA in which one strand of the DNA contains methylated DNA and the other is unmethylated and therefore a potential template for the assay. In order to address these issues that we have seen the template DNA was pretreated with DNA methyltransferase I (DNMTI), which fully methylates hemi-methylated DNA, thus eliminating this as a substrate for the assay. This pretreatment has not aided in the differentiation of genomic DNA from different tissues. Issues that have been seen in the development of these assays have fallen into three groups, outlined below.

Group I Primers: MSMB-EK2, WFDC, SKP2, UPK3A #1-2 and MMP9

Primers in Group I produced signals above threshold for blood, semen and saliva. Thus, these primers were not selective enough to identify semen. Primer and probe concentrations and temperature were adjusted to increase the levels of specificity (data not shown); however, significant separation between semen, saliva and blood peaks was not observed. To illustrate, smooth amplification curves were observed (~ ct value of 24) for blood, semen and saliva for

UPKA #1 primer set run at 60°C with 300nM primer and probe concentrations; nevertheless, all the plots crossed at the Ct value, making it impossible to differentiate the three body fluids from one another. Figure 12 is a representation plot of the ct value v. delta Rn for group I primers, as mentioned none of the samples crossed threshold.

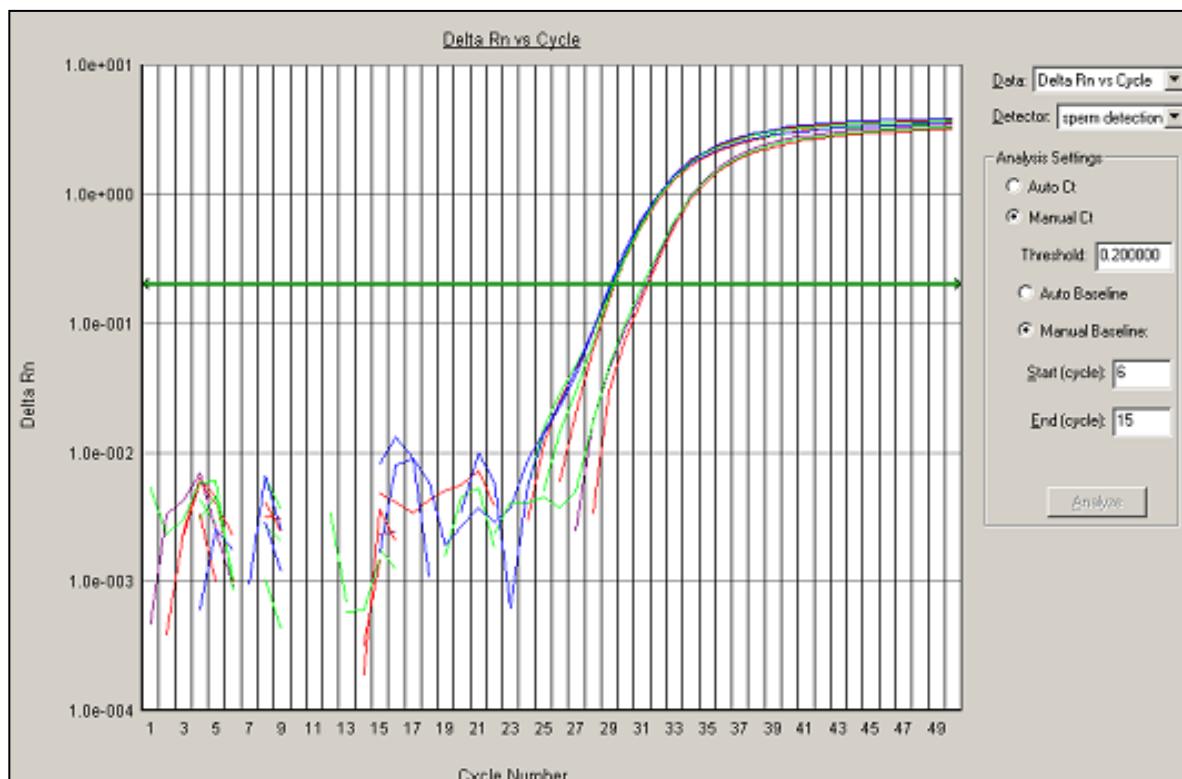


Figure 12: Representative plot of Group I primers. No tissue specificity is shown. Blood, semen and saliva DNA cross the threshold at approximately the same C_t value.

Group II Primers: UPK3A #3-4, CD52, SPINT2 #1-3

Group II primers failed to produce detectable PCR products resulting in none of the samples crossing threshold (figure 13). These primers were discontinued for the potential use in identifying body fluids following attempts to optimize annealing temperature.

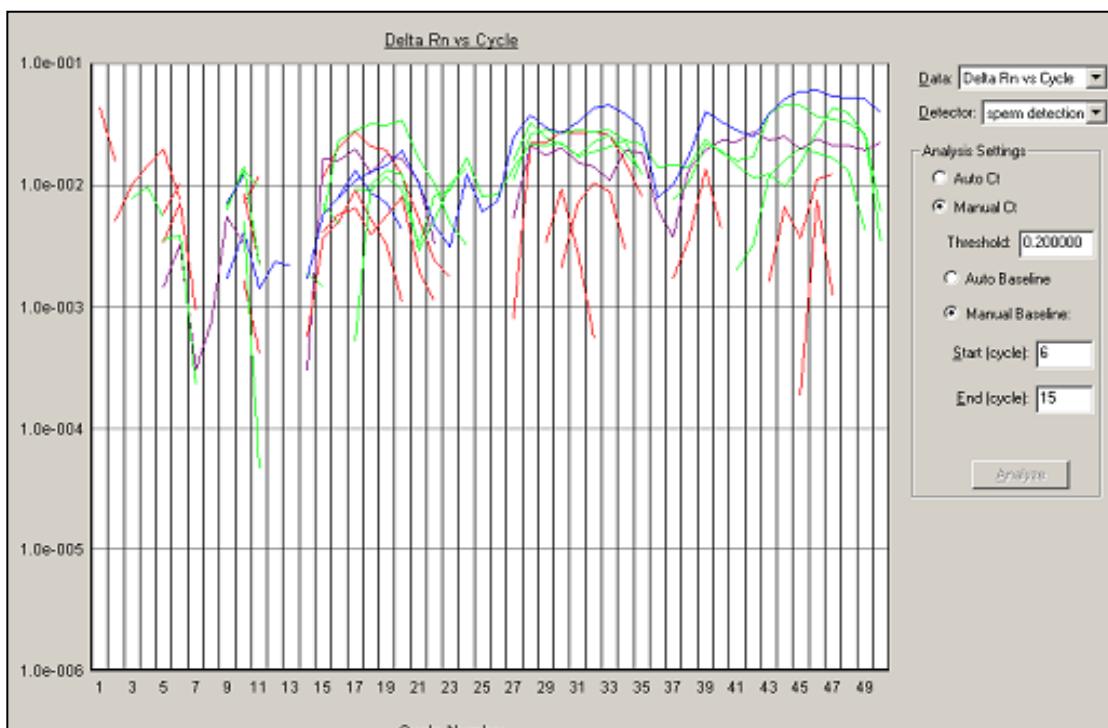


Figure 13: Representative plot of Group II primer illustrating failure to cross threshold

Group III Primers: MSMB-EK and MSMB-SC

Peak separation was observed in Group III primers. Manipulations were made to the primer/probe concentrations, temperature, buffer and DNMT treatment). Despite some increases in peak separation, complete selectivity for semen was not observed. In particular, blood, semen and saliva treated with Dnmt1 for the MSMB-SC primer set resulted in the greatest selectivity for semen (C_t 22) versus blood and saliva, whose curves appeared jagged and trailed just beneath the threshold. Little differentiation between non-DNMT treated saliva and semen was observed, C_t 22 and 20 respectively. Figure 14 is a representative plot of the C_t value v. delta Rn and shows differentiation between semen, saliva and blood peaks.

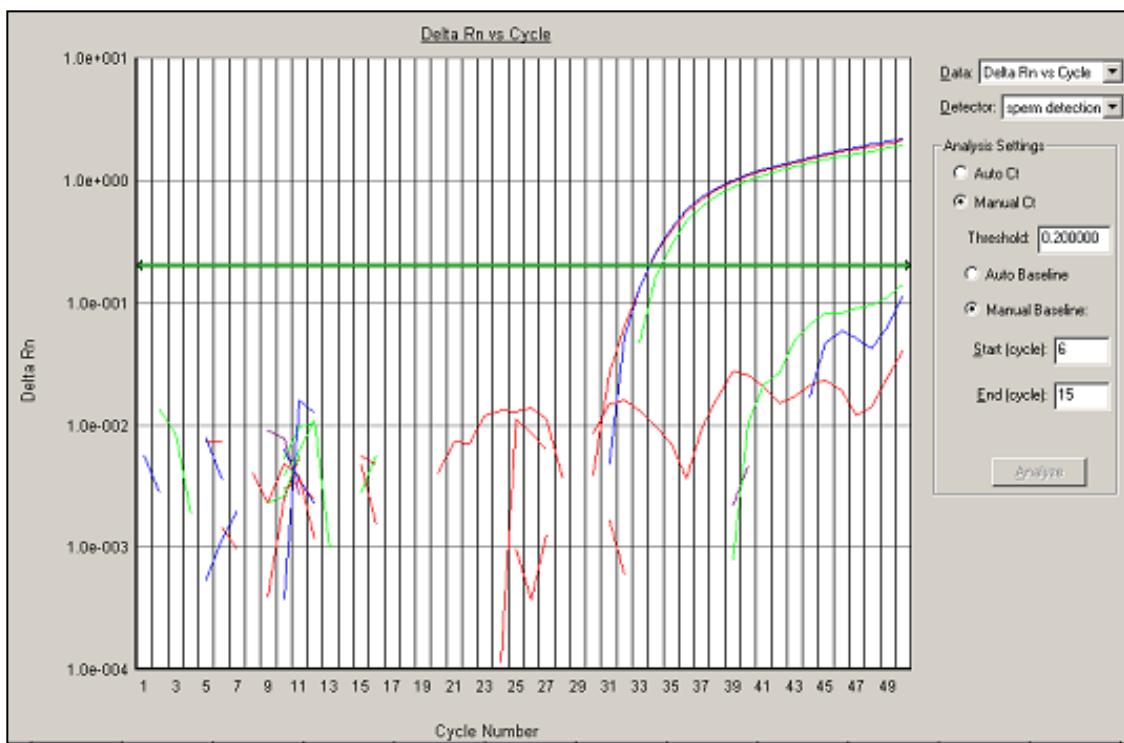


Figure 14: Representative plot of Group III primers. Notice that semen crosses the threshold, however blood and saliva do not.

Group III results were the hardest to interpret because they show clear separation of bisulfite treated semen DNA from the others, however when the bisulfite treated DNA from body fluids are mixed, there is a significant loss of sensitivity resulting in an assay that is not an acceptable method for the detection of semen in cases where the body fluids may be mixed. A possible reason for this finding is that once a sample is bisulfite treated the complexity of the template is reduced due to the conversion of the four base DNA code to essentially a three base code due to the conversion of cytosine to thymine. This might result in the primers annealing to multiple places on the genomic DNA, thereby reducing the efficiency of the reaction.

Discussion

This study has demonstrated a proof of concept for the use of quantitative PCR for the detection of sperm cells. Since sperm cells undergo a global demethylation during spermatogenesis (56, 57) they are a target-rich source for the development of methylation specific quantitative PCR assays that target unmethylated sequences. Unfortunately, the identification of unmethylated CpG islands in other tissues and cell types has been more problematic. None of the other primer sets and genes that were investigated in this study yielded conclusive evidence for the identification of other body fluids.

Given the number of failed assays, it will be important to develop a more targeted approach in order to develop the assay into one that can be utilized in forensic casework. Currently, a project is being considered in this laboratory to bisulfite treat various body fluids in order to obtain whole genome sequencing information to obtain methylation sequence data for relevant body fluids that can be compared to specifically target sequences that will be different in different body fluids. Currently available data available compares normal to matched tumor sequences and there is little information to be found for bisulfite sequence data in normal forensically relevant tissues and fluids. By comparing normal cells from different body fluids it should be possible to determine areas that are either fully unmethylated or fully methylated in a single body fluid and target those sequences for further study and development of methylation specific quantitative PCR assays. Additionally, population studies including bisulfite sequencing of the regions targeted by these assays must be obtained in order to perform the developmental validation necessary to implement this type of assay in casework.

Table 1: Primers and Probes Used in this Study

Body Fluid Target	Gene	Gene Name	Primers and Probe
Sperm	DPPA5	developmental pluripotency ntigen 5	Forward: 5'- GAAGTGGAAAGAGTTGGTAATT-3' Reverse: 5'-AAAACTCAAAAACAATAAAACCT-3' Probe: 5'-TTGGATTGTAGTTAGGGTGGTGGG -3'
Sperm	ADAD1	adenosine deaminase domain containing 1 (testis-specific)	Forward: 5'- GTTTTTTAGAAGGGTAAGGT -3' Reverse: 5'- TCACTTCAAACCACTCTACACA -3' Probe: 5'-TGATGGTGGTGTCCCCCTAGGTGATGTAAGATGTGGA-3'
Sperm	ADAM2	disintegrin and metalloproteinase domain-containing protein 2	Forward: 5'- TTTTGAGTAGTTGTGATTGTAGGT-3' Reverse: 5'- AAACACATATTCTTAAATACCACA-3' Probe: 5'- AGTAGAGATGGGGTTTTATTGTGTTAGGATGGTTGA-3'
Sperm	DDX4	DEAD(Asp-Glu-Ala-Asp) polypeptide 4 box	Forward: 5'- GAGTAGGAGGAGTTTGAAGTT-3' Reverse: 5'-CAAAAAAAATTATACACAAATAACTCA-3' Probe: 5'-AAFTTTTTGTTGGGGTTTAGAGTTATGGTGTAA-3'
Sperm	GALNTL5	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 5	Forward: 5'-TGGTTAGGATTGAAAATATT-3' Reverse: 5'-CATAAACCATCATATCCAATCCA-3' Probe: 5'-TTGGGAATATAGTTAATTGTAGTATTATGGTTGGATTGTT-3'
Sperm	GMCL1L	germ cell-less, spermatogenesis associated 1 pseudogene 1	Forward: 5'-TTAGAAAAAGGAATTGAAAATAATT-3' Reverse: 5'-CTAAATTAAAACATAAAATTACATCA-3' Probe: 5'-TTGGAAATAGTATTGAAAATTAGGTTAGTTAATT-3'
Sperm	KIF2B	kinesin family member 2B	Forward: 5'-AGGAGGTGAAAAAATAAAGATGGT-3' Reverse: 5'-AACATTAAAATTAAATTATATCCCA-3' Probe: 5'-TTGAGGAGTTTTGGGATTTGTTGAATATTGAGTTGAGAT-3'
Sperm	LYZL1	lysozyme-like 1	Forward: 5'-GGATTGTATGGTATTATGAGAGT-3' Reverse: 5'-ATAATCCCATTATCAACTTCTCA-3' Probe: 5'-AGATTAATAGTTTGTGTGGTAGATGTGGAAAGTTGAAGGA-3'

Body Fluid Target	Gene	Gene Name	Primers and Probe
Sperm	MS4A5	membrane-spanning 4-domains, subfamily A, member 5	Forward: 5'-AAAATGAAAGTGAAATAGTTAGGT-3' Reverse: 5'-CCCAACTCACTTCAACTTCCA-3' Probe: 5'-AGGTTAGGAGTTGAGATTAGGTTGGTTAATATGGTGAAA-3'
Semen	MSMB	microseminoprotein, beta-	Forward: 5'- Reverse: Probe:
Semen	MSMB	microseminoprotein, beta-	Forward: 5'-ATTTGAATTTAATAGGTGTTTGA-3' Reverse: 5'-TACCCAATAATCTAACCAATACAAT-3' Probe: 5'-TGGAGAGTTTAGTTATATTATTGAGTAGGT-3'
Semen	MMP9	matrix metallopeptidase 9	Forward: 5'-GGGTTTTTTTGTATAAGGATGAT-3' Reverse: 5'-CCAAACTCTACTTCAAACAAACA-3' Probe: 5'-AGGGTGAGTTGGGTGTGTTGGTTGT-3'
Semen	CD52	CD52 antigen, epididymal secretory protein E5	Forward: 5'-GTTTGTGATTGGGTATGGTGGTTAT-3' Reverse: 5'-AATCTCCTAACCTCATAATCCACCCA-3' Probe: 5'-AGGGTGAGTTGGGTGTGTTGGTTGT-3'
Semen	WFDC2		Forward: 5'-TTAAGGTGGTTGAAATTAGATTGT-3' Reverse: 5'-AAAAACAATATCCAACTATACA-3' Probe: 5'-AGATAAAGGTGTTGTTGAAATGTAGTTAAGGG-3'
Semen	SKP2	S-phase kinase-associated protein 2	Forward: 5'-TGAGTAGTATGTTGGAGTTGT-3' Reverse: 5'-ACAACTCATTACCTATAACATAACA-3' Probe: 5'-TGGGAATTGGGAGGTGAGTAGTTTG-3'
Semen	UPK3A	uroplakin 3A	Forward: 5'-GTTTTTATTATTTAGATGGGATT-3' Reverse: 5'-AATTTATAAAAAACAAATTCTACA-3' Probe: 5'-AAGGTGTTGAATTATTTGAAATTAAATTATTTAG-3'
Semen	SPINT2	serine peptidase inhibitor, Kunitz type, 2	Forward: 5'-TGTAGGTGGTGAGGGTGT-3' Reverse: 5'-ACACACACAATAAAACACTCA-3' Probe: 5'-GGTTTGGAAAGGTGATTTGGGG-3'
Semen	SPINT2	serine peptidase inhibitor, Kunitz type, 2	Forward: 5'-TGTGTTGAGGGTTTTGT-3' Reverse: 5'-ATAAAACTACATTCTCAATCAACCA-3' Probe: 5'-GGTGTAGTTGTGGGTTGAGGTGG-3'
Semen	SPINT2	serine peptidase inhibitor, Kunitz type, 2	Forward: 5'-TGTGTTGAGAAGGTTGG-3' Reverse: 5'-ATAAAACTACATTCTCAATCAACCA-3' Probe: 5'-TGTATTGATTGTGAGATTAAATGG-3'

Body Fluid Target	Gene	Gene Name	Primers and Probe
Semen	ACTL7B	actin-like 7B	Forward: 5'-AGTTGGTGTGGTTGGTAGGTT-3' Reverse: 5'-CATTACTATCCATCTACTCCTACAACAA-3' Probe: 5'-TGTTTTGGATATGGTATTATGTGAGATGTT-3'
Blood	CR1	complement component (3b/4b) receptor 1 (Knops blood group)	Forward: 5'-TTTAGGTGTAGGGGTTAAGTTG-3' Reverse: 5'-AAAACCAAAACTCAATTCTCAA-3' Probe: 5'-TGAGTGTAGTGGAAAGGTGTAGATGTTGAGT-3'
Blood	CD44	CD44 molecule (Indian blood group)	Forward: 5'-TTGAGGTAGTTTATTGTTAGTGG-3' Reverse: 5'-ACCAAAACTTATCCATAATATCCAAA-3' Probe: 5'-TGTTTTGTTTTGTTGGTTTTGTTG-3'
Blood	EPOR	erythropoietin receptor	Forward: 5'-AGTTTTGAGGTTGTAGTTGT-3' Reverse: 5'-CCAACCTAACATTAAAATCACATT-3' Probe: 5'-TGGGTGTGAATATTTTGTTGATTA-3'
Blood	EPOR	erythropoietin receptor	Forward: 5'-GGTTTTGTTGTTGAGATATTATGAT-3' Reverse: 5'-AAACTAACCTCACCCCTCTACACT-3' Probe: 5'-TGTTATGAGGTGGATGTTTGGTTGTAATGG-3'
Blood	MPO	myeloperoxidase	Forward: 5'-TGTTGTTAACGTTAACGTGGTTGT-3' Reverse: 5'-AAACACTAACACCAAAAAACTT-3' Probe: 5'-CATCCAAAACACCTCTCTAAACCCA-3'
Blood	MPO	myeloperoxidase	Forward: 5'-TGAGGAAGTATTGTTATGTATTGT-3' Reverse: 5'-AATCCTACTTACCTTCCAACACAAC-3' Probe: 5'-TGTATTGTTAACGTTTATTAAATGTTTTTG-3'
Blood	MPO	myeloperoxidase	Forward: 5'-AATTGATGGAGTAGTATGGTATGT-3' Reverse: 5'-TACAAACAAAAAATAAACCCACACA-3' Probe: 5'-TGATATTGGATGGGTGGTGTGTTGAG-3'
Blood	GP5	glycoprotein V (platelet)	Forward: 5'-GTTATATTTTGGTTGTTGGT-3' Reverse: 5'-TATTTAACATACCCCTAACACA-3' Probe: 5'-TGTGTAATAAAATTATGTATTTTAGGTGTG
Blood	BPGM	2,3-bisphosphoglycerate mutase	Forward: 5'-ATTTTTGGAAGGAAGTAGGGT-5' Reverse: 5'-TTTCACCACATTCAAATTCT -3' Probe: 5'-ATGTTGTTGTATTAGGGTTTGGTAGATTGGT -3'
Blood	AZU1	azurocidin 1	Forward: 5'-TTAGGAATTGGGGTTAGTATTGT-3' Reverse: 5'-AATGATTGATGTTGTTAGGTGA-3' Probe: 5'-TGTTATGATTGAGGTGGTGGGAGAGGTA-3'

Body Fluid Target	Gene	Gene Name	Primers and Probe
Blood	AZU1	azurocidin 1	Forward: 5'-TAGTTAGTTGGTTGGATGATTTGT-3' Reverse: 5'-AACTCTATCCTCCAATAAAACTACA-3' Probe: 5'-TGTGAGGGTGGATGGTGTGGGATTAG-3'
Blood	AZU1	azurocidin 1	Forward: 5'-AAAAATTAGTTGGGAGTGGTGGTGT-3' Reverse: 5'-ATTCCTATAATCCCAAACATAATCTCA-3' Probe: 5'-TGAGGTGGAAGGATGGTTGAGGTAGG-3'
Blood	AZU1	azurocidin 1	Forward: 5'-TTTTTGAGATAGAGTTTGTGTT-3' Reverse: 5'-AATACAAAATTAACCAAACATAATAACA-3' Probe: 5'-TGTGATTTGGTGGTTATTGTAATTGTTGTT-3'
Blood	AZU1	azurocidin 1	Forward: 5'-TTGGTTTGAATTGGTGGATTGATT-3' Reverse: 5'-CCAACACAAACATAACTCACACCCA-3' Probe: 5'-TTGTTGGTTGAGAAATAGTAGTTATTAAAT-3'
Blood	PADI4	peptidyl arginine deiminase, type IV	Forward: 5'-AATTTAGTATTTGGGAGGTTGAGGTAGGT-3' Reverse: 5'-TTCTATAACTACATACTACCACACCCA-3' Probe: 5'-TGAGATTATTTGGTTAATATGGTGAATGTT-3'
Blood	PADI4	peptidyl arginine deiminase, type IV	Forward: 5'-TTATTTGGTTAATATGGTGAATGTT-3' Reverse: 5'-CAATCTCAATTCACTACAACATCCA-3' Probe: 5'-TGGGTGGTAGTATGTAGTTAGTTAATGTT-3'
Blood	PADI4	peptidyl arginine deiminase, type IV	Forward: 5'-GTTAGGATGGTTTGATTGGTT-3' Reverse: 5'-ATTCAAAAAAAACCAACTAAACACA-3' Probe: 5'-TGTTTGTTAAAGTGTGGATTATA-3'
Blood	HBA1	hemoglobin, alpha 1	Forward: 5'-GTGGATGATATGTTAATGTGTTGTTGT-3' Reverse: 5'-CACCCCTCAACCAAATCAC-3' Probe: 5'-TGATTGTATGTATAAGTTGGGTGGATTGG-3'
Blood	HBA2	hemoglobin, alpha 2	Forward: 5'ATAAGTTTGGGTGGATTGGTTAAT-3' Reverse: 5'-AACACCATCTCACCCCTCAC-3' Probe: 5'-TTAAGGTGAGTGGTGGGTTGGAGTGA-3'
Saliva	AQP5	aquaporin 5	Forward: 5'-GGGTGAGGTGGGTTAGGATGTT-3' Reverse: 5'-TCCATACACAACCTCCCCCTTT-3' Probe: 5'-AAGGAGGAGTAAAAGTTGAAGAG-3'
Saliva	ZNF480	Zinc finger 480	Forward: 5'-GGATTGTGTGGAGTGAAGGTTAT-3' Reverse: 5'-CCACCTAACTCTCACAAAAACA-3' Probe: 5'-TTGGATTGAGGGTTATAGATTGAAATTGTTGTATTGTT-3'

Body Fluid Target	Gene	Gene Name	Primers and Probe
Saliva	ZNF480	Zinc finger 480	Forward: 5'-TTTTGTGAGAGTTAGGTGGTT-3' Reverse: 5'-ATAAACAAAATCCACCTAACAAA-3' Probe: 5'-TTTGTGTGGAGTTTTGTTAAGTTTTAGTGATGTTA-3'
Saliva	ZNF480	Zinc finger 480	Forward: 5'-TGTTAGGTGGATTTGGTTATGT-3' Reverse: 5'-AAATAAAATCACCTCCCCACACA-3' Probe: 5'-TTTGGAGTATAGTGTAAATTAGGAGAAGTTGTGTT-3'
Saliva	COL9A3	collagen, type IX, alpha 3	Forward: 5'-GAAGTTATTGGGAGGAGATATTGA-3' Reverse: 5'-CACAACAACCTAAACTAAAAACAC-3' Probe: 5'-AGAGTTGTGTGGGAAGTGTGGATTGGGATTTGG-3'
Saliva	COL9A3	collagen, type IX, alpha 3	Forward: 5'-AAGTAGTGTGGATAGTGATTTGG-3' Reverse: 5'-CTCCTCAACCCCAAATATAAAAACAA-3' Probe: 5'-TGTTGGGTATTGATTGGGTTTGGTATTGGGAT-3'
Saliva	COL9A3	collagen, type IX, alpha 3	Forward: 5'-AAGTTGGTTAGGATGGTATTGAT-3' Reverse: 5'-ACTATCCCCTAATATCACCCCA-3' Probe: 5'-TGAGTTTTGGTTGATGGAGAGAAAATTAGGTTTATT-3'
Saliva	COL9A3	collagen, type IX, alpha 3	Forward: 5'-AAGTTGGTTAGGATGGTATTGAT-3' Reverse: 5'-AAAACCACCCCAATCCC-3' Probe: 5'-TGAGTTTTGGTTGATGGAGAGAAAATTAGGTTTATT-3'
Saliva	COL9A3	collagen, type IX, alpha 3	Forward: 5'-TTTGTGAGTGATAATTGAAAGTAGTT-3' Reverse: 5'-AATAAACACCAACTATCAACTCTCA-3' Probe: 5'-TGGTGTAGGTTGTTGGGTTTGTGTTTGTAT-3'
Saliva	COL9A3	collagen, type IX, alpha 3	Forward: 5'-TTAGTGTATTGGGTTGAGATTGGT-3' Reverse: 5'-TAACAAACATAATCCTCTATCAACCA-3' Probe: 5'-TTATGTTGTTGGTGTAGGGTGGTGTGAGGT-3'
Saliva	COL9A3	collagen, type IX, alpha 3	Forward: 5'-TAGGTTATGGGGTATTGT-3' Reverse: 5'-AAAACCACCCCAATCCC-3' Probe: 5'-TGAGTTTTGGTTGATGGAGAGAAAATTAGGTTTATT-3'
Saliva	COL9A3	collagen, type IX, alpha 3	Forward: 5'-AGTTGAGGTAGAGTTGGTT-3' Reverse: 5'-ACTCCAAAATCACTATCCAACA-3' Probe: 5'-TGTTTTAGGTTTGTAGGGTATGGTTTTAGGTT-3'

Body Fluid Target	Gene	Gene Name	Primers and Probe
Saliva	COL9A3	collagen, type IX, alpha 3	Forward: 5'-GGAGGGGTTGGTATTTGT-3' Reverse: 5'-ACTCCAAAATCACTATCCAACA-3' Probe: 5'-TGTTTTAGGTTTTGTTAGGTATGGTTTTTAGGTT-3'
Saliva	COL9A3	collagen, type IX, alpha 3	Forward: 5'-TTGGTGGTATAGGATATGAGTT-3' Reverse: 5'-ACCAAACATCAACCCCCAA-3' Probe: 5'-TGTTGGTAGATTAGTGTAGTTAGGTTAGGTTGTGG-3'
Saliva	COL9A3	collagen, type IX, alpha 3	Forward: 5'-TGAGGTTAATAATTTTAGAGTTGT-3' Reverse: 5'-ACCAAACATCAACCCCCAA-3' Probe: 5'-TGTTGGTAGATTAGTGTAGTTAGGTTAGGTTGTGG-3'
Saliva	COL9A3	collagen, type IX, alpha 3	Forward: 5'-TGTATATATTTTTAGTTAGTTATTGT-3' Reverse: 5'-AAAACTACAATAACCAAAACA-3' Probe: 5'TGAAGGGAGAATTTTTTATTATTATTGTGGGAGAGATT-3'
Saliva	COL9A3	collagen, type IX, alpha 3	Forward: 5'-TGATTGTTTGATGATATTATTATGGGT-3' Reverse: 5'-AAAAAAACAACATACCAAAACTCC-3' Probe: 5'-TGTTGGGTGGTTGGGTTGGTT-3'
Skin	ELOVL3	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3	Forward: 5'-GTAGGTTATAGAGTGTAAAGTTAGT-3' Reverse: 5'-CACAAACAATAAACACACCCCCCTCCA-3' Probe: 5'-TGTTGGTTTTATTGGGTTGTAGTT-3'

Bibliography

1. Nelson, M., and N. I. o. J. (US. 2011. *Making Sense of DNA Backlogs, 2010: Myths Vs. Reality*. National Institute of Justice, Office of Justice Programs, US Department of Justice,
2. Hayeslip, D., S. Debus-Sherrill, and K. A. Walsh. 2012. Evaluation of the Forensic DNA Unit Efficiency Improvement Program. *urban.org*
3. Blumstein, A. 1986. *Criminal careers and "career criminals"*. Natl Academy Pr,
4. Vollack, K., B. Haak, R. Schwenzer, and W. Pflug. 2008. Implementation of a semi-automated processing system for DNA profiling of forensic casework samples. *Forensic Science International: Genetics Supplement Series* 1: 83-85.
5. Frumkin, D., A. Wasserstrom, B. Budowle, and A. Davidson. 2011. DNA methylation-based forensic tissue identification. *Forensic Science International: Genetics* 5: 517-524.
6. Schweers, B. A., J. Old, P. W. Boonlayangoor, and K. A. Reich. 2008. Developmental validation of a novel lateral flow strip test for rapid identification of human blood (Rapid Stain Identification™-Blood). *Forensic Science International: Genetics* 2: 243-247.
7. Pang, B., and B. K. K. Cheung. 2008. Applicability of two commercially available kits for forensic identification of saliva stains. *Journal of forensic sciences* 53: 1117-1122.
8. Brettell, T. A., and R. Saferstein. 1985. Forensic science. *Analytical chemistry* 57: 175-187.
9. Davidson, G., and T. B. Jalowiecki. 2012. Acid phosphatase screening - wetting test paper or wetting fabric and test paper? *Sci Justice* 52: 106-111.
10. Laux, D. L., and S. E. Custis. 2004. Forensic detection of semen III. Detection of PSA using membrane based tests: sensitivity issues with regards to the presence of PSA in other body fluids. *Midwestern Association of Forensic Sciences Newsletter* 33: 33-39.
11. Shaler, R. C. 2002. Modern forensic biology. *Forensic Science Handbook, Prentice Hall, Upper Saddle River, NJ* 529-546.
12. Crick, F. 1970. Central dogma of molecular biology. *Nature* 227: 561-563.
13. Juusola, J., and J. Ballantyne. 2005. Multiplex mRNA profiling for the identification of body fluids. *Forensic science international*

14. Juusola, J., and J. Ballantyne. 2007. mRNA Profiling for Body Fluid Identification by Multiplex Quantitative RT-PCR*. *Journal of forensic sciences*
15. Richard, M. L., K. A. Harper, R. L. Craig, A. J. Onorato, J. M. Robertson, and J. Donfack. 2012. Evaluation of mRNA marker specificity for the identification of five human body fluids by capillary electrophoresis. *Forensic Sci Int Genet* 6: 452-460.
16. Setzer, M., J. Juusola, and J. Ballantyne. 2008. Recovery and stability of RNA in vaginal swabs and blood, semen, and saliva stains. *Journal of forensic sciences* 53: 296-305.
17. Visser, M., D. Zubakov, and K. N. Ballantyne.... 2011. mRNA-based skin identification for forensic applications. *International Journal of ...*
18. Wang, Z., J. Zhang, H. Luo, Y. Ye, J. Yan, and Y. Hou. 2012. Screening and confirmation of microRNA markers for forensic body fluid identification. *Forensic Science ...*
19. Zubakov, D., E. Hanekamp, and M. Kokshoorn.... 2008. Stable RNA markers for identification of blood and saliva stains revealed from whole genome expression analysis of time-wise degraded samples. *International Journal of ...*
20. Vennemann, M., and A. Koppelkamm. 2010. mRNA profiling in forensic genetics I: Possibilities and limitations. *Forensic science international* 203: 71-75.
21. Meissner, A., T. S. Mikkelsen, H. Gu, M. Wernig, J. Hanna, A. Sivachenko, X. Zhang, B. E. Bernstein, C. Nusbaum, and D. B. Jaffe. 2008. Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature* 454: 766-770.
22. Schilling, E., and M. Rehli. 2007. Global, comparative analysis of tissue-specific promoter CpG methylation. *Genomics* 90: 314-323.
23. Song, F., J. F. Smith, M. T. Kimura, A. D. Morrow, T. Matsuyama, H. Nagase, and W. A. Held. 2005. Association of tissue-specific differentially methylated regions (TDMs) with differential gene expression. *Proceedings of the National Academy of Sciences of the United States of America* 102: 3336.
24. Bird, A. P. 1986. CpG-rich islands and the function of DNA methylation. *Nature* 321: 209.
25. An, J. H., A. Choi, K. J. Shin, W. I. Yang, and H. Y. Lee. 2012. DNA methylation-specific multiplex assays for body fluid identification. *Int J Legal Med*
26. Lee, H. Y., M. J. Park, A. Choi, J. H. An, W. I. Yang, and K. J. Shin. 2012. Potential forensic application of DNA methylation profiling to body fluid identification. *Int J Legal Med* 126: 55-62.

27. Madi, T., K. Balamurugan, R. Bombardi, G. Duncan, and B. McCord. 2012. The determination of tissue-specific DNA methylation patterns in forensic biofluids using bisulfite modification and pyrosequencing. *Electrophoresis* 33: 1736-1745.
28. Doi, A., I. H. Park, B. Wen, P. Murakami, M. J. Aryee, R. Irizarry, B. Herb, C. Ladd-Acosta, J. Rho, and S. Loewer. 2009. Differential methylation of tissue-and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. *Nature genetics* 41: 1350-1353.
29. Illingworth, R. S., and A. P. Bird. 2009. CpG islands--'a rough guide'. *FEBS Lett* 583: 1713-1720.
30. Holliday, R., and T. Ho. 2002. DNA methylation and epigenetic inheritance. *Methods* 27: 179-183.
31. Shen, L., Y. Kondo, Y. Guo, J. Zhang, L. Zhang, S. Ahmed, J. Shu, X. Chen, R. A. Waterland, and J. P. Issa. 2007. Genome-wide profiling of DNA methylation reveals a class of normally methylated CpG island promoters. *PLoS Genet* 3: 2023-2036.
32. Denis, H., M. N. Ndlovu, and F. Fuks. 2011. Regulation of mammalian DNA methyltransferases: a route to new mechanisms. *EMBO Rep* 12: 647-656.
33. Fleming, R. I., and S. A. Harbison. 2010. The development of a mRNA multiplex RT-PCR assay for the definitive identification of body fluids. *Forensic Science International: Genetics*
34. Kiefer, J. C. 2007. Epigenetics in development. *Dev Dyn* 236: 1144-1156.
35. Christensen, B. C., E. A. Houseman, C. J. Marsit, S. Zheng, M. R. Wrensch, J. L. Wiemels, H. H. Nelson, M. R. Karagas, J. F. Padbury, and R. Bueno. 2009. Aging and environmental exposures alter tissue-specific DNA methylation dependent upon CpG island context. *PLoS genetics* 5: e1000602.
36. Fitzpatrick, D. R., and C. B. Wilson. 2003. Methylation and demethylation in the regulation of genes, cells, and responses in the immune system. *Clin Immunol* 109: 37-45.
37. Gibney, E. R., and C. M. Nolan. 2010. Epigenetics and gene expression. *Heredity (Edinb)* 105: 4-13.
38. Razin, A., A. Levine, T. Kafri, S. Agostini, T. Gomi, and G. L. Cantoni. 1988. Relationship between transient DNA hypomethylation and erythroid differentiation of murine erythroleukemia cells. *Proc Natl Acad Sci U S A* 85: 9003-9006.

39. Aranyi, T., A. Varadi, I. Simon, and G. E. Tusnady. 2006. The BiSearch web server. *BMC Bioinformatics* 7: 431.
40. Kurkjian, C., S. Kummar, and A. J. Murgo. 2008. DNA methylation: its role in cancer development and therapy. *Curr Probl Cancer* 32: 187-235.
41. Luczak, M. W., and P. P. Jagodzinski. 2006. The role of DNA methylation in cancer development. *Folia Histochem Cytobiol* 44: 143-154.
42. Bottardi, S., A. Aumont, F. Grosveld, and E. Milot. 2003. Developmental stage-specific epigenetic control of human beta-globin gene expression is potentiated in hematopoietic progenitor cells prior to their transcriptional activation. *Blood* 102: 3989-3997.
43. Bartolomei, M. S., and A. C. Ferguson-Smith. 2011. Mammalian genomic imprinting. *Cold Spring Harb Perspect Biol* 3:
44. Trasler, J. M. 2009. Epigenetics in spermatogenesis. *Mol Cell Endocrinol* 306: 33-36.
45. Oppenheim, A., Y. Katzir, E. Fibach, A. Goldfarb, and E. Rachmilewitz. 1985. Hypomethylation of DNA derived from purified human erythroid cells correlates with gene activity of the beta-globin cluster. *Blood* 66: 1202-1207.
46. Yagi, S., K. Hirabayashi, S. Sato, W. Li, Y. Takahashi, T. Hirakawa, G. Wu, N. Hattori, N. Hattori, and J. Ohgane. 2008. DNA methylation profile of tissue-dependent and differentially methylated regions (T-DMRs) in mouse promoter regions demonstrating tissue-specific gene expression. *Genome research* 18: 1969-1978.
47. Ammerpohl, O., J. I. Martin-Subero, J. Richter, I. Vater, and R. Siebert. 2009. Hunting for the 5th base: Techniques for analyzing DNA methylation. *Biochim Biophys Acta* 1790: 847-862.
48. Rush, L. J., and C. Plass. 2002. Restriction landmark genomic scanning for DNA methylation in cancer: past, present, and future applications. *Anal Biochem* 307: 191-201.
49. Hayatsu, H. 2008. Discovery of bisulfite-mediated cytosine conversion to uracil, the key reaction for DNA methylation analysis--a personal account. *Proc Jpn Acad Ser B Phys Biol Sci* 84: 321-330.
50. Li, S., A. Haces, L. Stupar, G. Gebeyehu, and R. C. Pless. 1993. Elimination of band compression in sequencing gels by the use of N4-methyl-2'-deoxycytidine 5'-triphosphate. *Nucleic Acids Res* 21: 2709-2714.
51. Sulewska, A., W. Niklinska, M. Kozlowski, L. Minarowski, W. Naumnik, J. Niklinski, K.

- Dabrowska, and L. Chyczewski. 2007. Detection of DNA methylation in eucaryotic cells. *Folia Histochem Cytobiol* 45: 315-324.
52. Liu, X., X. Yu, D. J. Zack, H. Zhu, and J. Qian. 2008. TiGER: a database for tissue-specific gene expression and regulation. *BMC Bioinformatics* 9: 271.
53. Zhou, X., B. Maricque, M. Xie, D. Li, V. Sundaram, E. A. Martin, B. C. Koebbe, C. Nielsen, M. Hirst, P. Farnham, R. M. Kuhn, J. Zhu, I. Smirnov, W. J. Kent, D. Haussler, P. A. Madden, J. F. Costello, and T. Wang. 2011. The Human Epigenome Browser at Washington University. *Nat Methods* 8(12): 989-990.
54. Li, L. C., and R. Dahiya. 2002. MethPrimer: designing primers for methylation PCRs. *Bioinformatics* 18: 1427-1431.
55. Gill, P., A. J. Jeffreys, and D. J. Werrett. 1985. Forensic application of DNA 'fingerprints'. *Nature* 318: 577-579.
56. Carrell, D. T., and S. S. Hammoud. 2010. The human sperm epigenome and its potential role in embryonic development. *Mol Hum Reprod* 16: 37-47.
57. Nagai, H., Y. S. Kim, T. Yasuda, Y. Ohmachi, H. Yokouchi, M. Monden, M. Emi, N. Konishi, M. Nogami, K. Okumura, and K. Matsubara. 1999. A novel sperm-specific hypomethylation sequence is a demethylation hotspot in human hepatocellular carcinomas. *Gene* 237: 15-20.