REPORT TITLE

Automating the Differential Digestion Method in the Analysis of Sexual Assault Cases using Selective Degradation

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AUTHOR(S)

Helena G. Wong, Jennifer S. Mihalovich, George Sensabaugh

ABSTRACT

Sexual assault evidence samples require the use of a specific method known as a differential digestion to separate sperm from non-sperm cells. The application of a degradative agent to selectively remove non-sperm DNA from mixed samples is of great interest to the forensic science community as this modification allows automation of the differential digestion process, saving time and labor. An automated differential digestion protocol was developed using a DNase I digestion step. The protocol utilizes 96-well plates and incorporates microscope slide preparations, resulting in high efficiency and high throughput. Initial evaluations of the DNase process revealed lower DNA yield and inferior STR DNA typing quality compared to the conventional differential digestion. Optimization of the concentrations of the divalent ions magnesium and calcium and the quantity of DNase used increased both the DNA yield and quality of the STR DNA typing results. This automated differential digestion using selective degradation with DNase I was tested on samples stored for extended periods of time (up to 60 years) and on samples exposed to multiple freeze/thaw cycles, or heat and humidity. All samples subjected to the selective degradation method produced results similar to samples processed by the conventional differential digestion method. STR DNA typing data from all examined samples illustrated that the automated differential digestion protocol with selective degradation using DNase I is capable of producing sperm fractions with no or minimal epithelial cell carryover.

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EXECUTIVE SUMMARY

The differential digestion protocol was successfully automated using a selective degradation method. Selective degradation replaces the multiple wash and centrifugation steps of the conventional differential digestion method by using a DNase I treatment to digest and eliminate all residual non-sperm DNA in the sperm fraction. The automated differential digestion protocol utilizes the SlicPrep™ 96 well plate which includes a 96 well spin basket for samples, and a U-shaped collar which elevates the spin basket creating additional space required for the centrifugation steps. Tween 80 was selected as the lysis buffer detergent replacing sodium dodecyl sulfate (SDS). The epithelial cell fraction digestion was reduced from one hour to 30 minutes. Decreased digestion time did not affect the DNA yield of the epithelial cell fraction. The DNase incubation step was performed at 56°C for 15 minutes. The $CaCl_2$ and $MgCl_2$ concentrations were optimized to 5mM and 90mM respectively and the quantity of DNase used was optimized to 15 units to achieve a DNA yield and high quality STR DNA typing data comparable to the conventional differential digestion protocol. The volume of PBS for the initial soak step was set at 650µL to accommodate the space available when the U-shaped collar is inserted. To maximize the release of cellular material from the substrate into solution, a manual toothpick agitation step was incorporated into the protocol.

The use of DNase I in the selective degradation differential digestion under optimized parameters resulted in DNA of comparable quantity and quality as that obtained by the conventional differential digestion protocol. Aged and environmentally compromised samples were not negatively affected by this selective degradation protocol.

The selective degradation differential digestion using DNase I eliminated residual non-sperm DNA in the sperm fraction at an efficiency similar to the conventional differential digestion protocol; the separation efficiency for both methods is less than 100%. Epithelial cell DNA carry-over into the sperm fraction was detected more frequently in samples that contained low sperm cell DNA input. This result was observed in both the sensitivity study and the mixed semen study. In samples where the sperm donor contribution was very low, allelic drop out was observed and the detected alleles in the sperm fraction

could not be de-convoluted due to similar peak height ratios between alleles from the sperm donor and alleles from epithelial cell DNA carryover.

In the sensitivity study, full DNA profiles were obtained from samples with male DNA yield as low as approximately 150pg. Samples with less than 100pg of male DNA resulted in low-level partial DNA profiles. In the samples where no sperm were observed microscopically, the STR DNA typing data revealed no or few detected alleles. The reproducibility study demonstrated consistency in the manipulation and preparation of samples by the liquid handler, VERSA 1100. No contamination during robotic sample manipulation and liquid handling processes was observed.

The automated differential digestion enables laboratories to process sexual assault evidence kits in a high efficiency and high throughput manner. The automated process allows a full plate of 96 sexual assault evidence samples to be processed in about 8 hours with very minimal analyst interaction. Using the conventional manual differential digestion process, the same number of samples would take about six times as long and would require the full engagement of the analyst. The minimal hands-on application of the automated method will also reduce the possibility of contamination and sample switching. Implementation of the automated differential digestion process will assist laboratories in significantly reducing the backlog in sexual assault evidence kit analysis.

INTRODUCTION

Many forensic laboratories are faced with an increasing demand for analysis of sexual assault evidence. The time consuming and labor intensive work that is required for DNA analysis, combined with an insufficient number of analysts to meet annual case requests in many laboratories, quickly results in an overwhelming backlog. This high demand is leading laboratories to transition to automation as a solution for processing a large number of cases in a highly efficient manner. Although many areas of DNA analysis have adopted automation, the process which separates sperm DNA from non-sperm DNA in sexual assault evidence samples remains a time consuming manual task.

Evidence items from sexual assault cases typically involve mixtures of DNA from the victim and assailant. In order to effectively interpret the DNA profiles obtained from evidence items containing sperm, the victim's non-sperm cellular material, which is often predominantly epithelial cells, must be separated from the suspect's sperm cells in a process called a differential digestion. First introduced in 1985[1], the differential digestion method takes advantage of the presence of disulfide bonds which protect the sperm heads and make them resistant to lysis by sodium dodecyl sulfate (SDS) and proteinase K (pro K). Epithelial cells are preferentially lysed by SDS and pro K while the sperm heads remain intact. The supernatant, which contains the lysed epithelial cells and other non-sperm cellular material, is removed into a separate tube; this is termed the epithelial cell (or non-sperm) fraction. The remaining pelleted material termed the sperm fraction, which contains the intact sperm heads, is cleaned of any residual non-sperm cell DNA by a series of centrifugation and wash steps. Finally, the sperm heads are lysed by addition of SDS, pro K, and dithiothreitol (DTT) which disrupts the disulfide bonds protecting the sperm cell membrane. The differential digestion is an effective process for separation of DNA from sperm cells

and DNA from non-sperm cells. However, it is a time consuming and laborious manual process, and complete separation of the epithelial cell DNA from the sperm cell DNA is not always achieved.

Slight modifications have been made to the differential digestion since its introduction [2, 3, 4]; however, the process has remained relatively unchanged. A new technique termed "selective degradation" has recently been proposed as a modification to the differential digestion process. This approach uses an agent to degrade any residual non-sperm cell DNA remaining in the sperm fraction after lysis; the unlysed sperm cells remain unaffected by this agent [5]. Selective degradation replaces the need for multiple centrifugation and wash steps, making this method amenable to automation[5]. Various platforms of the selective degradation method have been introduced [6]. In this article, DNase I was utilized as the degradative agent.

DNase is routinely used in the molecular biology field to degrade and eliminate unwanted DNA. However, using an enzyme that degrades DNA on evidence samples is concerning to many given the invaluable and often irreplaceable nature of evidence. DNA from evidence samples is often limited in quantity and potentially previously compromised by environmental and/or chemical degradation processes, exacerbating the risk of destroying potentially informative DNA.

The first phase of this research project focused on optimization of the selective degradation differential digestion process. This included selection of a detergent, digestion times and temperatures, reagent concentrations, and DNase inactivation. The second phase of this research focused on transitioning the optimized selective degradation protocol to an automated robotic platform. The reproducibility and sensitivity of the automated method was examined and results were compared to the conventional differential digestion method. The use of DNase on mock forensic samples was examined for its effects on DNA yield, efficiency of separation of sperm and non-sperm DNA, and quality of STR DNA typing results. In addition, the effect of DNase on aged and environmentally compromised samples was examined.

METHODS

Samples

1. Semen Samples

Sperm was obtained from neat semen samples donated by laboratory staff. The seminal fluid was removed by centrifugation and the remaining sperm cells were reconstituted in phosphate buffered saline (PBS). This allowed for consistent aliquots of cells for sample comparison. The DNA concentration of the sperm samples was determined by DNA quantitation using real time PCR. Dilutions of these neat sperm samples were made to target the desired concentrations for various experiments.

2. Vaginal Samples

Epithelial cells were obtained from vaginal swabs donated by laboratory staff. The epithelial cells were recovered by soaking the swabs in PBS and the DNA concentration was determined by DNA quantitation using real time PCR. Neat epithelial cell samples were used in all experiments.

3. Mock Sexual Assault Samples

Mock sexual assault samples were prepared by combining 15 µL of each of the prepared sperm and epithelial cell solutions (see above). Due to the large number of samples generated in this research, multiple sperm and epithelial cell solutions were prepared thus the starting concentrations of the solutions may vary. However, to ensure consistent quantitation results, samples within a set that were compared to each other all had the same starting sperm and epithelial cell concentrations. To represent the conditions often observed in typical sexual assault evidence, samples were prepared such that low quantity of sperm cells were mixed with an excess of epithelial cells. Initial manual experiments (Phase 1) optimizing the reagent concentrations and incubation conditions were performed using liquid samples to eliminate the factor of cells sticking onto swabs. After achieving optimal parameters, the process was transitioned from a manual single-tube format to an automated 96-well plate format (Phase 2). Samples were prepared in the same way as the manual experiments with the exception of using sterile cotton swabs as the substrate for sperm and epithelial cells. Cuttings of the swabs were placed into a 96 deep-well plate (Promega Slicprep^{TM} 96 device).

4. Aged Semen Stains

Eleven semen stains spotted onto fabric swatches between the years of 1952 and 1991 were obtained from Forensic Analytical Specialties, Inc (Hayward, CA). Six of the stains have been stored at room temperature while the other four have been stored frozen at -20°C.

Table 1: Table of the eleven samples that were obtained from Forensic Analytical Specialties, Inc. 'RT' means that the sample was stored at room temperature. 'FZ' means that the sample was stored in frozen condition.

Year	Storage Condition	# of Stains		Year	Storage Condition	# of Stains
1952	RT	1		1985	RT	2
1977	RT	1		1985	FZ	2
1979	RT	1		1991	RT	1
1980	FZ	2		1991	FZ	1

5. Environmentally Compromised Samples

Environmentally compromised samples were simulated by subjecting sperm cell spotted on oral swabs to ten freeze/thaw cycles. The samples were repeatedly taken in and out of the freezer for hours at a time for five days. Another set of swabs were exposed to heat in a humidity chamber for five days at 75°C and 80% relative humidity.

6. Unknowns

Seven mixed samples were obtained from previous proficiency tests prepared by Collaborative Testing Services (CTS) stored at -20°C. The seven selected samples consisted of semen and blood mixtures on fabric swatches.

Table 2: Table of the seven CTS proficiency tests that were used and the associated item numbers that were examined.

CTS Number	Item Number		CTS Number	Item Number
06-571	4		08-573	4
06-575	3		08-575	3
07-574	3		09-571	3
07-576	4			

7. Controls

   Reagent blanks were incorporated into each set of samples to control for contamination during the extraction process. Positive and negative amplification controls were ran with each amplification set.

Reagents

- Phosphate Buffered Saline (0.01M, pH 7.4) – Sigma Aldrich P3813, RT
- Stain Extraction Buffer, RT
  - 10mM Tris HCl – Sigma Aldrich T3038
  - 10mM EDTA – Sigma Aldrich 03690
  - 100mM NaCl – Sigma Aldrich S9625
  - 2% SDS – SERI B122
- Tween 80 Buffer, RT
  - 20mM Tris HCl – Sigma Aldrich T3038
  - 1mM EDTA – Sigma Aldrich 03690
  - 2% Tween 80 – Sigma Aldrich P5188
- DNase I (1 Kunitz Unit (U)/µL), Frozen
  - 1 U/µL DNase I – Sigma Aldrich D5025
  - 40% Glycerol – Sigma Aldrich G6279
  - DEPC Treated Water – Sigma Aldrich 95284
- $MgCl_2$ and $CaCl_2$ Salt Solution, RT/Refrigerated
  - 90mM $MgCl_2$ – Sigma Aldrich 68475
  - 5mM $CaCl_2$ – Sigma Aldrich 21115
  - DEPC Treated Water – Sigma Aldrich 95284
- EDTA (0.5M) – Sigma Aldrich 03690, RT/Refrigerated
- Proteinase K (20mg/mL) – Sigma Aldrich P2308, Frozen
- Dithiothreitol (1M) – Sigma Aldrich D9779, Frozen
- Christmas Tree Stain – SERI R540

Selective Degradation Digestion

Initial experimental conditions were based on published data describing the selective degradation process [5]. In the method published by Garvin, samples were digested using a lysis buffer containing Triton X-100 and pro K. After removing the epithelial cell fraction, DNase digestion was achieved by the addition of 450 U of DNase and 25µL of a solution containing 125mM $CaCl_2$ and $MgCl_2$. After one-hour of sample incubation at 56°C, the DNase was inactivated by the addition of 20µL of 0.5mM EDTA. The sperm fraction was lysed by the addition of lysis buffer and DTT.

Microscopic Examinations

Microscope slides were prepared on an eight well microscope slide prior to and after the initial digestion step (**Figure 5**). Aliquots of the samples, consisting of re-suspended cellular material, were stained with

the Christmas Tree stain and examined with brightfield microscopy. The expected concentration of sperm-derived male DNA per sample was calculated by assuming 80 fields of view at 400x magnification, using 3µL of sample eluted into a final extract volume of 50µL, and with an expectation of ~3.5pg of DNA per haploid sperm cell.

DNA Extraction and Quantitation

The epithelial (or non-sperm) cell fractions and sperm cell fractions were extracted on the Qiagen EZ1 Advanced XL BioRobot® using the Investigator Kit Large Volume Protocol which utilizes a silica bead-based extraction method. DNA extracts were quantitated using the Plexor® HY kit (Promega) on a 7500 Real Time PCR System (Applied Biosystems) and were analyzed using the Plexor Analysis Software v1.5.4.18. The Plexor® HY Kit provides simultaneous quantitation of autosomal (total human) and Y-chromosomal (male) DNA [7]. Sperm fraction DNA concentrations were evaluated based on the male DNA yield. The sperm fraction autosomal DNA yield was not used because epithelial cell DNA carryover may be present, which would provide a less accurate reflection of the recovered sperm cell DNA.

DNA Amplification and STR Genotyping

Samples were normalized to a concentration of 0.15 ng/µL of total human DNA in 10µL and were amplified using the AmpF*l*STR® Identifiler® Plus PCR Amplification Kit on a GeneAmp® 9700 PCR thermal cycler (Applied Biosystems) for 28 cycles. PCR products were separated by capillary electrophoresis using an ABI® 3130 Genetic Analyzer (Applied Biosystems) using 5 second injections. STR DNA typing data were analyzed using GeneMapper® ID 3.2.1 software (Applied Biosystems) with a 50 RFU analytical threshold and 150 RFU stochastic threshold. The acceptable peak height ratio was 60% based on internal laboratory validation study across all loci. All procedures were performed according to manufacturer's instructions.

RESULTS

Phase I: Optimization of Reaction Conditions

SELECTION OF DETERGENT

The addition of DNase to the differential digestion procedure requires changing the detergent in the lysis buffer from SDS (ionic detergent) to a non-ionic detergent. The sodium ions in SDS inhibit DNase by competing with divalent ions (such as Mg^{2+} and Ca^{2+}) for a site that either directly or indirectly affects the active site on DNase [8, 9, 10]. Three non-ionic detergents, Triton X-100, Tween 80, and IGEPAL CA 630, were selected for evaluation of compatibility with DNase, cell lysis efficiency, and compatibility with microscope slide sample preparation and staining.

Two sets of four identically prepared samples containing both sperm and epithelial cells were digested with the three non-ionic detergents and with SDS. Microscopic examination of the re-suspended post epithelial cell digest demonstrated the absence of intact epithelial cells and the presence of sperm cells in all samples. The sperm fractions were subjected to DNase treatment to remove any residual epithelial cell DNA. Quantitation results showed no statistical difference in the epithelial cell fraction DNA yield

between each of the four detergents (**Figure 1**). However, the sperm fraction showed significant differences in DNA yield between the SDS and non-ionic detergent samples **(Figure 2)**. The samples digested with SDS resulted in approximately four times more in DNA yield than sample digested with non-ionic detergents. The significant decrease in DNA yield could either indicate poor lysis efficiency of the non-ionic detergents or potential inhibition or degradation caused by DNase. Poor lysis efficiency of the non-ionic detergents was unlikely since the epithelial cell fractions yielded similar results between all four detergents. An additional set of four samples containing only sperm cells was digested with the three non-ionic detergents and with SDS to verify whether poor lysis efficiency was the cause. Samples were not subjected to DNase treatment to control for variable factors. No statistical difference was detected in the DNA yield between samples digested with each of the four detergents **(Figure 3)**. These results demonstrated the ability of each non-ionic detergent to efficiently lyse cells and provide the same yield in DNA as the currently used detergent, SDS. Further research determined that the concentration of the DNase, Mg^{2+}, and Ca^{2+} directly affected the DNA yield. Optimization of these reagents to achieve high DNA yield will be discussed in later sections.

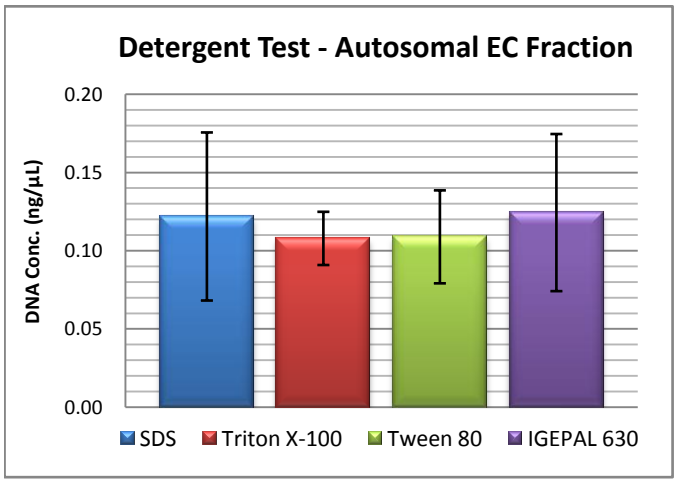


Figure 1: Autosomal DNA yield of the epithelial cell fraction of samples digested with SDS and the three non-ionic detergents. Error bars represent one standard deviation.

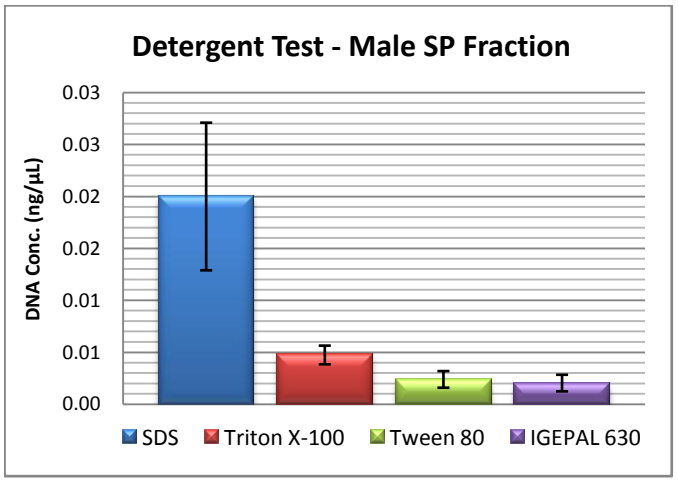


Figure 2: Male DNA yield of the sperm cell fraction of samples digested with SDS and the three non-ionic detergents. Error bars represent one standard deviation.

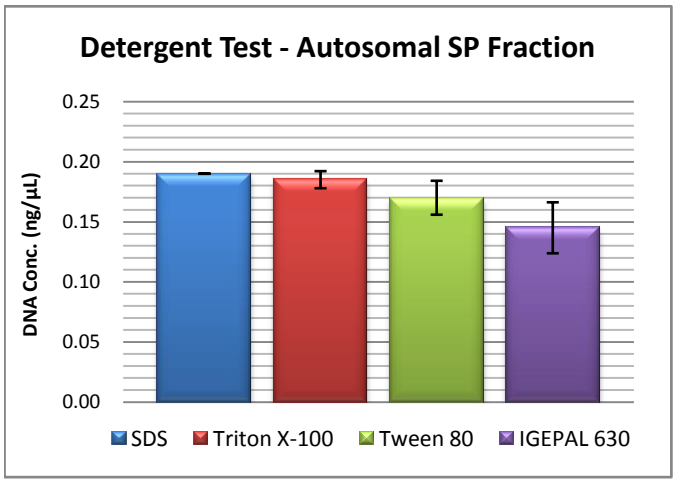


Figure 3: Autosomal DNA yield of the sperm cell fraction of samples digested with SDS and the three non-ionic detergents. Error bars represent one standard deviation.

The sperm fractions of the samples digested with the three non-ionic detergents resulted in clean single source male STR DNA profiles. The sperm fractions of the SDS digested samples exhibited an approximately 1:5 female-to-male mixture based on peak heights. **(Figure 4)**. The presence of epithelial DNA carryover indicated that DNase was inactive in the SDS sample and was not able to eliminate the epithelial DNA. This indicates that SDS inhibits the activity of DNase. Successful elimination of the epithelial DNA in the non-ionic detergent samples demonstrated the compatibility of the non-ionic detergents with DNase activity.

Figure 4: Electropherogram of a sample digested with SDS. A mixture between the epithelial cell donor and the sperm cell donor is observed. Data is scaled to 1600 RFUs.

Microscopic examination of sexual assault evidence for sperm cells is an important step in confirming the presence of semen. The process of microscope slide preparation includes heat fixing 3µL of sample onto a slide and staining with Christmas Tree Stain. The selective degradation process does not require washing the sperm pellet and thus the detergent remains in the sample. Therefore, an important factor in detergent selection is the ability of the cell suspension to readily dry on a microscope slide. Samples containing SDS, Triton X-100, 0, and IGEPAL 630 did not completely dry on microscopes slide during the heat fixation step. The interaction that occurs between the detergents and the dyes results in a loss of surface tension, indicated by the inability of the dyes from the Christmas Tree stain to maintain a beaded, spherical shape over the well **(Figure 5)**. The inability to heat fix samples to the slide poses a problem for microscopic examination of the cellular material because the wash steps during the staining process could result in the loss of unfixed cells and could lead to contamination between wells. Tween

80 was selected as the detergent for the lysis buffer in the differential digestion process due to its cell lysis efficiency and compatibility with microscope slide preparation.

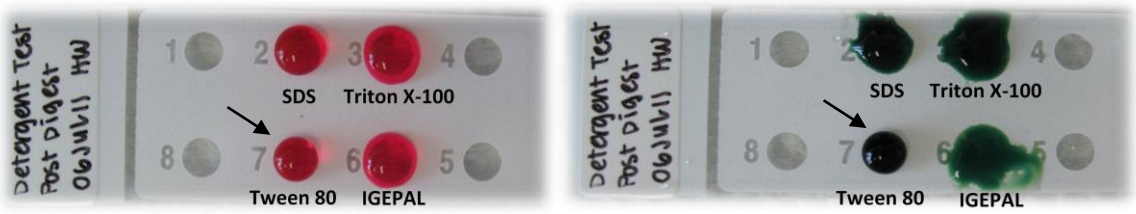


Figure 5: Microscope slide preparations of samples containing SDS, Triton X-100, Tween 80 and IGEPal 630 using the Christmas Tree stain. Samples were stained with nuclear fast red for ~15 minutes, washed with water, followed by staining with picroindigocarmine green for ~15 seconds and washed with ethanol. The sample containing Tween 80 was the only one that heat dried on the slide, as indicated by a well-formed spherical beaded shape.

DIGESTION TIMES AND TEMPERATURES

The epithelial cell fraction of three sets of six samples containing both sperm and epithelial cells were subjected to digestion with a lysis buffer containing Tween 80 and pro K for 60-minute, 30-minute, and 15-minute periods. In the latter, after the initial digestion, the epithelial cell fraction supernatant was removed and the sample was re-digested for an additional 15 minutes with fresh lysis buffer and Pro K. The DNase digestion and sperm fraction digestion was held constant for 15 minutes in all experiments. Quantitation data showed no statistical difference in DNA yield between the epithelial cell fractions of all three digestion times **(Figure 6).** This indicated that at a minimum of 15 minutes, all epithelial cells present in the samples were completely digested based upon microscopic examinations and additional digestion times provided no additional cell lysis. The 30-minute epithelial cell digestion was selected as the digestion time for all subsequent experiments due to having the shortest digestion time with the least number of sample manipulations.

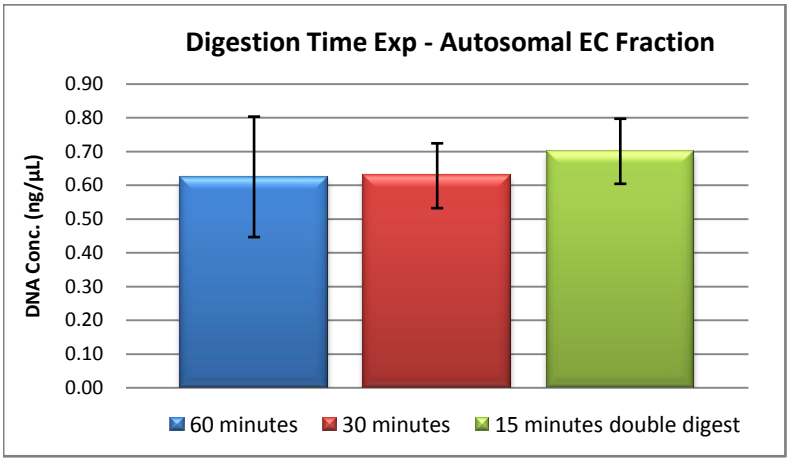


Figure 6: Autosomal DNA yield of the epithelial cell fraction of samples digested for various times. Error bars represent one standard deviation.

DNase I remains active from ambient temperature (~25°C) to 60°C in solutions ranging from pH 5 to 7 [11]. Most literature recommends DNase incubation at a temperature of 37°C because the most common application of DNase is RNA purification and the more fragile nature of RNA requires a lower incubation temperature. DNA is often incubated at 56°C in forensic applications, therefore the DNase incubation step was evaluated at 37°C and 56°C to determine whether DNase activity was affected by the elevated temperature.

One set of six identically prepared samples containing both epithelial and sperm cells were digested for 30 minutes. The epithelial cell DNA supernatant was removed and DNase was added to the sperm fraction. The sperm fractions were incubated for 15 minutes. Half of the sample set was incubated at 37°C and the other half of the set was incubated at 56°C. No statistical difference was observed in DNA yield between DNase incubation at 37°C and 56°C **(Figure 7)**. DNase activity and its ability to eliminate residual epithelial DNA was not affected by the difference in temperatures, as demonstrated by clean sperm fraction STR DNA profiles with no epithelial cell DNA carryover **(Figure 8)**. To maintain a constant temperature range throughout the entire digestion protocol, the DNase incubation temperature of 56°C was selected.

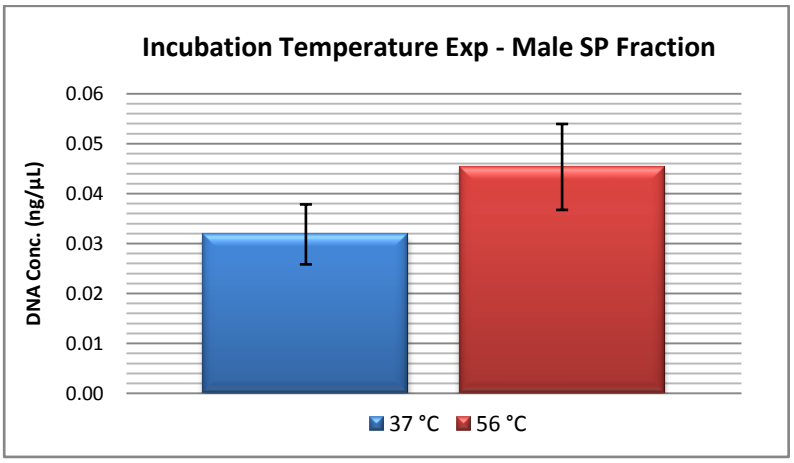


Figure 7: Male DNA yield of the sperm cell fraction of samples digested at 37°C and 56°C. Error bars represent one standard deviation.

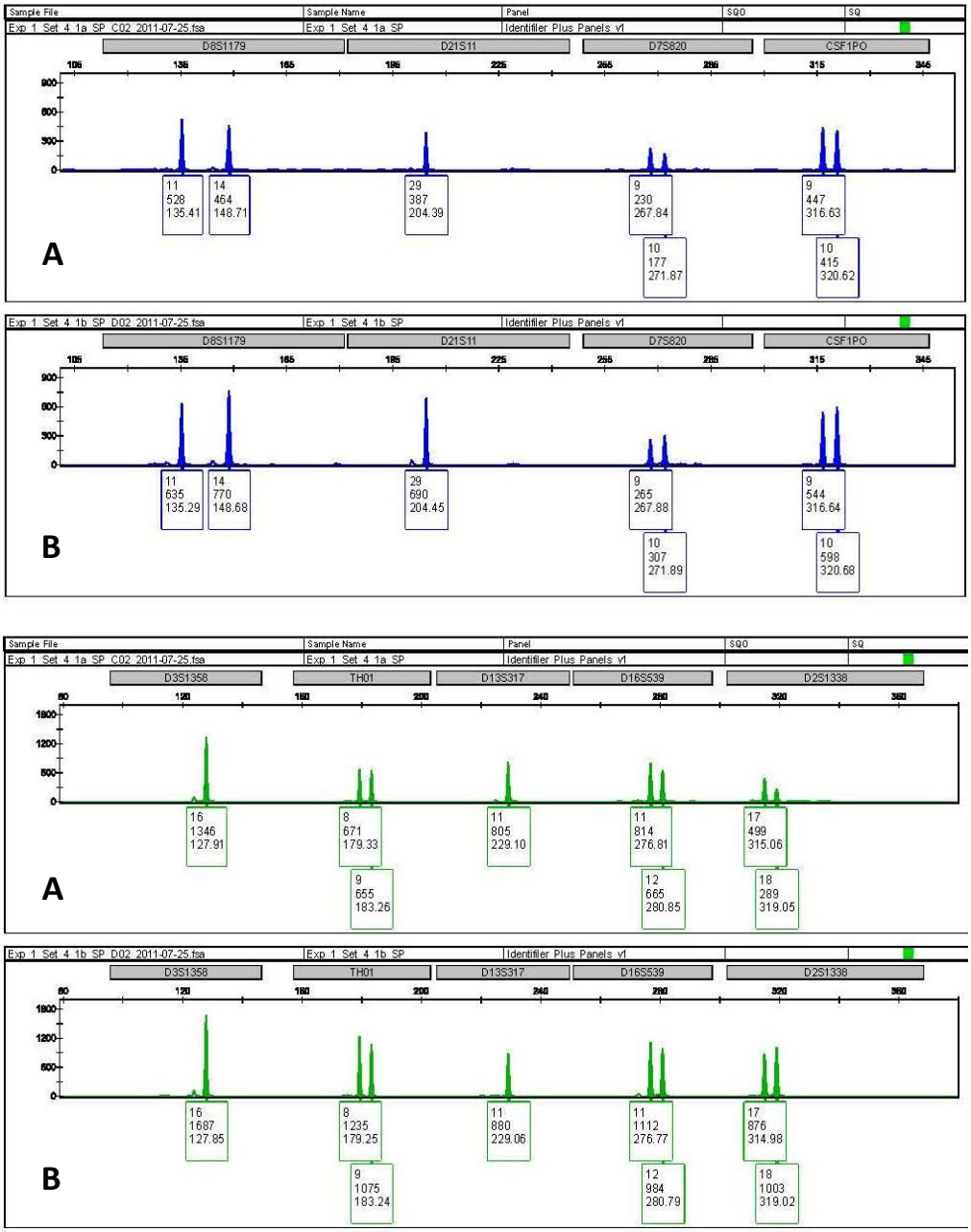


Figure 8: Electropherogram of two samples with different DNase incubation temperatures. The sample on the top (A) was incubated at 37°C and the sample on the bottom (B) was incubated at 56°C. Both samples yielded clean, single source profiles. Data on the blue dye was scaled to 1000 RFUs and the green dye was scaled to 2000 RFUs.

REAGENT CONCENTRATION OPTIMIZATION

The initial reagent concentrations used were based on published data describing the selective degradation process [5]. Quantitation and STR DNA typing results from the first sets of experiments (detergent tests and digestion times & temperatures) indicated the need for optimization of the reagent concentrations to achieve high DNA yield and high DNA typing quality. Experiments were performed to optimize the concentrations of DNase and divalent cations (Mg^{2+} and Ca^{2+}). DNA quantitation and STR typing data were evaluated to determine the optimal reagent concentrations for producing single source profiles with high DNA yield and high STR DNA typing quality. Twelve sets of two to four samples containing both sperm and epithelial cells were prepared. The first six sets of samples were aimed at optimizing the $CaCl_2$ and $MgCl_2$ concentration. The last six sets of samples were aimed at optimizing the DNase concentration and the $MgCl_2$ concentration. DNase concentrations were tested at 1, 2, 5, 10, 15, 18, and 360 (U). $MgCl_2$ concentrations were tested at 10, 25, 45, 90, 125, and 180 mM while $CaCl_2$ concentrations were tested at 1, 2, 3, 4, 5, 10, 25, and 125 mM.

Evaluation of DNA Quantitation Yield

The initial experiments using selective degradation by DNase I in the differential digestion process resulted in a significant decrease in DNA yield of the sperm fraction. Only 30% of the male DNA from the sperm fraction was recovered compared to the same sample digested with the conventional differential digestion method. This significant loss in DNA may be the difference between a full DNA profile, a partial profile, or no profile depending on the starting DNA concentration of the sample. Many sexual assault evidence samples contain low levels of sperm and such a lowered recovery rate would not warrant the use of an automated selective degradation differential digestion process as crucial data may be lost.

The most probable explanation for the loss of sperm DNA was incomplete deactivation of DNase prior to sperm lysis. Any remaining DNase activity can potentially destroy the sperm DNA once the sperm cells have been lysed. The activity of DNase is directly related to the divalent ion concentration [12]. As a result, multiple experiments were performed to optimize the concentrations of $MgCl_2$ and $CaCl_2$. The concentrations experimented upon were selected based on recommended divalent ion concentrations for DNase applications in various DNase I product information guides [11, 21, 22]. **Figure 9** illustrates the various combinations of $MgCl_2$ and $CaCl_2$ concentrations that were tested. Results showed a general increase in the sperm fraction DNA yield as the concentration of Ca^{2+} decreased; the Mg^{2+} concentration did not have a significant effect on the DNA yield. Samples containing 1mM Ca^{2+} gave the highest DNA yield and were comparable to DNA yields obtained by the conventional differential digestion method. However, the STR DNA typing data revealed a 1:1 mixture of male and female DNA, indicating the lack of DNase activity. This demonstrated that excessive DNase activity and the incomplete DNase inactivation was the cause to the decrease in DNA yield.

Samples containing 5mM Ca^{2+} produced the second highest sperm fraction DNA yield and clean single source STR DNA profiles **(Figure 9)**. From these results, it was concluded that 1mM Ca^{2+} was insufficient for activation of DNase and a minimum of 5mM Ca^{2+} was required for DNase activity. Additional experiments were performed using 1 to 5mm Ca^{2+} to determine the optimal Ca^{2+} concentration which produced the highest DNA yield and the cleanest sperm fraction DNA profile. The Mg^{2+} concentration

was held constant at 10mM. Samples with 5mM Ca^{2+} produced clean, single source male STR DNA profile with no epithelial cell DNA carry-over. Samples with 1mM and 2mM Ca^{2+} each resulted in mixtures while samples with 3mM and 4mM Ca^{2+} had few occurrences of low level epithelial cell DNA carry-over; this is illustrated in **Table 3** with the number of alleles attributable to epithelial cell DNA carry-over at variable Ca^{2+} concentrations. A concentration of 5mM $Ca^{2+}$was chosen as it provided the cleanest profile while maintaining an acceptable DNA yield.

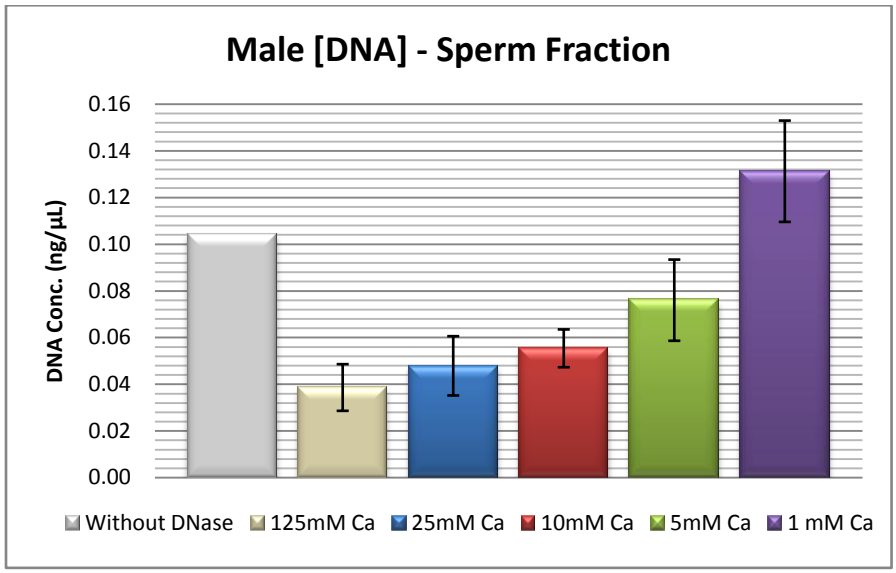


Figure 9: Male DNA yield of the sperm cell fraction of samples with varying $CaCl_2$ and $MgCl_2$ concentrations. The $MgCl_2$ concentration did not seem to have an effect on the DNA yield. However, a decrease in $CaCl_2$ concentration resulted in an increase in DNA yield. Error bars represent one standard deviation.

Table 3: Two sets of six samples digested with varying concentrations of Ca^{2+}. The average number of alleles in the sperm fraction belonging to carry-over from epithelial cell DNA is shown. Complete carry-over of the epithelial cell DNA is represented by 19 detected non-overlapping epithelial cell donor alleles.

Calcium Concentration	Number of Alleles from EC Carry-over
Without DNase	19
1mM	19
2mM	19
3mM	8
4mM	2
5mM	0

Evaluation of STR DNA Typing Quality

Evaluation of the STR DNA typing data of samples digested with the original reagent concentrations revealed poor data quality. The targeted DNA input for amplification of 1.5ng typically results in STR profiles with satisfactory peak height levels of approximately 1000 RFU for heterozygous loci. However, low peak height levels ranging from ~100-500 RFU and peak height imbalances of less than the acceptable 60% were observed when 1.5ng of DNase digested samples were amplified and typed

(Figure 10). The STR DNA profiles of the samples subjected to the selective degradation differential digestion process showed peak heights that averaged 3-4 times lower than samples subjected to the conventional differential digestion process. While both methods resulted in the same DNA yield for equivalent DNA inputs, a decrease in peak heights indicated a negative effect of the selective degradation process on STR DNA amplification and typing. In addition, there were some occurrences of mixtures in the sperm fraction STR DNA typing data. Since these samples contained higher epithelial cell DNA concentrations, it is possible that the Mg^{2+} and Ca^{2+} concentrations need additional optimization in order to eliminate all residual epithelial cell DNA.

One possible cause for the low peak height levels in the samples subjected to selective degradation with DNase is inhibition. The reagents in the selective degradation process that could potentially inhibit the STR DNA typing process are DNase, EDTA, $MgCl_2$, or $CaCl_2$. EDTA can inhibit the PCR process by chelating the Mg^{2+} ions necessary for DNA polymerase activity [13]. Ca^{2+} ions can also inhibit the PCR process by competing with Mg^{2+} as a cofactor for the DNA polymerase used in amplification [14]. Experiments were conducted to determine if these reagents caused inhibition in the STR DNA typing process. Separate blank samples containing lysis buffer were spiked with either DNase (360 units, inactive due to lack of divalent activators), EDTA (20µL of 0.5M), or $MgCl_2$ & $CaCl_2$ (25 µL of a solution containing 10mM $MgCl_2$ and 5mM $CaCl_2$), followed by the addition of DNA extracts. The samples containing 1.5 ng of DNA were amplified and typed. STR DNA typing data from all three samples were of satisfactory peak height levels of at least 1000 RFU and showed no signs of inhibition. As a result, inhibition from DNase, EDTA, or $MgCl_2$ and $CaCl_2$ was eliminated as the cause of the poor STR DNA typing data.

Degradation of the samples by DNase is another possible cause for the low peak heights. The average amplicon size for the Plexor HY quantitation kit is approximately 100-150bp [7]. The average amplicon size for the Identifiler Plus amplification kit is approximately 100-360bp [15]. Short DNA fragments resulting from degradation may still be sufficient for amplification during quantitation due to the small amplicon targets. However, the amount of DNA of a sufficient length available for successful amplification by Identifiler Plus may be significantly less. Thus, the actual amount of amplifiable DNA input in a degraded sample may be significantly less than expected from the quantitation data. Since allele peak heights are directly related to the amount of DNA input, lower than expected quantities of amplifiable DNA may result in low-level typing data.

Lowering the DNase digestion temperature to 37°C and decreasing the DNase digestion time to 5 minutes did not improve the STR DNA typing data. The amount of DNase used was decreased from 360 units to 18 units to further explore the possibility of degradation due to DNase activity. Each aliquot of DNase was 18U/µL so the least amount of DNase that can be used without having to prepare new aliquots was 18 units. Decreasing the amount of DNase resulted in significantly improved STR DNA typing data **(Figure 11)**. The peak height levels of the sample digested with 18 units of DNase were approximately three times higher than the same sample digested with 360 units of DNase. However, a mixture was obtained. The sperm fraction of the sample digested with 360 units of DNase produced a low level single source profile. Increasing the concentration of Mg^{2+} from 10mM to 90mM resulted in a single source profile for the sperm fraction of the sample digested with 18 units of DNase.

These results demonstrate that the amount of DNase used has a significant effect on the quality of the STR DNA typing data. At high concentrations of DNase, lowered peak heights and peak height imbalances may result. The ratio of Mg^{2+} to DNase is important for optimizing DNase activity necessary for achieving clean single source DNA profiles. Lower amounts of DNase require a higher concentration of Mg^{2+} to eliminate all residual epithelial DNA. Lowering the amount of DNase and increasing the Mg^{2+} concentration for selective degradation does not affect the DNA yield of the sperm fraction.

Figure 10: Electropherogram of a sample prepared with the selective degradation differential digest method using 360 units of DNase, 10mM $MgCl_2$ and 5mM $CaCl_2$. Data is scaled to 1000 RFUs. The peak heights in this profile are low at approximately 100-550 RFUS, with significant peak height imbalances of less than the acceptable 60% at some loci.

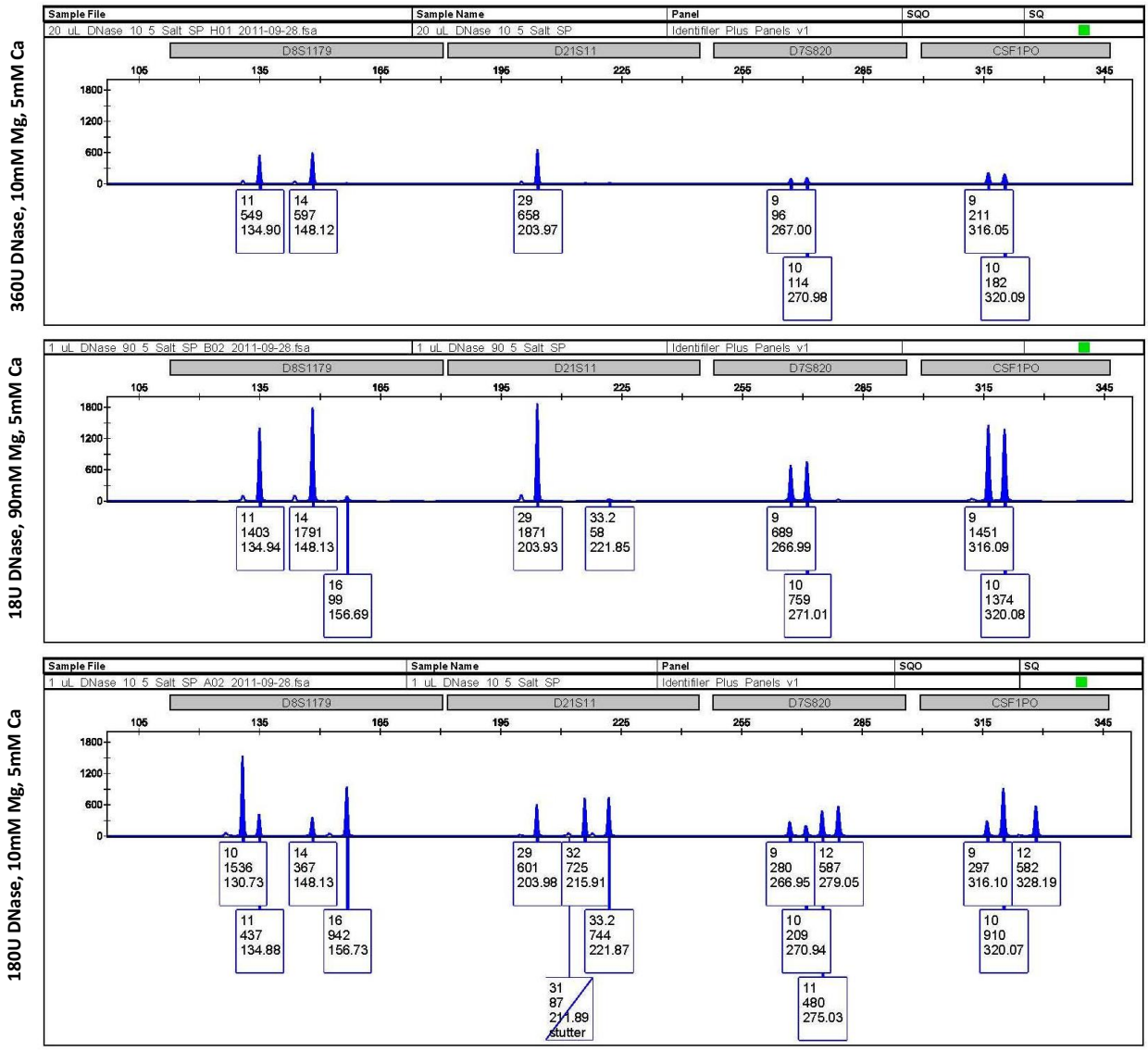


Figure 11: STR DNA profiles of samples with the same starting epithelial cell and sperm concentrations digested with varying amounts of DNase and Mg^{2+}. Each sample had 1.5ng of DNA input for amplification. Data scaled to 2000 RFUs. The first profile was prepared with the initial DNase and divalent ion concentrations, resulting in very low peak heights. The second profile was prepared with the optimal DNase and divalent ion concentrations, showing significant improvement in peak height and minimal epithelial cell DNA carry-over. The third profile was prepared with decreased DNase concentration but the same divalent ion concentrations as the first profile. While the first profile resulted in a single source profile, the third profile resulted in significant epithelial cell DNA carry-over, indicating that the

DNase Inactivation

Inactivation of DNase prior to sperm cell lysis is important to prevent the loss of sperm cell DNA. DNase can be inactivated by the addition of reducing agents, SDS, and metal chelators such as EDTA[16, 17]. DNase may also be heat-inactivated at temperatures above 65°C for 5-10 minutes [18, 19]. The activity of DNase is directly dependent on the presence of Mg^{2+} and Ca^{2+} divalent ions. Thus, removal of these divalent ion activators by the addition of EDTA was selected as the DNase inactivation method. Experiments consisting of two sets of six samples were performed to determine if the addition of heat was necessary to completely inactivate the DNase. EDTA was added to the sperm fractions after the

DNase digestion step and was allowed to chelate the Mg^{2+} and Ca^{2+} ions for 10 minutes at room temperature and at 65°C. It was observed that heat inactivation did not contribute to any differences in the sperm fraction DNA yield, indicating that the DNase was completely inactivated by EDTA prior to heating. The same results were seen in both sets of samples **(Figures 12 and 13)**. No difference was observed in the STR DNA typing data between the heat inactivated samples and the non-heat inactivated samples **(Figure 14)**.

DNase inactivation with EDTA was confirmed by preparing a blank sample that contained the reagents required for the selective degradation process all the way up to the EDTA inactivation step. A positive control sample containing only TE^{-4} was prepared. The reagent blank and positive control samples were spiked with the same quantity of extracted DNA. A negative control was prepared by subjecting the same quantity of extracted DNA through the DNase digestion process. The negative control was to verify that the DNase digestion procedure was performing properly. Quantitation results showed no statistical difference in the DNA yield between the blank sample and the positive control **(Table 4)**. This indicated that the DNase in the blank sample was completely inactivated by the addition of EDTA and therefore no loss of DNA was detected in the DNA extract. The negative control showed no detectable amount of DNA yield, confirming that the DNase was active and did eliminate all of the DNA extract. As a result, addition of EDTA without heat was chosen as the method for DNase inactivation.

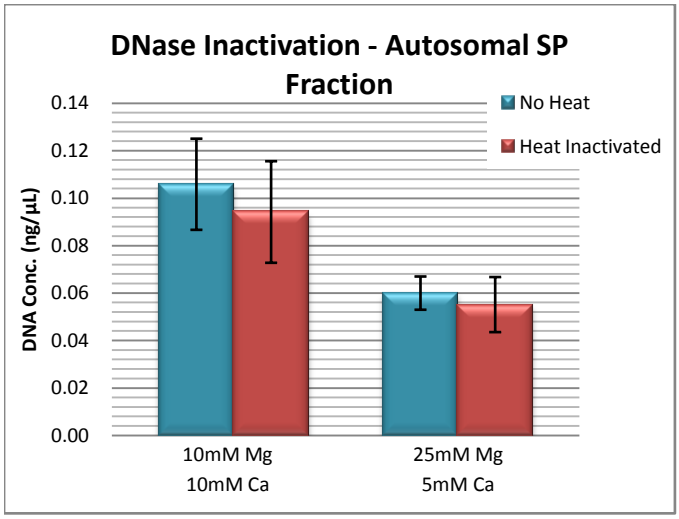


Figure 12: Autosomal DNA yield of the sperm cell fraction of two sets of samples. Half of each set of sample was subjected to heat inactivation and the other half was not. Error bars represent one standard deviation.

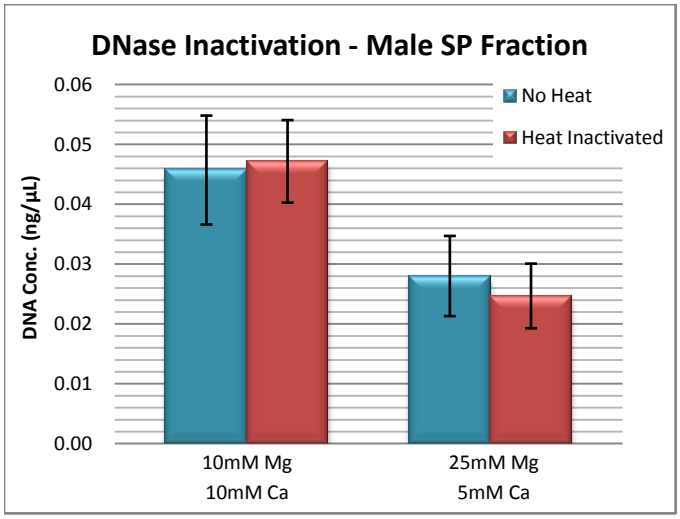


Figure 13: Male DNA yield of the sperm cell fraction of two sets of samples. Half of each set of sample was subjected to heat inactivation and the other half was not. Error bars represent one standard deviation.

Table 4: Autosomal and Male DNA concentrations of three samples testing DNase Inactivation. The negative control served to show that the DNase and divalent ion concentrations used were sufficient to activate the DNase and eliminate the DNA extract that was added prior to the DNase digestion. The Auto/Y Ratio of the negative control is irrelevant as the autosomal and male DNA detected in the autosomal and male is so insignificant. The positive control showed the DNA concentration of the DNA extract that was used. The blank sample demonstrated that after adding EDTA to the DNase and divalent ions solution, the DNase was completely inactivated and thus the DNA extract that was added afterwards yielded the same concentration as the positive control sample.

DNA Spiked Samples	Autosomal [DNA] (ng/µL)	Male [DNA] (ng/µL)	Auto/Y Ratio
Negative Control	0.0003	0.0002	1.2
Positive Control	6.5000	6.7000	1.0
Blank Sample	5.1000	6.7000	0.8

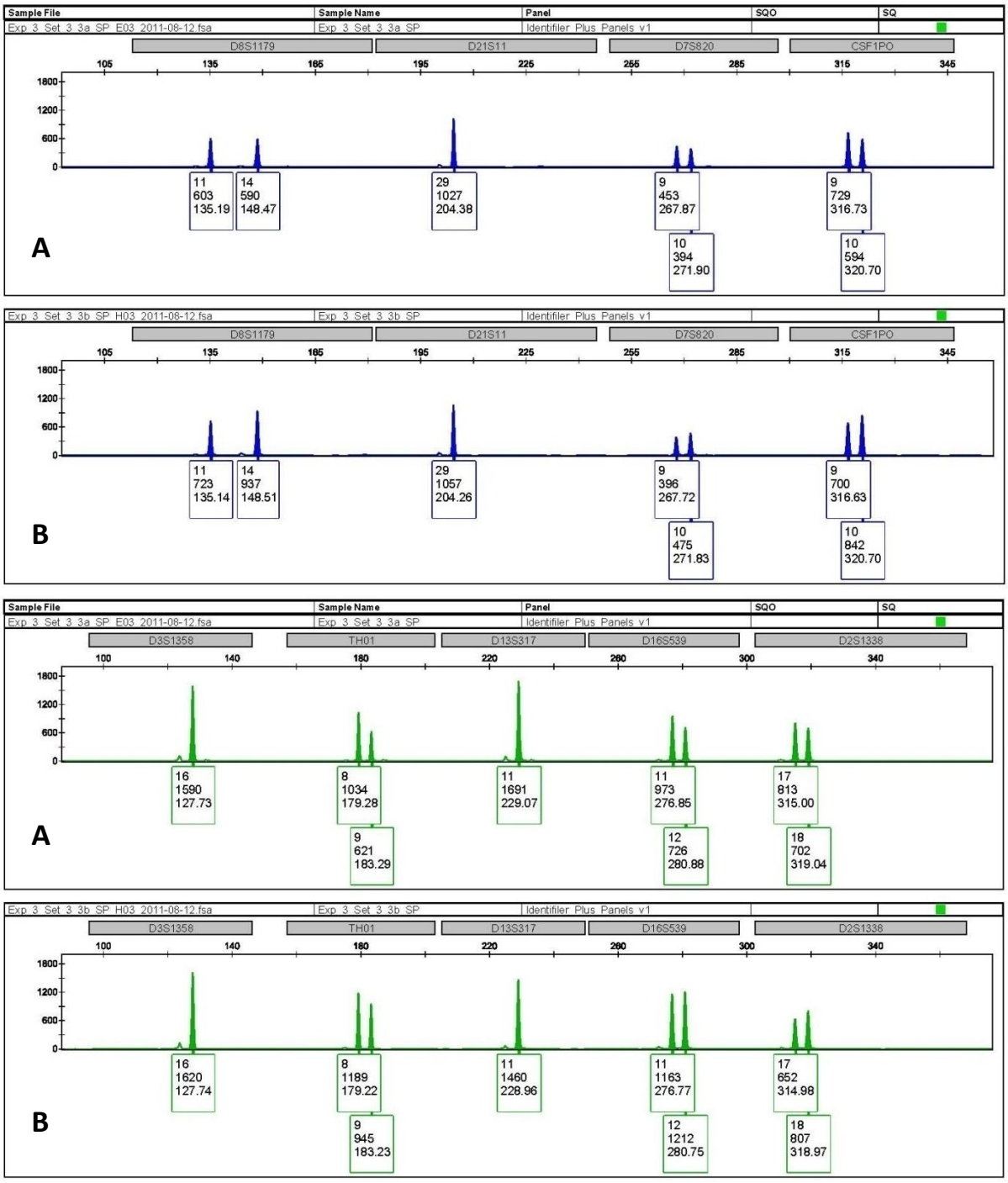


Figure 14: Electropherogram of two identical samples. The top sample (A) was subjected to heat inactivation with EDTA and the bottom sample (B) was subjected to only EDTA inactivation without heat. Data is scaled to 2000 RFUs.

OPTIMIZED SELECTIVE DEGRADATION DIFFERENTIAL DIGESTION PROCESS

Optimization of the selective degradation differential digestion process was achieved, producing results with high DNA yield and high STR DNA typing data quality. The optimized protocol is as follows:

Digest samples with 510 µL of a 2% Tween 80 buffer solution and 10 µL of pro K (20 mg/mL). Incubate samples with agitation at 56°C for 30 minutes using a dry block heating and shaking incubator (Eppendorf Thermomixer® R). Following the incubation, centrifuge the samples for 3 minutes at 2000 x g. Remove the supernatant containing lysed epithelial cell material and transfer to a separate tube; this is now called the epithelial cell (or non-sperm) fraction. Add 290 µL of a 2% Tween 80 buffer solution, 25 µL of a solution containing $CaCl_2$ (5mM) and $MgCl_2$ (90mM), and 15 µL of DNase I (1U/µL) to the sperm fraction and incubate at 56°C for 15 minutes to destroy any residual non-sperm DNA. Add 20 µL of EDTA (0.5M) and let sit for 10 minutes at room temperature to inactivate DNase I. Digest the sperm fraction adding 10 µL of pro K (20mg/mL) and 20 µL of DTT (1M) and incubate at 56°C for 15 minutes.

Phase 2: Transition to Automation

ROBOTIC PARAMETERS

Automation of the selective degradation differential digestion protocol employed the VERSA 1100 liquid handler (Aurora Biomed; **Figure 15**) with VERSAware software and Slicprep™ 96 plates (Promega). The VERSA 1100 includes an on-deck heating and shaking unit used for all automated incubation and swab agitation steps. A four channel pipettor head was used for liquid handling and for preparation of microscope slides. The VERSA 1100 was employed for transferring samples from the 96-well plate to individual 2.0mL tubes for DNA extraction on the EZ1 Advanced XL BioRobot® (Qiagen). Pauses are incorporated into the protocol to allow for manual intervention required for two centrifugation steps (6 minutes at 1450 x g) and for removing sample tubes and microscope slides.

The Slicprep™ 96 device is a 2.2mL 96 deep well plate that includes a 96 well spin basket insert and a U-shaped collar. Sample substrates are placed inside of the spin basket which has seven 1mm perforations which allow the flow of liquid in and out of the insert during incubation. The U-shaped collar is used to create space for the centrifugation steps by raising the spin basket by approximately 1cm [20].

Figure 15: VERSA 1100 liquid handler by Aurora Biomed.

Sample agitation is required for efficient release of biological material into solution because epithelial and sperm cells may adhere quite strongly to various substrates. In the conventional manual differential digestion, a sterile toothpick was used to manually agitate the swab after the PBS soak and the epithelial cell digestion steps. A concern with the transition from manual to robotic preparations was the capability of the robotic shaking platform to efficiently release epithelial and sperm cells from swabs.

Experiments were performed to compare the DNA yield from replicate samples prepared manually using toothpick agitation and robotically on the VERSA 1100 using the shaker. The selective degradation method was used in both processes. Half of a cotton swab was placed into each well of the Slicprep™ plate. After 500µL of PBS was added, the plate was placed on the shaker and the samples were agitated for 30 minutes at 2000 rpm at room temperature.

DNA quantitation results showed that robotic preparation resulted in a DNA yield approximately 10% of the yield obtained by manual preparation **(Figure 16)**. These results indicate inefficient release of the cellular material from the swab. One possible cause was that half of a swab filled up most of the interior of the narrow Slicprep™ Plate wells, resulting in inefficient space for agitation of the sample. In addition, 500µL of PBS was insufficient to completely submerge half of a swab in the plate well.

To resolve these problems, the halved cotton swabs were cut into 4 to 5 smaller pieces. The volume of PBS was increased to 1000µL to allow for complete submersion of the sample substrate. The shaking speed was increased to 2500 rpm. After changing these parameters, the DNA yield of samples agitated by shaking on the robot was now 90% of the samples agitated manually with a toothpick **(Figure 16)**. These results demonstrate that the volume of PBS in which the swabs are extracted and the size of the substrate placed inside the Slicprep™ plates are crucial for maximizing the release of cellular material during the shaking process.

However, a 1000µL volume exceeds the ability of the SlicPrep™ plate insert to lift the sample above the liquid level during the centrifugation process. The volume of PBS was therefore reduced to 700µL to accommodate the centrifugation process. Decreasing the volume of PBS resulted in robotically prepared samples with a DNA yield approximately 30% of manually prepared samples (**Figure 16)**. Further experiments were performed to determine if an overnight PBS soak, a manual toothpick agitation step, or inserting a toothpick during the shaking step would increase the number of cells released **(Table 5)**. An overnight PBS soak and inserting a toothpick during the shaking step did not increase the number of cells released. As expected, a manual toothpick agitation step did increase the number of cells released, providing DNA yields that were comparable to the manual selective degradation differential digestion process. As a result, incorporation of a manual agitation step may be required to increase cellular release from the swab and the resulting DNA yield.

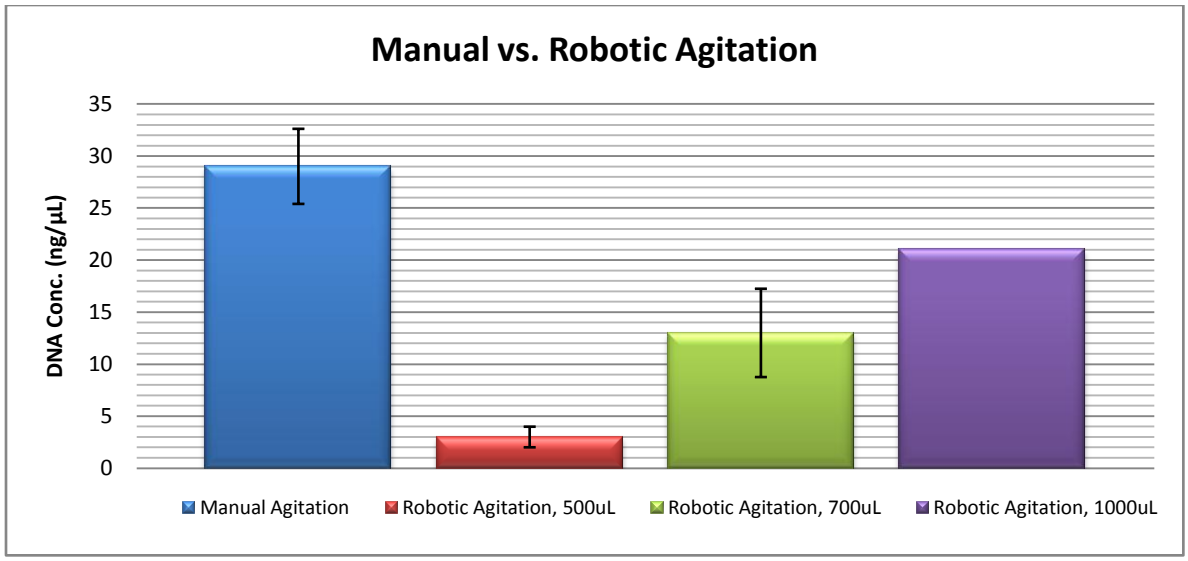


Figure 16: Average Male DNA yield of the sperm fraction of samples digested manually and robotically using the selective degradation method. Error bars represent one standard deviation. The "Manual Agitation" sample was agitated manually with a toothpick. The "Robotic Agitation, 500µL" sample was half of a cotton swab soaked in 500µL PBS and shaken for 30 minutes on the robot at 2000 rpm. Both of the "Robotic Agitation, 700µL" and "Robotic Agitation, 1000µL" samples were half of cotton swabs cut into 4-5 smaller pieces and were soaked in either 700µL or 1000µL PBS. Both samples were also shaken for 30 minutes at 2500 rpm. The 1000µL sample consisted of only one sample.

Table 5: Autosomal and Male DNA concentrations of samples digested manually and robotically using the selective degradation method. Manually digested samples were agitated by a toothpick. Robotically digested samples were agitated on a shaking unit on the VERSA 1100. The 'overnight PBS soak' sample was soaked in PBS overnight, then digested on the robot. The 'toothpick in well' sample had a toothpick inside the well of the SlicPrep™ during the shaking incubation step. The robotically digested 'toothpick agitation' sample was agitated on a shaking unit for 30 minutes but was agitated additionally by a toothpick after the shaking step.

Sample/Technique	Autosomal [DNA] (ng/µL)	Male [DNA] (ng/µL)	Auto/Y Ratio
Manual Digestion, Toothpick Agitation	18.0000	25.0000	0.7
Robotic Digestion, 500µL	8.1000	9.9000	0.8
Robotic Digestion, 700µL	14.0000	16.0000	0.9
Robotic Digestion, 1000µL	16.0000	21.0000	0.8
Robotic Digestion, overnight PBS soak	5.4000	7.7000	0.7
Robotic Digestion, Toothpick in well	9.6000	12.0000	0.8
Robotic Digestion, Toothpick Agitation	14.0000	19.0000	0.7

Conventional vs. Selective Degradation Method

After optimization of reagent concentrations and digestion conditions was achieved, three sets of twenty samples containing epithelial and sperm cells were used to evaluate the manual conventional differential digestion versus both the manual and robotic selective degradation differential digestion methods. DNA quantitation and STR DNA typing results from all three sample sets were compared.

The DNA quantitation results showed no statistical difference in DNA yield between the manual conventional differential digestion method and the manual selective degradation method **(Figure 17)**. This demonstrates that under the optimized parameters, the use of DNase does not result in any detectable loss of sperm cell DNA. However, in comparing the quantitation results of the manual selective degradation method with the robotic selective degradation method, an approximately 70% decrease in epithelial and sperm cell DNA yield was observed. The decrease in yield was due to the reduced release of cellular material from the substrate by robotic handling, even when the substrate was cut into smaller pieces and the optimized conditions of shaking at 2500 rpm for 30 minutes were used. **Table 5** demonstrates that incorporating a manual agitation step to the robotic selective degradation method produces similar DNA yield to the manual selective degradation method. As a result, a manual agitation step prior to centrifugation is recommended to maximize the release of cellular material from the substrate.

The STR DNA typing data for the conventional differential digestion method, the manual selective degradation method, and the robotic selective degradation method all produced clean sperm fractions with satisfactory peak height levels **(Figure 18)**. No signs of inhibition or degradation were observed.

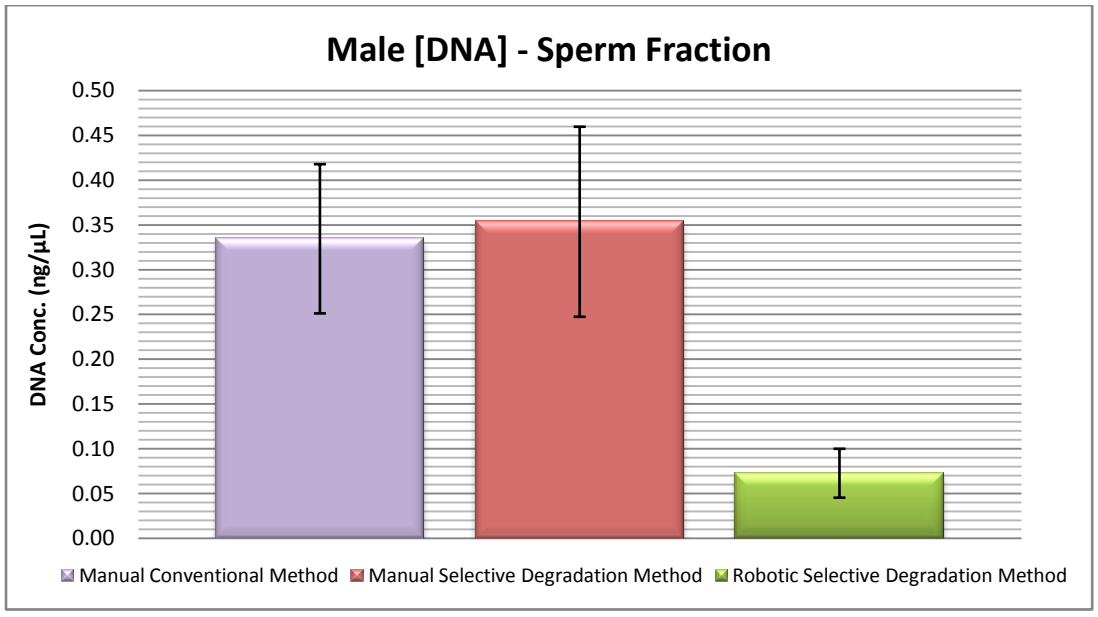


Figure 17: Male DNA yield comparing the three differential digestion methods: manual conventional, manual selective degradation, and robotic selective degradation. Error bars represent one standard deviation.

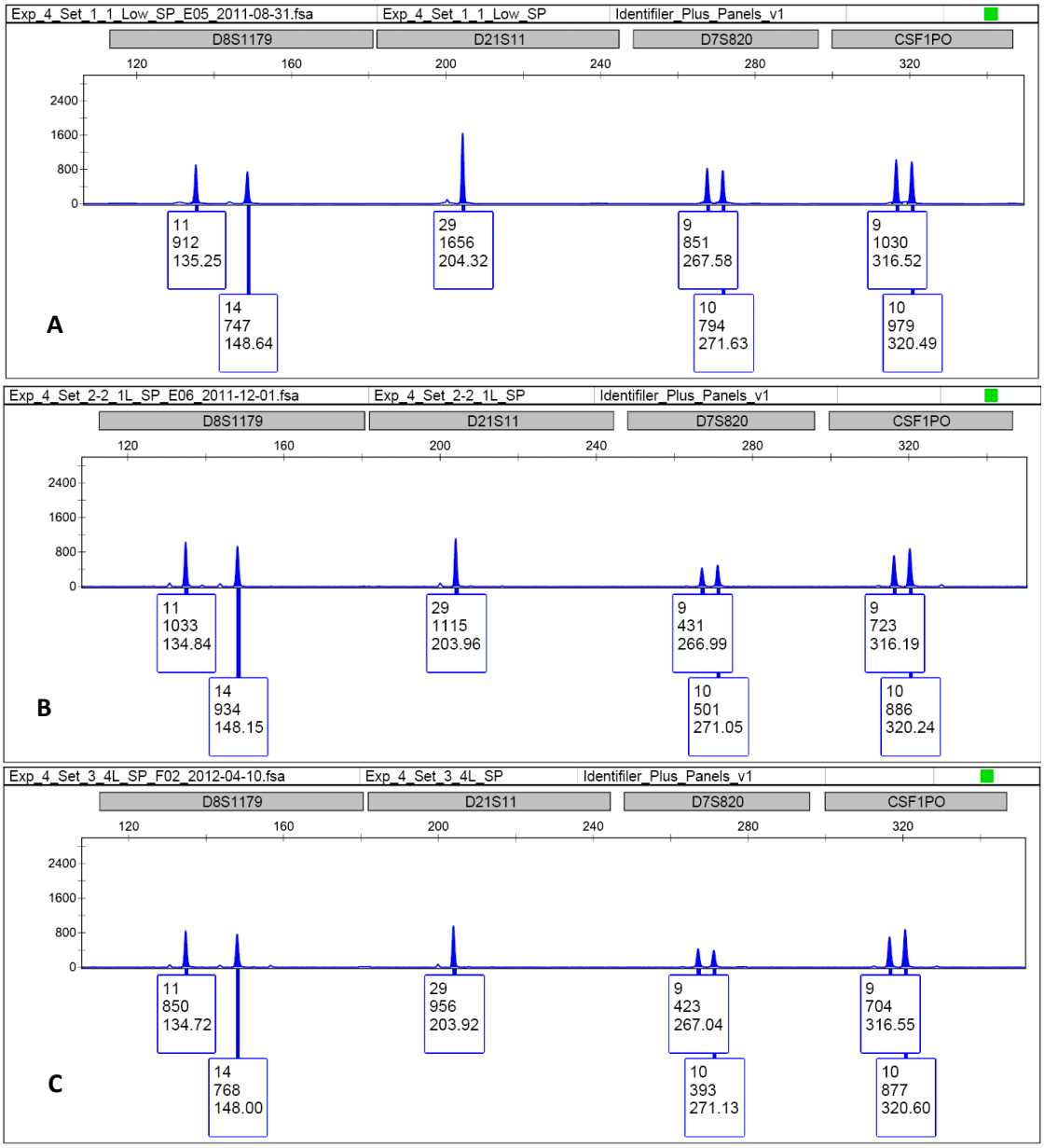


Figure 18: Electropherogram of three samples. The first sample (A) was digested using the conventional differential digestion method. The second sample (B) was digested manually using the selective degradation differential digestion method. The third sample (C) was digested robotically using the selective degradation differential digestion method. All data scaled to 3000 RFU.

AGED AND ENVIRONMENTALLY COMPROMISED SAMPLES

All of the preceding experiments employed freshly obtained cellular material. However, forensic samples are often exposed to environmental conditions such as extreme temperature shifts, light, and heat and humidity that could potentially lead to degradation of the DNA. The age of the samples may also affect the quality and condition of the cells and DNA. As a result, sperm samples subjected to harsh environmental conditions were studied to determine if physically compromised sperm samples would be lysed prior to the addition of DTT resulting in loss of sperm cell DNA due to destruction by DNase. Additionally, samples stored for an extended period of time at room temperature and -20°C were tested to determine if the age and storage conditions of the sperm sample affected the quality of DNA quantitation and STR typing results when using the selective degradation method.

Environmentally compromised samples were simulated by subjecting sterile cotton swabs containing epithelial cells and sperm cells through ten freeze/thaw cycles. Another set of swabs were kept in a humidity chamber for five days at 75°C and 80% relative humidity. The aged samples consisted of eleven neat semen stains on white cotton cloths from 1952-1991, stored at room temperature and -20°C. Three cuttings were taken from each stain.

Samples were subjected to both the conventional manual conventional differential digestion process and the robotic selective degradation process to compare the results of each method. Epithelial cell digestion of both sets of samples was conducted robotically. Samples were agitated using only the shaker. After removal of the epithelial cell fraction supernatant, the sperm pellets on the plate were re-suspended and split for further processing. Half of the sperm cell suspension was transferred to tubes for continued processing by the conventional differential digestion method, while the remaining half of the sperm cell suspension was retained on the plate and subjected to the robotic selective degradation process.

DNA quantitation data of aged and environmentally compromised samples showed no statistical difference in the male DNA yield for the samples prepared robotically with a DNase selective degradation step compared to the samples prepared manually by the conventional differential digestion method. There was no appreciable difference in the quality of the STR DNA typing data between the manually and robotically prepared samples **(Figure 19)**. Most of the samples resulted in full DNA profiles with satisfactory peak heights. Compromised or aged samples that resulted in low partial profiles did so regardless of whether the manual conventional differential digestion method or the robotic selective degradation preparation was used. These results indicated that environmentally compromised and aged sperm samples were able to withstand DNase treatment and produced equivalent results to samples processed by the conventional differential digestion method.

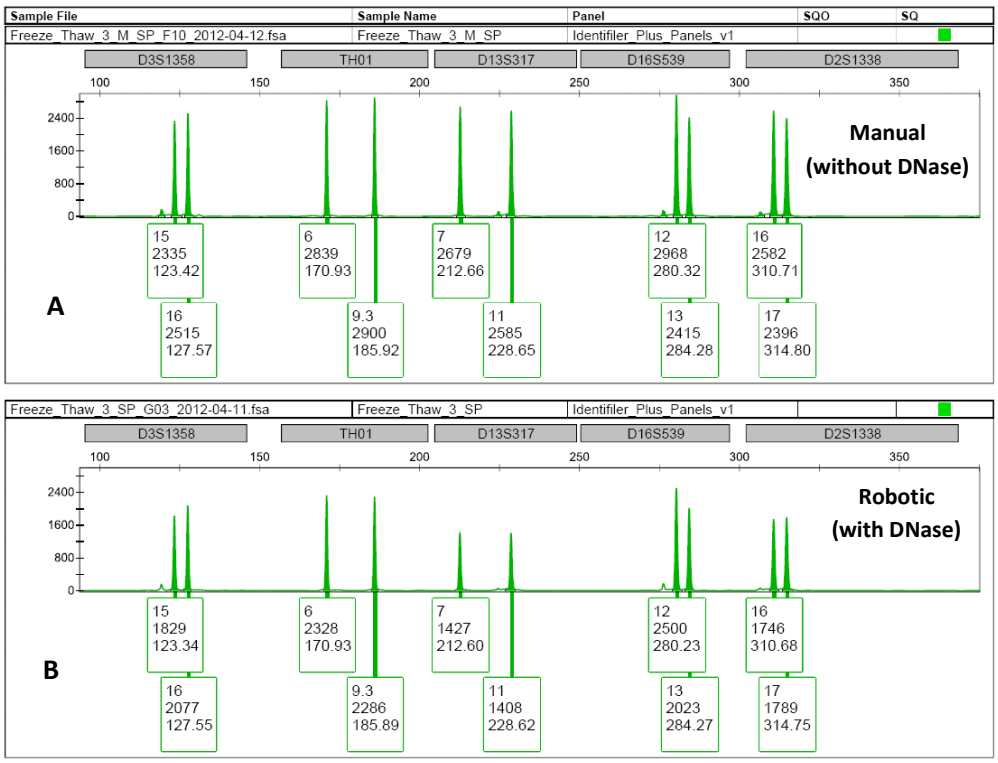


Figure 19: Electropherogram of sperm samples that were subjected to 10 freeze/thaw cycles. Samples were prepared manually without DNase (A) and robotically with DNase (B). Data scaled to 3000 RFUs.

The samples subjected to a heat and humidity treatment showed an average yield of approximately 75pg in the epithelial and sperm cell fractions. The STR DNA typing data for both fractions resulted in detection of either few alleles or no alleles **(Figure 20)**. Similar results were obtained whether the samples were prepared by the conventional differential digestion method or the selective degradation method. It was concluded that subjecting cellular material to constant high heat and humidity will cause severe degradation of the sample.

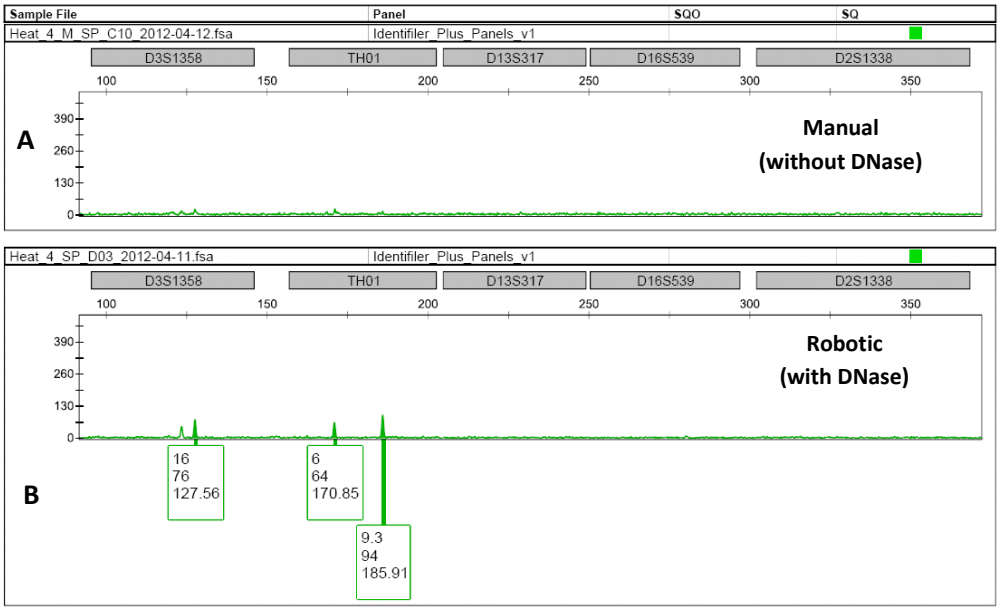
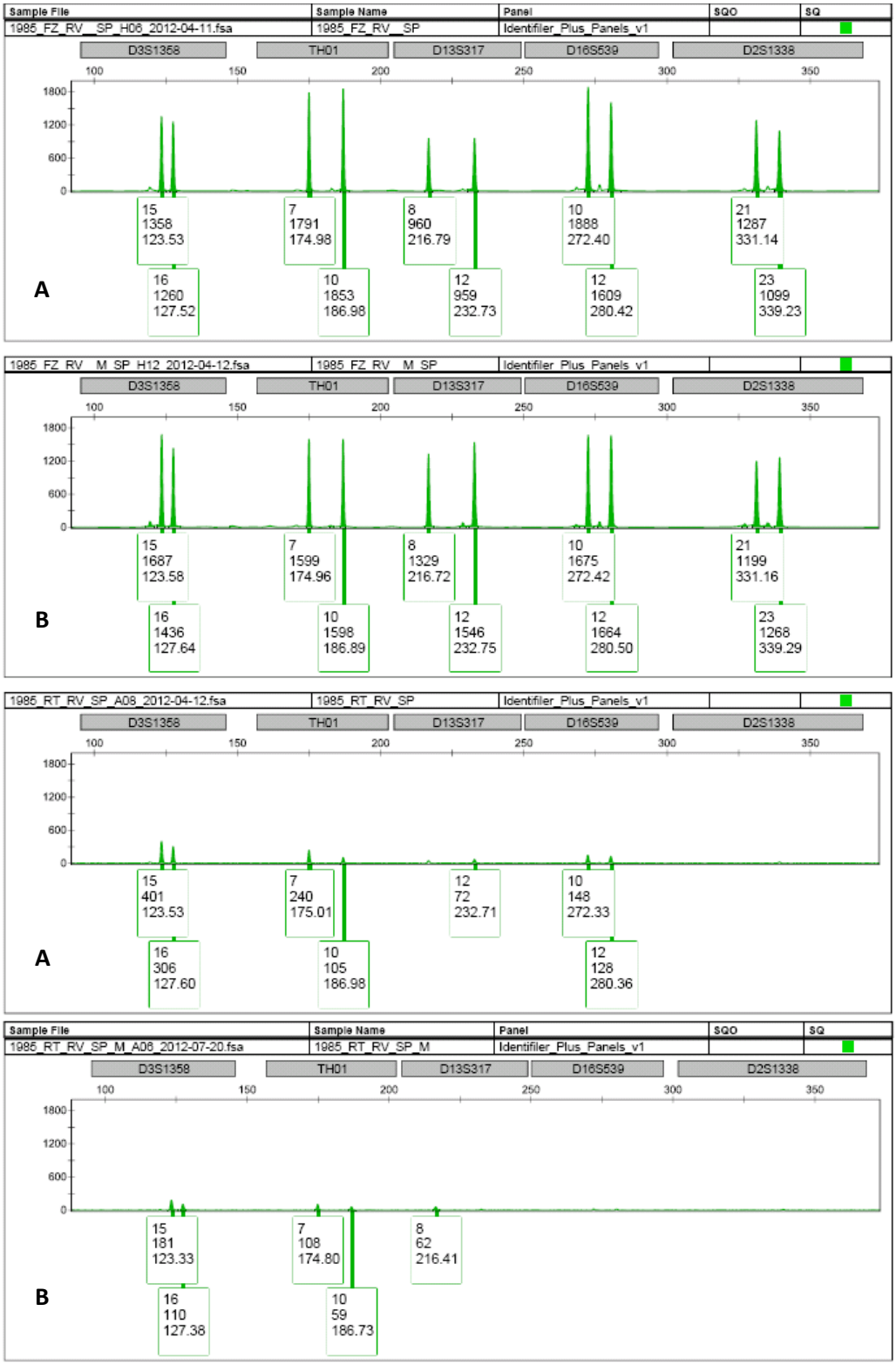


Figure 20: Electropherogram of sperm samples that were incubated for 5 days at 75°C and 80% relative humidity. Samples were prepared manually without DNase (A) and robotically with DNase (B). Data scaled to 500 RFUs.

The aged sperm samples obtained between 1952 and 1991 produced full STR DNA typing profiles for samples dating as far back as 1980, which was the first year with frozen samples **(Figure 21)**. The general trend indicated that samples stored frozen produce full STR DNA typing profiles with satisfactory peak heights while samples stored at room temperature for the same length of time may produce low partial profile, or result in detection of few or no alleles. There were four exceptions out of 15 samples where the frozen samples did not provide a full STR DNA typing profile. However, for these samples the STR DNA typing results were consistent with the low DNA quantitation results; less than 100pg were amplified for each of these four samples. The decrease in DNA yield is most likely a result of unequal sampling as opposed to degradation. One room temperature sample resulted in a full DNA profile but with lower RFU signals **(Figure 22)**.



Robotic Frozen

Manual Frozen

Robotic Room Temp

Manual Room Temp

Figure 21: Electropherogram of paired semen stains from 1985 stored at -20°C and at room temperature. Samples were prepared robotically with DNase (A) and manually without DNase (B). Data scaled to 2000 RFUs.

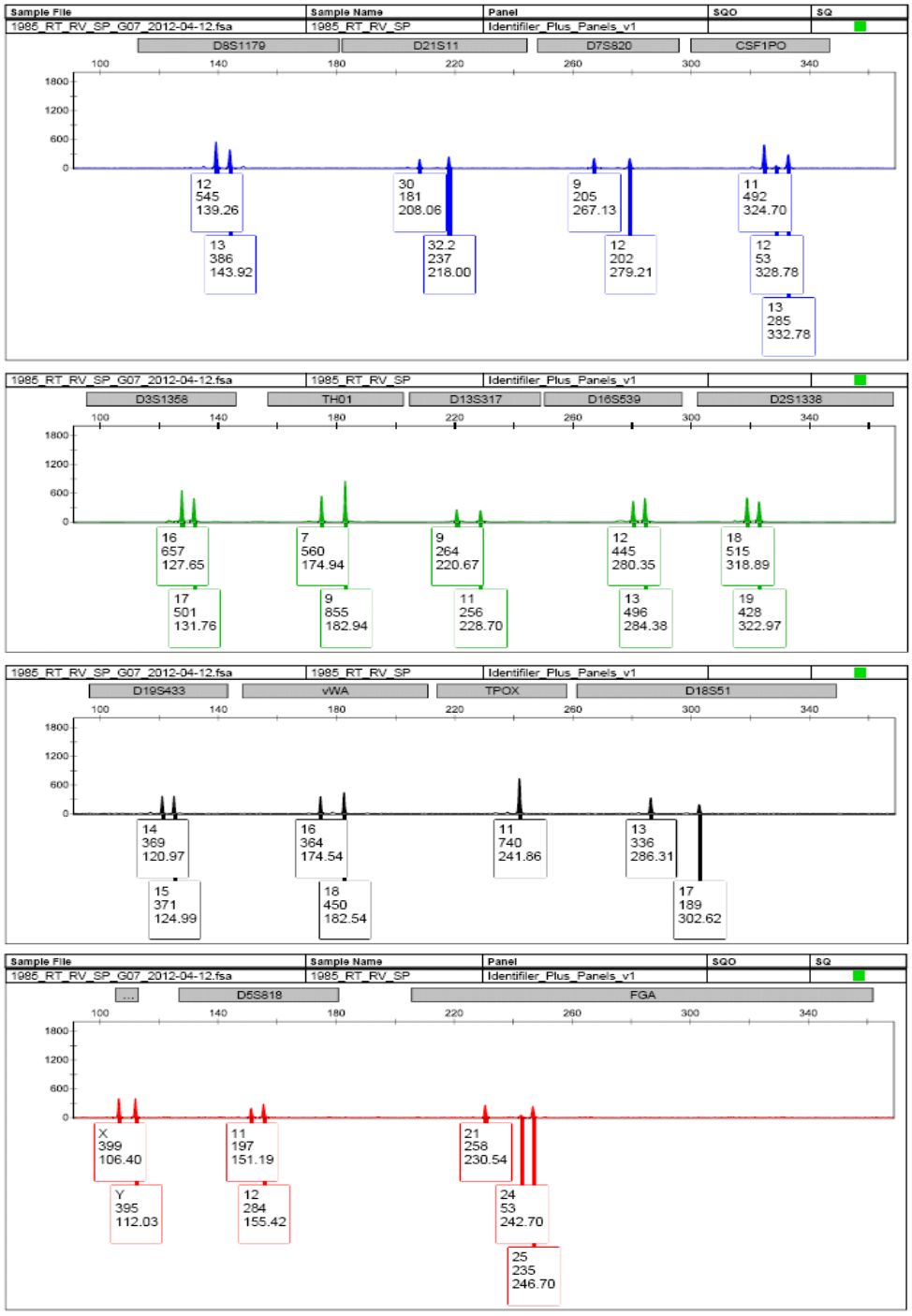


Figure 22: Electropherogram of a sample from 1985 stored at room temperature digested robotically. This is the only room temperature sample that resulted in a full DNA profile. Data scaled to 2000 RFUs.

MIXED SEMEN STUDY

Many forensic samples contain more than one sperm donor. A study on mixed semen samples was performed to replicate these types of casework samples. The mixed semen samples, consisting of two sperm donors present at unequal levels, were digested using the selective degradation differential digestion protocol.

In the evaluation of the STR DNA typing data, a clear major and minor sperm donor ratio was observed in the sperm fractions. More than four alleles were detected at some loci and were determined to result from epithelial cell DNA carryover. The epithelial cell fraction resulted in clean single source profiles with satisfactory peak height levels.

The sperm fraction DNA profile for sample 1 exhibited the lowest peak height levels. All of the alleles for the major sperm donor were detected. However, drop out of three of the minor sperm donor alleles was observed. Eight alleles foreign to the major and minor sperm donors were detected and were determined to be epithelial cell DNA carryover.

The sperm fraction DNA profile for sample 2 showed satisfactory peak height levels. All of the alleles for the major sperm donor were detected. However, drop out of three of the minor sperm donor alleles was observed. Eleven alleles foreign to the major and minor sperm donors were detected and were determined to be epithelial cell DNA carryover.

The sperm fraction DNA profile for sample 3 showed moderate peak height levels. All of the alleles for the major sperm donor and minor sperm donor were detected. Ten alleles foreign to the major and minor sperm donors were detected and were determined to be epithelial cell DNA carryover.

The sperm fraction DNA profile for sample 4 showed high peak height levels. All of the alleles for the major sperm donor and minor sperm donor were detected. Three alleles foreign to the major and minor sperm donors were detected and were determined to be epithelial cell DNA carryover.

The major-to-minor donor ratio for samples 1, 2, and 3 was approximately 4:1 based upon peak heights. In these samples, where the DNA concentration of the minor sperm donor was low, a significant amount of epithelial cell DNA carryover was detected **(Figure 23)**. In addition, the minor sperm donor and the epithelial cell donor alleles could not reliably be distinguished by peak height. As demonstrated by the sensitivity study (refer to next section), DNase is not 100% efficient in eliminating all trace epithelial cell DNA and the effect is more apparent with lower concentrations of sperm cell DNA.

The major-to-minor donor ratio for sample 4 was approximately 3:1 based upon peak heights. The slightly higher contribution of DNA from the minor sperm donor was demonstrated by the increased minor sperm donor peak heights **(Figure 24)**. The higher DNA input from the minor sperm donor also resulted in a reduction of detectable epithelial cell DNA carryover. In addition, the alleles corresponding to epithelial cell DNA carryover were just above the analytical threshold and were readily distinguishable from the minor sperm donor by peak height. Significant peak height imbalances were observed for the

major sperm donor at a few loci. The donor ratio proportion between the major and minor sperm donor was relatively consistent throughout most of the loci but some inconsistencies were observed in the larger loci.

The DNA profiles obtained from the mixed semen sample study successfully reflected the types of profiles which may be obtained in casework samples. The uneven mixtures revealed a clear major sperm donor and a minor sperm donor. Not all minor sperm donor alleles were detected in all samples. Epithelial cell DNA carryover was observed in all profiles. However, a significantly higher rate of epithelial cell DNA carryover was observed in samples where the minor sperm donor DNA input was lower. This observation demonstrates that the quantity of DNA in the sperm fraction directly affects the amount of detectable epithelial cell DNA carryover.

The peak heights of the alleles attributable to epithelial cell DNA carryover were below 200 RFU with an average peak height of 100 RFU for samples with a low minor sperm donor DNA input. There was one allele attributable to epithelial cell DNA carryover at 283 RFU; however, this allele was at a stutter position and the high peak height could be a result of stutter contribution. The low peak heights of the alleles from epithelial cell DNA carryover indicate that the DNase was performing as expected; without DNase treatment, the amount of epithelial cell DNA carryover is expected to be significantly higher. The detection of alleles attributable to epithelial cell DNA carryover was consistent with previous observations that DNase is not 100% efficient in eliminating all residual epithelial cell DNA.

In the samples with low minor sperm donor DNA input, the detected minor alleles could not reliably be attributed to the minor sperm donor or epithelial cell DNA carryover. Allelic drop out from the minor sperm donor was observed at some loci and the peak height levels of the alleles attributable to epithelial cell DNA carryover were similar in height to the alleles from the minor sperm donor. When DNA input is low in a mixture it is important to keep in mind that allelic drop out may occur and that deduction of individual minor donor profiles may not be possible. However, in differentially separated samples, comparison of the two fractions can aid in data interpretation. Therefore in samples where alleles from epithelial cell DNA carryover is indistinguishable from a minor sperm donor by peak heights, the alleles from the epithelial cell fraction can be used to distinguish alleles attributable to epithelial cell DNA carryover from true minor donor alleles in the sperm fraction.

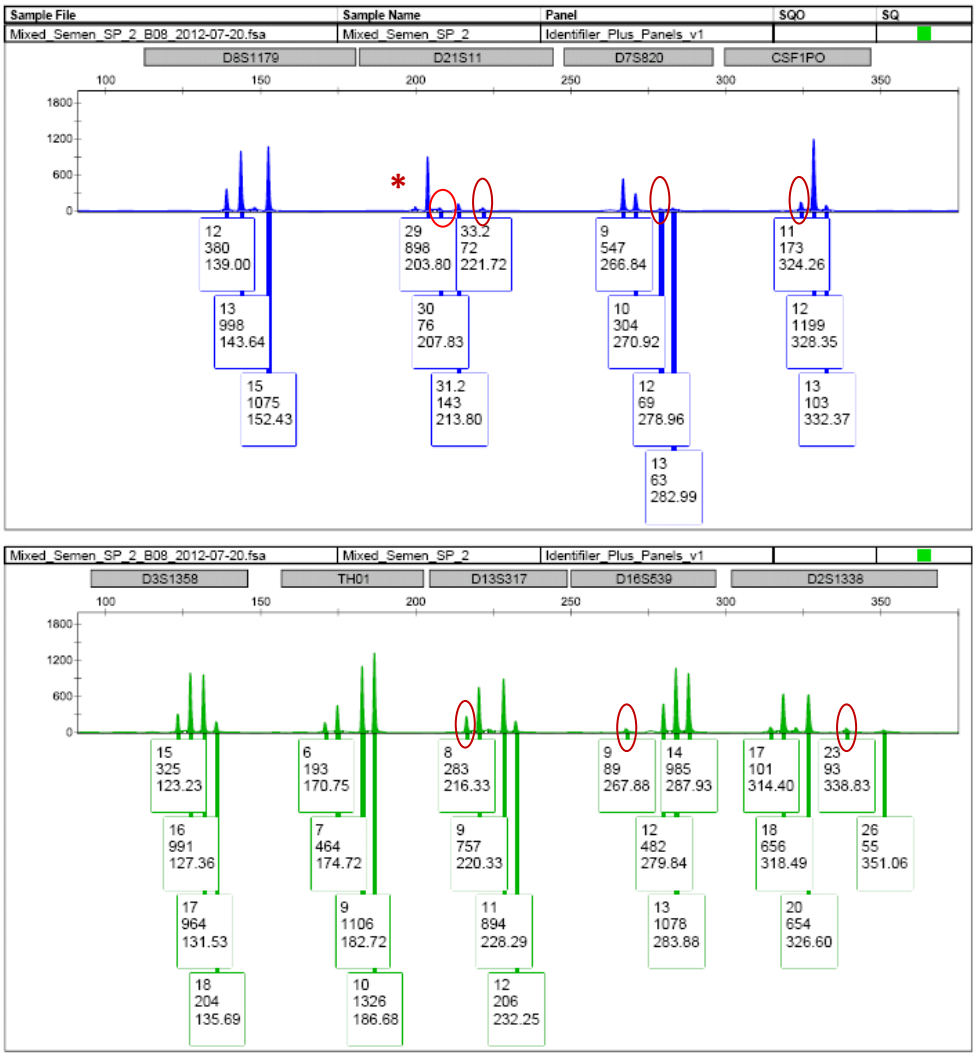


Figure 23: Electropherogram of sample 2 from the mixed semen study. The minor sperm donor proportions are low in this sample and drop out from the minor donor is observed, indicated by an asterisk (*). The alleles foreign to the both sperm donors are circled in red. Data is scaled to 2000 RFUs.

Figure 24: Electropherogram of sample 4 from the mixed semen study. The minor sperm donor proportions are higher in this sample. The alleles foreign to the both sperm donors are circled in red. Data is scaled to 2500 RFUs.

SENSITIVITY STUDY

The sensitivity study consisted of four sets of sperm and epithelial cell mixtures. Each set consisted of a two-fold serial dilution of sperm cells mixed with a constant number of epithelial cells. The concentration of the neat sperm sample used for the serial dilutions was determined by taking 15µL of the sample for extraction and quantitation. Dilutions of the neat sperm sample were made with PBS to target concentrations of 1.0 ng/µL to 0.001 ng/µL. Two sets of the sperm and epithelial cell mixtures were spotted onto swab substrates to replicate casework conditions and test the sensitivity of the VERSA 1100. The other two sets of the sperm and epithelial cell mixtures were processed directly as liquid samples to determine the sensitivity of the selective degradation differential digestion protocol. By processing the cell mixture directly, without substrate, the efficiency of the cell elution from the substrate is removed as a factor.

The liquid samples produced higher DNA yields than the swab samples. The swab samples yielded only about 20% of the DNA compared to the liquid samples. These results are consistent with previous experiments. The swab samples were agitated only by shaking and thus did not recover all of the cells that were pipetted onto the swabs. Full DNA profiles were obtained from samples with male DNA yield as low as approximately 150pg **(Figure 25)**. The quality and peak height level of the DNA profiles were dependent upon and relative to the DNA input. Samples with 1.5 ng DNA input produced DNA profiles with satisfactory peak heights. However, the peak heights of the samples with lower DNA concentrations demonstrated low peak height levels. Samples with 100pg of DNA or less resulted in low partial DNA profiles **(Figure 26)**. In the samples where no sperm were observed microscopically, the STR DNA typing data resulted in either no or few alleles detected **(Table 6)**. There is a general trend for microscopic examinations to estimate a lower DNA yield than the actual yield.

Significant epithelial cell DNA carry-over in the STR DNA typing data of the sperm fraction was observed in samples with very low male-to-female DNA ratios. These results suggest that the DNase is not 100% effective in eliminating all trace amounts of epithelial cell DNA. In samples with higher quantities of male DNA, the level of epithelial cell DNA carryover declined to undetectable levels due to the higher male-to-female DNA ratios.

Sample	# Sperm Observed	Expected Male [DNA] By Manual Calculation (ng/µL)	Estimated Male [DNA] By Quantitation (ng/µL)	# Alleles Observed (Out of 24)
Dilution 1	17	0.0198	0.0770	24
Dilution 2	10	0.0117	0.0420	24
Dilution 3	2	0.0023	0.0130	24
Dilution 4	1	0.0012	0.0019	7 (4 Shared)
Dilution 5	0	0.0000	0.0002	3 (All Shared)
Dilution 6	0	0.0000	0.0000	0
Dilution 7	0	0.0000	0.0002	0
Dilution 8	0	0.0000	0.0000	0
Dilution 9	0	0.0000	0.0000	0
Dilution 10	0	0.0000	0.0000	0

Table 6: Sensitivity study of the robot. The DNA concentrations shown in the table reflect the results of one set of ten swab consisting of a two-fold serial dilution. Microscopic examinations of the cellular material were used to evaluate the approximate number of sperm present in 3ul of each sample. The number of sperm observed is shown in column 2. The expected DNA concentration in column 3 was manually calculated based on the number of sperm observed. Calculations are discussed in the "Methods" section. The male DNA concentration in column 4 represents the estimated DNA concentration of the sperm fraction as determined by DNA quantitation with Plexor HY. A full DNA profile of the sperm fraction consisted of 24 alleles. In low level profiles, alleles that are shared between the sperm fraction and the epithelial cell fraction are shown in parentheses.

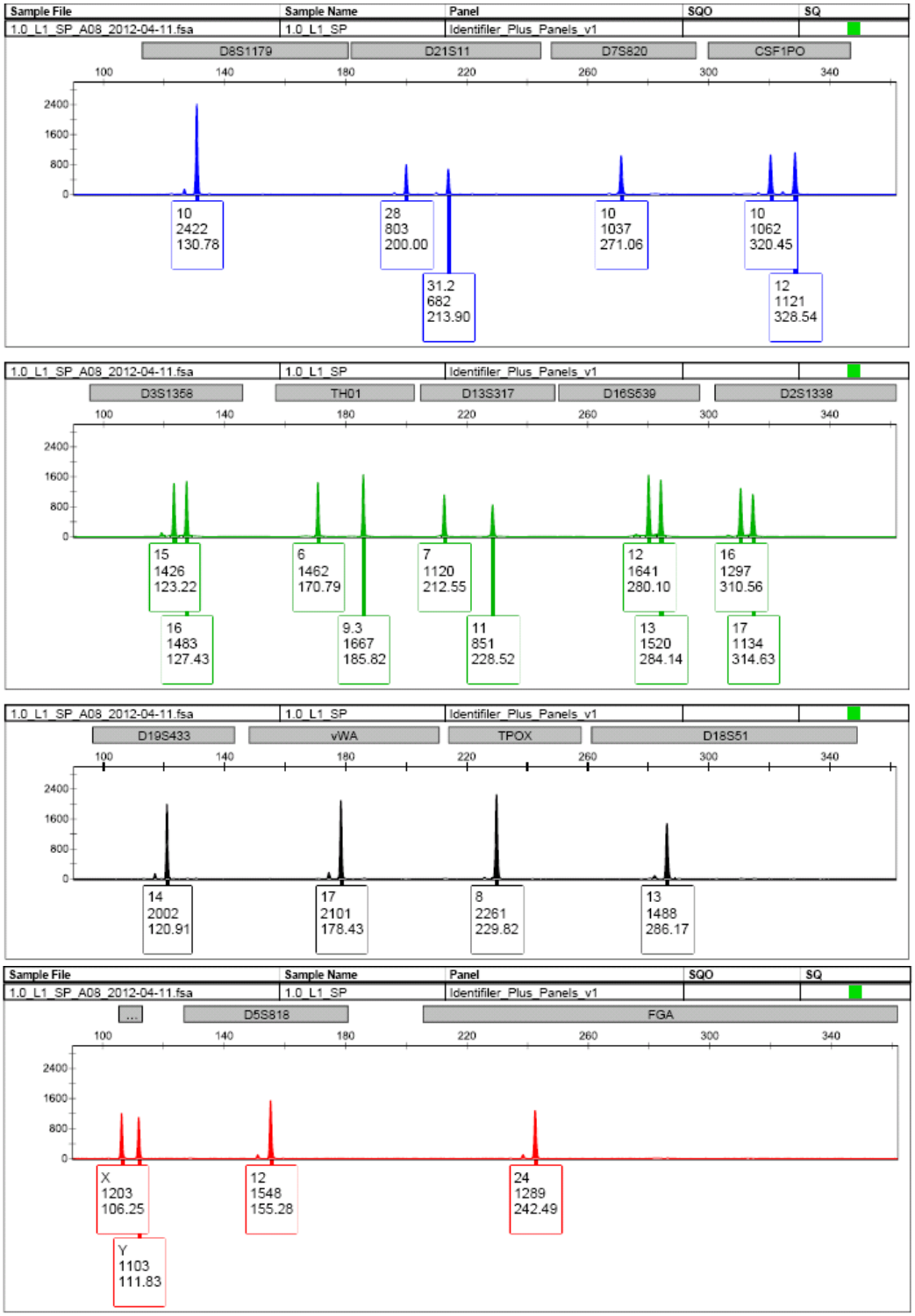


Figure 25: Electropherogram from the sensitivity study. The sample above is Dilution 1, liquid sample. The profile is a single source male profile with no signs of epithelial carryover. The quality and peak height level is also very satisfactory. Data is scaled to 3000 RFUs.

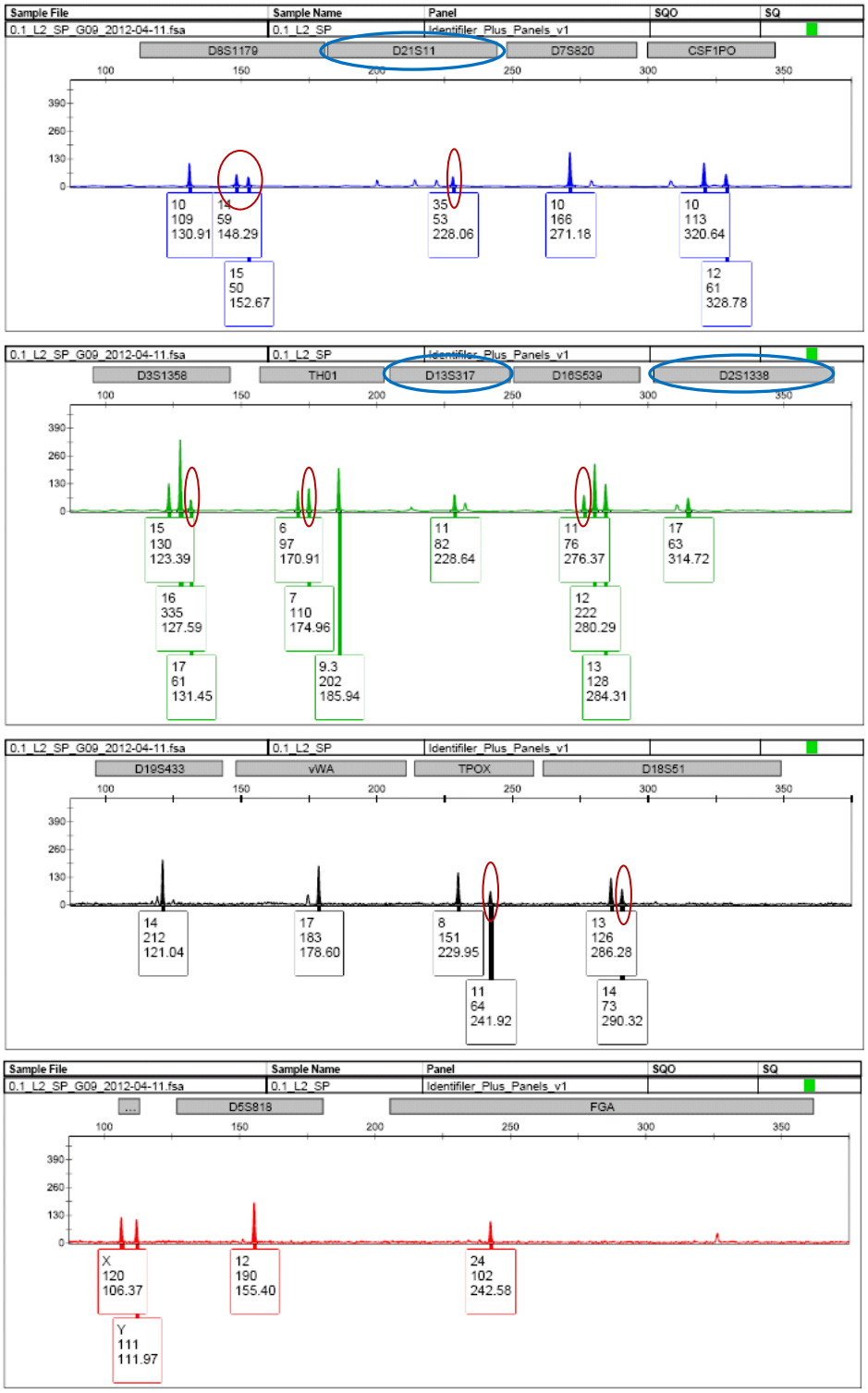


Figure 26: Electropherogram from the sensitivity study. The sample above is Dilution 4, liquid sample. The male DNA input for this sample was 100pg. The profile is a low, partial profile with epithelial carryover. Alleles foreign to the sperm donor is circled in red. Loci where an allele from the sperm donor has drop-out is circled in blue. Data is scaled to 500 RFUs.

Reproducibility Study

The reproducibility study consisted of two sets of eight replicate samples made with 15 µL of the isolated sperm cell sample. One set of samples were spotted onto swab substrates and the other set was processed directly as liquid samples. Quantitation data from the reproducibility studies demonstrated that the robot was very consistent in the manipulation and preparation of the samples **(Figure 27)**. The coefficient of variation (CV) was about 20% for eight replicate samples. Given a 20% variation in the quantitation system alone, the results of the study were acceptable in demonstrating reproducibility [7]. In addition, the reproducibility between similar samples in other studies also demonstrated that the robot was consistent in achieving reproducible results.

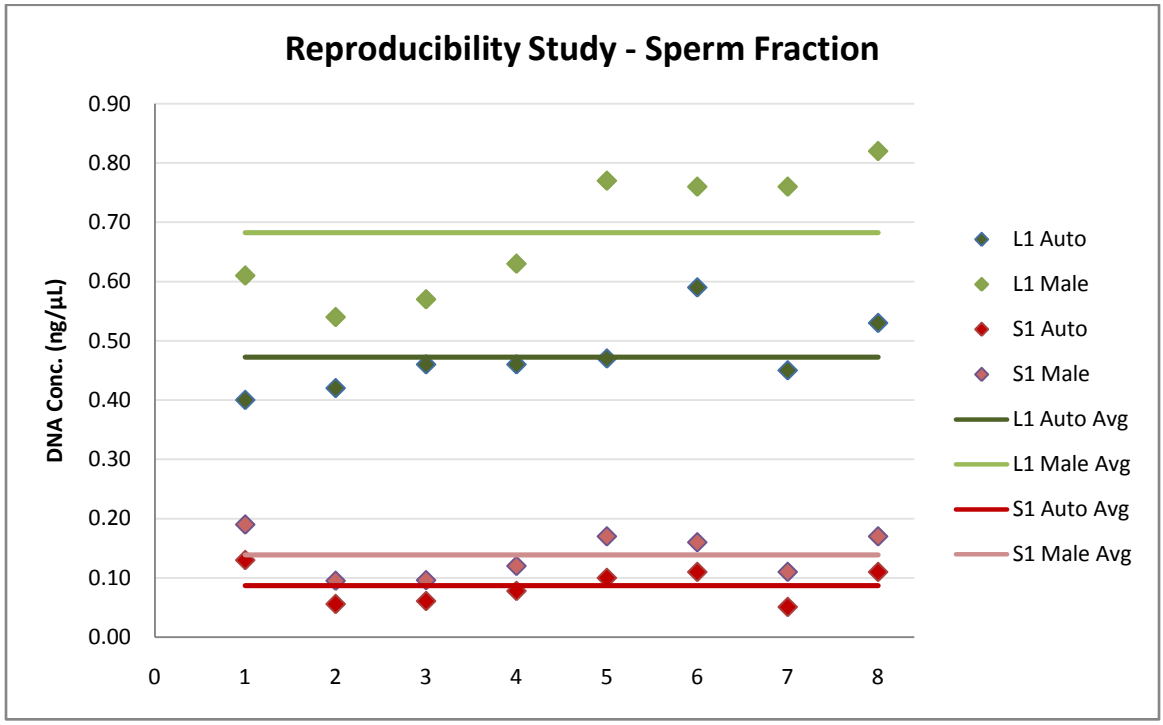


Figure 27: Graph of the reproducibility study. The study consisted of two sets of eight samples (L1 and S1). The graph shows the autosomal DNA concentration and the male DNA concentration. The horizontal line across each set of data represents the average value for that particular set of data.

Contamination Study

A contamination study consisting of blank samples situated throughout a 96-well plate was performed. Potential sources of contamination in the automated process include samples splashing during the shaking process, pipette tips leaking during robotic liquid handling, and manually removing the spin basket insert from the SlicPrep™ plate.

Out of the 56 blank samples in the contamination study, ten samples had detectable DNA. Seven of the ten samples showed DNA yield of less than 50 pg total in 50µL samples (0.001 ng/µL). This data, which is at the detection limit of the quantitation system, is consistent with data from typical quantitation runs and could be attributed to the detection of environmental contaminants due to an extremely sensitive quantitation system which provides consistent DNA detection down to 3.8 pg[13]. In three of the ten

samples, greater than 50 pg of total DNA was detected. The highest DNA yield detected was 205 pg total in a 50 µL sample (0.0041 ng/µL). DNA amplification and DNA typing using 10 µl of these then samples were conducted; the highest DNA input was 41pg. STR DNA typing data showed observance of some peaks below 50 RFU in all three samples, two alleles were detected in one sample **(Figure 29)**, and another sample had one detectable allele. No allelic activity was detected in the seven samples that had less than 10pg of DNA input.

All of the protocol steps in which potential contamination could be introduced, such as removing and inserting the Slicprep™ plate insert, occurred prior to the removal of the epithelial cell fraction. However, none of the blanks in the EC fraction exhibited any detectable DNA indicating the contamination was introduced after the removal of the epithelial cell fraction. The most probable explanation for the contaminated blanks from the sperm fraction is that after removal of the epithelial cell fraction, half of the aged and environmentally compromised samples were manually removed and placed into tubes for a manual differential digestion. The three blanks with more than 50pg of DNA were surrounded by such samples, illustrated by **Figure 28**. The few alleles detected in the blank samples (#25 and 28) were also concordant with the 1980 FZ 4 sample. In addition to the fact that 95% of the blanks had no detectable DNA, the three contaminated samples most likely resulted from contamination during the manual manipulation as opposed to during robotic manipulations. The manual manipulation that was performed during the contamination study is not part of the standard protocol.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Rep S1	Rep S7	Blank 6	Normal 1	Heat 3	Blank 14	CTS 07-574	1952 RT 1	Blank 24	1980 FZ 4	1985 FZ 6	1985 RT 9
B	Rep S2	Blank 3	Rep L4	Normal 2	Blank 11	Freeze Thaw 4	CTS 07-576	Blank 21	1979 RT 3	1980 FZ 5	1985 FZ 7	1985 RT 9
C	Blank 1	Rep S8	Rep L5	Blank 9	Heat 4	Blank 15	Blank 19	1952 RT 1	1979 RT 3	Blank 27	1985 FZ 7	1991 RT 10
D	Rep S3	Rep L1	Blank 7	Normal 3	Freeze Thaw 1	Blank 16	CTS 08-573	1977 RT 2	Blank 25	1980 FZ 5	1985 FZ 7	1991 RT 10
E	Rep S4	Blank 4	Rep L6	Normal 4	Blank 12	Blank 17	CTS 08-575	Blank 22	1979 RT 3	1980 FZ 5	1985 RT 8	1991 RT 10
F	Blank 2	Rep L2	Rep L7	Blank 10	Freeze Thaw 2	CTS 06-571	Blank 20	1977 RT 2	1980 FZ 4	Blank 28	1985 RT 8	1991 FZ 11
G	Rep S5	Rep L3	Blank 8	Heat 1	Freeze Thaw 3	Blank 18	CTS 09-571	1977 RT 2	Blank 26	1985 FZ 6	1985 RT 8	1991 FZ 11
H	Rep S6	Blank 5	Rep L8	Heat 2	Blank 13	CTS 06-575	1952 RT 1	Blank 23	1980 FZ 4	1985 FZ 6	1985 RT 9	1991 FZ 11

Figure 28: Diagram of the contamination study plate set-up. Samples in blue were the aged and environmentally compromised samples that had half of its volume manually removed for manual preparation without DNase. Blanks 20, 25, and 28 had more than 50 pg of total DNA detected and are circled in red.

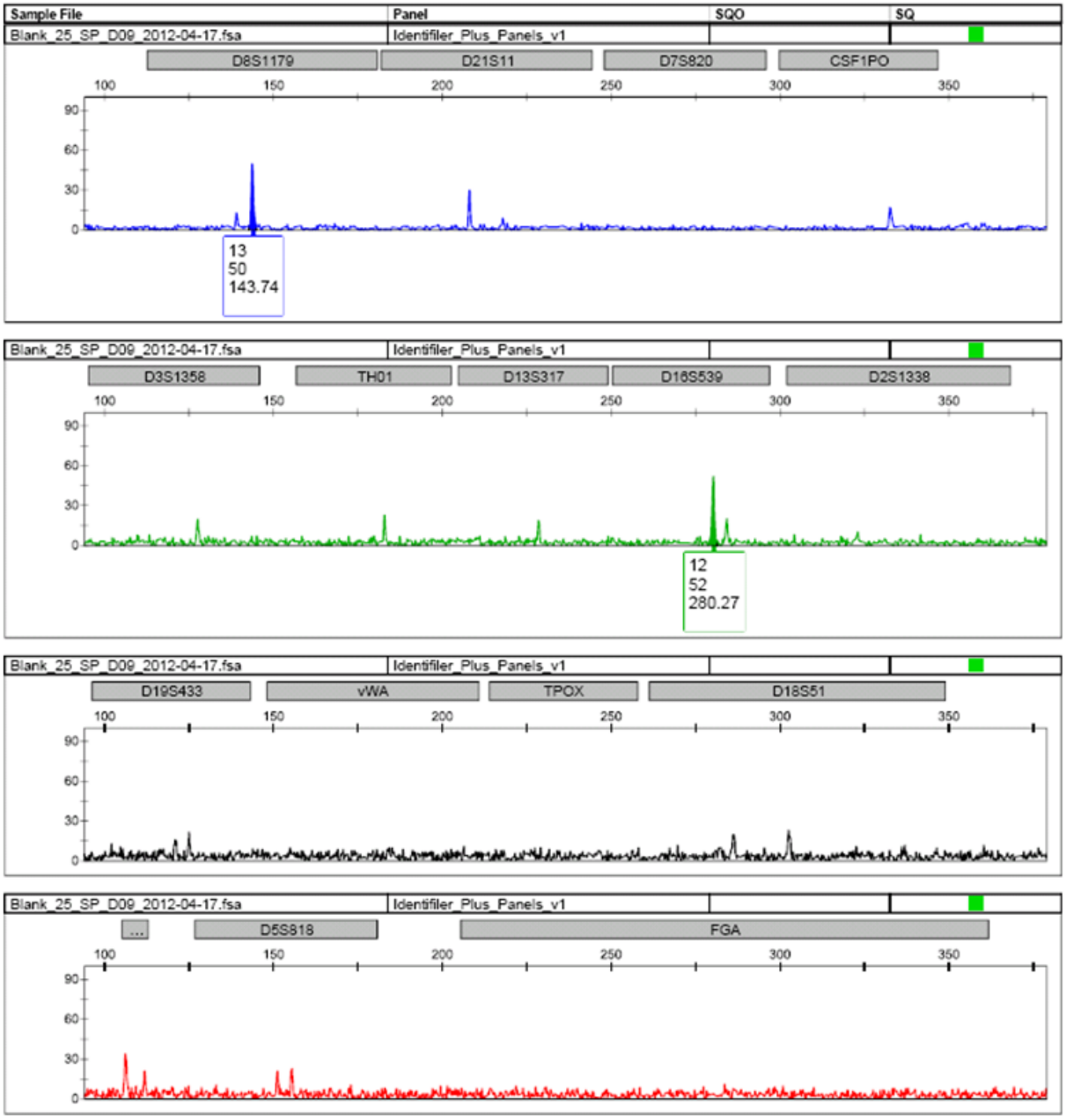


Figure 29: Electropherogram of a blank sample from the contamination study that had two detectable alleles. This blank sample yielded the highest DNA concentration out of all of the blank samples. 41pg of DNA input was amplified in this sample. Data is scaled to 100 RFUs.

UNKNOWN SAMPLES

Seven unknown samples from previous proficiency tests from the Collaborative Testing Services consisting of either blood or a mixture of blood and semen were processed using the automated selective degradation differential digestion process. The STR DNA typing data was evaluated and compared to the known STR results provided on the Manufacturer's Information sheet on the CTS Forensics website. The epithelial cell fractions for two samples (06-575 and 09-571) were male, single source samples. All alleles were detected and were concordant with the STR results provided by the Manufacturer's Information. The remaining five samples were all mixtures of no more than two donors. The alleles foreign to the epithelial cell fractions were concordant with the male donor of the sample. All alleles were detected and were consistent with the female or male donor. No signs of contamination or drop out were detected in any of the seven samples. The quality and the peak height level of the epithelial cell fractions were satisfactory.

The STR profiles of the sperm fractions were all male, single source samples. Only one profile (07-574) exhibited the presence of one EC carryover allele. The profiles were evaluated and compared to the known STR results and all samples were concordant with the male fraction profile. No signs of contamination or drop out were detected in any of the seven samples. The quality and the peak height level of the sperm fractions were satisfactory.

	Samples	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338
CTS 06-571	EC Fraction	13,14,15	28,29,30	10,11,12	10,11,12	14,16,18	6,9,9.3	9,11	11,12,13	16,19,25,26
	Manufacturer Results Item 4 – Blood	13,14	28,30	10,11	10,11	14,18	6,9.3	9,11	11,11	16,19
	SP Fraction	15	28,29	10,12	11,12	16	9,9.3	9,11	12,13	25,26
	Manufacturer Results Item 4 – Semen	15,15	28,29	10,12	11,12	16,16	9,9.3	9,11	12,13	25,26
		D19S433	vWA	TPOX	D18S51	AMEL	D5S818	FGA		
	EC Fraction	13,14,15	16,17,18	8,10,11	13,14,18	X,Y	9,11	19,20,23,25		
	Manufacturer Results Item 4 – Blood	14,15	17,18	10,11	13,14	X,X	9,11	20,23		
	SP Fraction	13	16,18	8	13,18	X,Y	11	19,25		
	Manufacturer Results Item 4 – Semen	13,13	16,18	8,8	13,18	X,Y,	11,11	19,25		

	Samples	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338
CTS 06-575	EC Fraction	8,12	31,33.2	8,12	11,12	16,17	9,9.3	11,12	11,13	17,22
	Manufacturer Results Item 3	8,12	31,32.2	8,12	11,12	16,17	9,9.3	11,12	11,13	17,22
	SP Fraction	8,12	31,33.2	8,12	11,12	16,17	9,9.3	11,12	11,13	17,22
	Manufacturer Results Item 3	8,12	31,32.2	8,12	11,12	16,17	9,9.3	11,12	11,13	17,22
		D19S433	vWA	TPOX	D18S51	AMEL	D5S818	FGA		
	EC Fraction	15,15.2	17,19	11	14,15	X,Y	10,12	22,23		
	Manufacturer Results Item 3	15,15.2	17,19	11,11	14,15	X,Y	10,12	22,23		
	SP Fraction	15,15.2	17,19	11	14,15	X,Y	10,12	22,23		
	Manufacturer Results Item 3	15,15.2	17,19	11,11	14,15	X,Y	10,12	22,23		

	Samples	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338
CTS 07-574	EC Fraction	13,14	31.2,32.2,33.2,35	10,11,13	10,11,12	16	7,8	12,13	11,13	17,18,20,21
	Manufacturer Results Item 3 – Blood	13,14	32.2,35	10,13	10,11	16,16	7,7	12,12	11,13	20,21
	SP Fraction	13,14	31.2,33.2,32.2	10,11	12	15,16	6,8	11,13	11,13	17,18
	Manufacturer Results Item 3 – Semen	13,14	31.2,33.2	10,11	12,12	15,16	6,8	11,13	11,13	17,18
		D19S433	vWA	TPOX	D18S51	AMEL	D5S818	FGA		
	EC Fraction	13,14,15.2,16.2	15,16,18	8,10,12	13,16,18	X,Y	11,12	22,23		
	Manufacturer Results Item 3 – Blood	15.2,16.2	15,16	10,12	16,18	X,X	11,11	22,22		
	SP Fraction	13,14	16,18	8	13,15	X,Y	11,12	21,23		
	Manufacturer Results Item 3 – Semen	13,14	16,18	8,8	13,15	X,Y	11,12	21,23		

	Samples	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338
CTS 07-576	EC Fraction	15	29,30,32,32.2	8,10	10,11,12	15,16,17,18	6,7,9.3	13	11,13	22,23,25
	Manufacturer Results Item 4 – Blood	15,15	29,32	8,10	10,12	15,17	7,9.3	13,13	11,13	22,25
	SP Fraction	12,15	30,32.2	11,12	11	16,18	6,9	12,13	13	23,25
	Manufacturer Results Item 4 – Semen	12,15	30,32.2	11,12	11,11	16,18	6,9	12,13	13,13	23,25
		D19S433	vWA	TPOX	D18S51	AMEL	D5S818	FGA		
	EC Fraction	13,14	14,16	8,9,10	12,15	X,X	12	19,20,21		
	Manufacturer Results Item 4 – Blood	13,14	14,16	8,10	12,15	X,X	12,12	19,20		
	SP Fraction	14	14,17	8,9	15,17	X,Y	12	19,21		
	Manufacturer Results Item 4 – Semen	14,14	14,17	8,9	15,17	X,Y	12,12	19,21		

	Samples	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338
CTS 08-573	EC Fraction	11,12,13	30	8,10,11	10,11,12,13	15,18	6,7,9,9.3	11,12	11,12,14	17,21,22
	Manufacturer Results Item 4 – Blood	12,13	30,30	8,10	10,11	18,18	6,9.3	11,12	12,14	17,22
	SP Fraction	11,13	29,31.2	11	12,13	15	7,9	11	11,12	16,21
	Manufacturer Results Item 4 – Semen	11,13	29,31.2	11,11	12,13	15,15	7,9	11,11	11,12	16,21
		D19S433	vWA	TPOX	D18S51	AMEL	D5S818	FGA		
	EC Fraction	14,15	17,18	8,9,11	15,18,19	X,Y	9,12	19,20,22		
	Manufacturer Results Item 4 – Blood	14,15	17,18	11,11	15,18	X,X	12,12	19,20		
	SP Fraction	15	16,17	8,9	18,19	X,Y	9,11	22,24		
	Manufacturer Results Item 4 – Semen	15,15	16,17	8,9	18,19	X,Y	9,11	22,24		

	Samples	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338
CTS 08-575	EC Fraction	13,14	28,29,30,32.2	8,10,11,12	8,12	15	6,7,9	8,12	11,12	17,23
	Manufacturer Results Item 3 – Blood	13,14	28,29	8,11	8,12	15,15	6,9	8,12	11,11	17,23
	SP Fraction	14	30,32.2	10,12	12	14,15	6,7	12	12	17,23
	Manufacturer Results Item 3 – Semen	14	30,32.2	10,12	12,12	14,15	6,7	12,12	12,12	17,23
		D19S433	vWA	TPOX	D18S51	AMEL	D5S818	FGA		
	EC Fraction	14	16,17,18	8,9	12,19,20	X,Y	11,12,13	21,23,25		
	Manufacturer Results Item 3 – Blood	14,14	16,18	8,9	19,20	X,X	11,12	23,25		
	SP Fraction	12,14	16,17	8	12,14	X,Y	12,13	21,22		
	Manufacturer Results Item 3 – Semen	12,14	16,17	8,8	12,14	X,Y	12,13	21,22		

	Samples	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338
CTS 09-571	EC Fraction	9,11	30,31	8,12	11,12	14,18	9,9.3	12	12,14	17
	Manufacturer Results Item 3	9,11	30,31	8,12	11,12	14,18	9,9.3	12,12	12,14	17,17
	SP Fraction	9,11	30,31	8,12	11,12	14,18	9,9.3	12	12,14	17
	Manufacturer Results Item 3	9,11	30,31	8,12	11,12	14,18	9,9.3	12,12	12,14	17,17
		D19S433	vWA	TPOX	D18S51	AMEL	D5S818	FGA		
	EC Fraction	13,14	17,18	8	14,16	X,Y	11,12	20,22		
	Manufacturer Results Item 3	13,14	17,18	8,8	14,16	X,Y	11,12	20,22		
	SP Fraction	13,14	17,18	8	14,16	X,Y	11,12	20,22		
	Manufacturer Results Item 3	13,14	17,18	8,8	14,16	X,Y	11,12	20,22		

CONCLUSIONS

Through use of the selective degradation method, automation of the differential digestion process was achieved without compromising the quantity and quality of the recovered DNA. The conventional differential digestion is a time consuming manual process; automation of this process reduces analyst bench-time thereby increasing the time available for data interpretation, report writing, and casework review. With the increase in demand for casework per analyst and reduced turn-around time, many laboratories will find that automation of the differential digestion process will aid in the reduction of sexual assault evidence backlogs. Using the automated selective degradation differential digest method, a full plate of 96 sexual assault evidence samples can be processed in approximately 8 hours with minimal analyst interaction. Processing the same number of samples by the conventional differential digestion method would take approximately six times as long and would require the full engagement of the analyst. The minimal number of manual manipulation steps of the automated method also reduces the possibility of contamination and sample switching.

Although the conventional manual differential digestion process is an effective method of separating sperm cells from non-sperm cells, carryover of the epithelial cell DNA is often observed in sperm fraction STR DNA profiles, complicating interpretation of the DNA typing results. Despite the cleaner separation of the epithelial cell DNA from the sperm fraction that may be attained by increasing the number and stringency of the washes, consideration must be given to the increased risk of loss of sperm cells during

each wash step. However, selective degradation by an enzyme such as DNase I, can efficiently remove residual epithelial cell DNA without appreciable loss of sperm cell DNA. The clean single source male DNA profile which may be obtained can be evaluated in a simple and straightforward manner, saving analyst time which would be required for interpretation if a DNA mixture was obtained.

Although the initial evaluation of the use of DNase for selective degradation of residual epithelial cell DNA during the differential digestion process led to observations of reduced DNA yield and poor STR DNA typing data, optimization of the protocol resulted in significant improvements such that the data obtained was comparable to the conventional manual differential digestion method. The selective degradation procedure employing DNase is capable of producing high DNA yield and high quality STR DNA typing data, with easily interpretable sperm fraction DNA profiles. However, specific parameters associated with the use of DNase must be carefully evaluated to avoid the potential loss of DNA evidence due to the action of DNase I. These parameters include, but are not limited to, the amount of DNase used and the magnesium and calcium divalent cation concentrations. Experiments performed on aged semen stains, and samples subjected to multiple freeze thaw cycles, or heat and humidity, also demonstrated that the DNase treatment did not have an appreciable negative effect on the DNA yield or quality of STR DNA typing results on environmentally compromised samples. All samples which produced a full DNA profile with satisfactory peak heights using the conventional differential digestion method also produced a DNA profile of comparable quality using the automated selective degradation method. Evaluation of the automated differential digestion method demonstrated the ability of the robot to recover DNA reproducibly. However, the reduced efficiency of the release of cellular material from the sample substrate without a manual agitation step remains a disadvantage as analyst intervention and manual sample agitation is required. Future research may focus on utilizing dissolvable swabs in place of cotton swabs to maximize the release of cellular material into solution. Another area of potential future research is investigation into the use of sodium hydroxide, as described by Hudlow and Buoncristiani [6], in place of the traditional lysis buffer, pro K, and DTT used in the differential digestion.

The automated selective degradation differential digestion process has been validated at the Oakland Police Department Criminalistics Laboratory. Manuals and protocols have been written and this process has been implemented since the beginning of 2013. The biology unit staff is currently being trained on the process and will receive competency testing prior to implementation of this process in casework.

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DISSEMINATION OF RESEARCH FINDINGS

This research paper has been presented at the 2013 American Academy of Forensic Science Meeting in Washington D.C.

This research has been published as Helena Wong's thesis paper for her Master of Science degree in Forensic Science at the University of California, Davis (Automating the Differential Digestion Method in the Analysis of Sexual Assault Cases using Selective Degradation, Wong H., Sept 8, 2012). Ms. Wong presented this research at the UC Davis seminar meeting in May 2012. The Jan Bashinski Criminalistics Graduate Thesis Grant was also awarded in 2012 for presentation of this research project at the 2013 AAFS meeting.

The methods and procedure to this research paper has been provided to Harris County Institute of Forensic Science, Strand DNA Testing Laboratory, Orange County California Criminalistics Laboratory, Michigan State Police Department, and Philadelphia Police Department.

Differential Digestion using Selective Degradation

The following reagents are used for this protocol:

- Phosphate Buffered Saline, pH 7.4
- Tween 80 (2% Tween 80, 20mM Tris HCl, 1mM EDTA)
  - Sigma Aldrich (P5188)
- Proteinase K (20mg/mL)
- Dithiothreitol (1.0 M)
- DNase I (1 U/µL) – (40% glycerol, brought up to volume with DEPC treated water)
  - DNase I: Sigma Aldrich (D5025-150KU)
  - DEPC treated water: Sigma Aldrich (95284)
- $CaCl_2$ and $MgCl_2$ solution (5mM and 90mM, respectively)
  - CaCl2 : Sigma Aldrich (21115)
  - MgCl2: Sigma Aldrich (68475)
- EDTA (0.5M)
  - Sigma Aldrich (03690)

1. Cut the substrate or swab and place into a 2.0mL dolphin tube.

2. Add a volume of PBS such that the substrate is fully submerged. Typically, add 200µL (for a whole swab) or 100µL (for half of a swab). Incubate for a minimum of 30 minutes at 4°C. If aqueous extract is not required for body fluid screening or conventional typing tests (e.g. amylase, acid phosphatase, p30), the substrate can be soaked in a larger volume of PBS.

3. Agitate samples using a toothpick to loosen the cells from the substrate. Using a sterile toothpick, transfer the substrate to an insert, place the insert in the dolphin tube and centrifuge for approximately 3 minutes at 12K x g to pellet the cells. This step is designed to release the cellular material from the substrate for initial microscopic evaluation. However, not all of the cellular material may be released from the substrate during this step.

4. Retain the substrate in a new, sterile and appropriately labeled tube for step 5. If needed, transfer all but ~30µL of the supernatant to another new, sterile and appropriately labeled tube. This is the "aqueous extract"; store it frozen (-10 to -20°C). If aqueous extracts are not needed, discard the supernatant. **Retain the tube containing the cellular pellet**. This is the pre-digested sample.

5. Re-suspend the cellular pellet in the remaining ~30µL of PBS and transfer 3µL (10%) to a microscope slide. Heat the slide to fix the cellular material to the slide. Stain the slide with Christmas Tree Stain. Label the pre-digest material microscope slide appropriately. Evaluate the "pre-digest" cellular material microscopically.

6. Transfer the substrate from the corresponding insert back into the tube containing the remaining resuspended cellular pellet from step 5. Add 510µL of Tween 80 and 10µL of Pro K. *(Note: Tween 80*

is used instead of SEB because the SDS in SEB contains sodium ions which interferes with and inhibits DNase activity)

7. Assure that the substrate is submerged and incubate for ~30 minutes at 56°C.

8. Agitate the substrate with a sterile toothpick to loosen the sperm cells from the substrate. Using a sterile toothpick, transfer the substrate to an insert, place the insert in the dolphin tube and centrifuge for approximately 3 minutes at 12K x g to pellet the cells. This centrifugation will pellet non-digested cellular material that may include sperm heads. The supernatant will contain the contents of the cellular material that has been lysed during the Tween 80/ProK digestion.

9. Carefully transfer all but ~30µL of the supernatant to a separate appropriately labeled 2.0mL tube. This is the epithelial cell (EC) fraction (or non-sperm fraction). Add 400µL of MTL buffer to the EC fraction digests. Proceed to DNA extraction of the EC fractions using the BioRobot® EZ1 or store at 4°C to extract at a later time.

10. The remaining pellet is the sperm cell (SP) fraction. Re-suspend the cellular pellet in the remaining ~30µL of Tween 80 and transfer 3µL (10%) to a microscope slide. Heat the slide to fix the cellular material to the slide. Stain the slide with Christmas Tree Stain. Label the pre-digest material microscope slide appropriately. Evaluate the "post-digest" cellular material microscopically.

11. Residual EC DNA is eliminated by adding DNase I. Add 290µL of Tween 80, 25µL of $CaCl_2$ and $MgCl_2$ solution, and 15µL of DNase I. Incubate at 56°C for 15 minutes. DO NOT VORTEX the samples!

12. To inactivate the DNase prior to the sperm cell lysis, add 20µL EDTA and leave at room temperature for ~10 minutes.

13. To lyse the sperm cells, add 10µL of Pro K and 20µL of DTT and incubate at 56°C for 15 minutes.

14. Transfer the sperm fraction supernatant to an appropriately labeled 2.0mL tube. Add 400µL of MTL buffer to the EC fraction digests. Proceed to DNA extraction of the EC fractions using the BioRobot® EZ1 or store at 4°C to extract at a later time.