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The Use And Development Of Laser Microdissection To Separate Spermatozoa From Epithelial Cells For Str Analysis

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THE USE AND DEVELOPMENT OF LASER MICRODISSECTION TO SEPARATE SPERMATOZOA FROM EPITHELIAL CELLS FOR STR ANALYSIS

by

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ABSTRACT

Short Tandem Repeat (STR) analysis has become a valuable tool in identifying the source of biological stains, particularly from the investigation of sexual assault crimes. Difficulties in analysis arise primarily in the interpretation of mixed genotypes when cell separation of the sexual assailant's sperm from the victim's cells is incomplete. The forensic community continues to seek improvements in cell separation methods from mixtures for DNA typing. This report describes the use of laser microdissection (LMD) for the separation of pure populations of spermatozoa from two-donor cell mixtures. In this study, cell separation was demonstrated by microscopic identification of histologically stained spermatozoa and female buccal cell mixtures, and STR analysis of DNA obtained from the separated sperm cells. Clear profiles of the male donor were obtained with the absence of any additional alleles from the female donor. Five histological stains were evaluated for use with LMD and DNA analysis: hematoxylin/eosin, nuclear fast red/picroindigocarmine, methyl green, Wright's stain, and acridine orange. Hematoxylin/eosin out-performed all other stains however nuclear fast red/picroindigocarmine could be used satisfactorily with STR analysis. In addition, three DNA isolation methods were evaluated for LMD collected cells: QIAamp® (Qiagen), microLYSIS® (Microzone Ltd.) and Lyse-N-Go™ (Pierce Chemical Co.). MicroLYSIS performed poorly, yielding low levels of PCR product. Lyse-N-Go™ extraction was effective for the recovery of DNA from LMD collected sperm cells while QIAamp® isolation performed best for the recovery of DNA from LMD collected epithelial cells. LMD is shown to be an effective, low-manipulation separation method that enables the recovery of sperm while excluding epithelial cell DNA.

I would like to dedicate this work to my husband, Bob. I greatly appreciate the support you have given me and believing in my research. I know it has been a long process. I thank you for encouraging me to accomplish my goals. I love you with all my heart!

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CHAPTER 1. INTRODUCTION

STR analysis has become a valuable tool in identifying the source of biological stains particularly in the investigation of sexual assault crimes. Typically the male sperm cells are the component of interest, while the victim's cells from a vaginal, rectal, oral or other body swab complicate the genotyping of the assailant.

In cases where biological stains from two or more individuals cannot be separated a multifaceted statistical analysis is required with data interpretation that involves some subjectivity by the analyst. Typically, this is more time consuming for the forensic practitioner and challenging for an expert witness to successfully convey to a jury, potentially lessening the power of the evidence. The introduction of DNA evidence involving mixed genotype interpretation has several vulnerabilities to judicial attack. The legal weakness of this type of evidence stems from either the technical factors involved or from the lack of a scientific standard for a uniform mixture interpretation model. Mixture interpretation is complicated by a number of technical factors including, but not limited to, allele overlap, stochastic fluctuation, low quantity of DNA, degraded template, three-allele patterns, microvarients [Ladd et. al., 2001] and strand slippage stutter [Moxon et al., 1999]. The Scientific Working Group for DNA Analysis Methods have set recommendations for mixture analysis but allows laboratories and practitioners scientific freedom to develop validated methods of mixture interpretation [SWGDAM, 2000], and the National Research Counsel has set guidelines on the evaluation of forensic DNA evidence [NRC II, 1996]. In addition, the National Institute of Standards and Technology (NIST)

has sponsored extensive research on the analysis of mixed stains [Duewer et al., 2001; Kline et al., 2003]. The variability of methods used for mixture interpretation can vary not only from lab to lab, but within a lab, and even from case to case when conducted by a single analyst. The most common methods used are "Qualitative Statement-No Calculation", "Inferring Genotypes-Match Probability", "Exclusion Probabilities" and "Likelihood Ratio Estimates" [Ladd et al., 2001]. When choosing to infer genotypes, there are again different approaches and levels of stringency applied, some mathematical others subjective. This variety of methods used by forensic scientists indicates that a single interpretation model is inadequate for all mixture scenarios, and that mixture de-convolution does require the scientist to select the method that best conveys the facts. However, this subjectivity in analysis provides other experts and attorneys with an opening to challenge the validity of the results and evidence. Some Courts have ruled the "Qualitative Statement-No Calculation" method to be inadmissible [Nelson v State, 1993; US v Yee, 1991] and the debate over proper interpretation guidelines continues both within US Courts and between practicing forensic scientists.

The purpose of this project was not to propose another mixture interpretation model or support the theoretical basis for an existing method. Instead, the goals of this project would eliminate the need for mixture interpretation in many cases by addressing the problem early in the line of evidence analysis, during sperm identification. The development of a method capable of fully separating spermatozoa from epithelial cells before DNA analysis would result in more readily interpretable typing patterns improving the chances for a successful individualization. As a result, testimony in these cases would be more straightforward, and better understood by the judicial system facilitating the adjudication of crimes.

The current method of preferential lysis [Yoshida et al., 1995; Gill et al., 1985] has been the forensic standard for separating sperm cells from epithelial and blood cells for nearly 20 years. This method utilizes cell-specific differences in membrane chemical composition by first lysing the non-sperm cells without disrupting the sperm cells, then washing away any residual unwanted DNA from the intact sperm cells. Although this method can generally provide two cellular fractions, one comprising of sperm cell DNA and the other of non-sperm DNA, the separation is not always complete, and there may be carryover from one fraction to another making eventual genotype interpretation and further statistical analysis challenging. This challenge is frequently encountered in cases where the ratio of victim cells to assailant sperm cells is large or where there are very low numbers of sperm. Additional limitations to this technique are the pre-mature lysis and loss of sperm cells in the first digestion and the multiple liquid transfers and washing steps reducing cell recovery. This method therefore may require substantial numbers of sperm cells in the starting material to obtain a complete genotype.

Flow cytometry was introduced as a superior method to separate sperm cells from vaginal epithelial cells [Schoell et al., 1999] in the late 1990s. This fluorescence-activated cell sorting (FACS) approach relies on differences in the cell size, shape, surface phenotype, cytoplasm and DNA content. It has been reported that flow cytometry has improved sensitivity relative to preferential lysis in identifying male DNA in a mixture. However, this high sensitivity requires a low concentration of cellular debris in the vaginal lavage. FACS was also successful in separating sperm from a mixture as low as 1:160 sperm/vaginal cell ratio, which provides superior resolution over the preferential lysis method. Unfortunately, FACS also requires a large number of target cells as starting material (6000 sperm in 1 million vaginal cells). In addition, the

starting cell suspension requires a lengthy, multi-step florescent immunostaining process before initiating cytometry. There are no reports applying FACS to analysis of non-clinical samples, such as samples from post-mortem collection or crime scene samples which could be a problem as larger amounts of released DNA from degraded cells would be expected in these types of samples.

A microchip-based sperm and epithelial cell separation method utilizes the differential physical properties of cells that result in settling of the epithelial cells to the bottom of a reservoir and subsequent adherence to the glass substrate [Horsman et al., 2005]. The flow rate can be used to separate the sperm cells from the epithelial cell-containing mixture. Semen donor profiles using AmpF*l*STR COfiler amplification were clearly obtained from the male fractions, but the presence of female DNA in the sperm fraction was evident - most likely to epithelial cell DNA free floating in the sample. This separation procedure can be performed under 30 minutes and has the potential of being automated. However, the sperm recovery using gravity-driven flow was less than 5% and only increased to ~25% by extending the flow time to 70 minutes. The authors suggest that using a negative pressure flow instead may improve this yield, although, at this method may not be suited for samples with minute numbers of sperm.

Membrane filtration was another separation method introduced as an alternative to the preferential lysis method [Chen el al., 1998]. This filtration method was developed to cleanly separate spermatozoa from epithelial cells based upon differences in size and shape. This is a very simple and rapid technique with separation based on choice of the appropriate filter pore size and either low speed centrifugation or vacuum filtration. In this study 70% of sperm cells in the mixed cell sample penetrated the filter into the collection tube. This provides a larger sperm

yield from that of preferential lysis. However, a 1.0-2.0% of intact epithelial cells also penetrated through the filter into the collected sperm, and epithelial cell DNA released from lysed cells contaminated the filtrate. The investigator then performed this separation on a 1:1 mixture followed by genotyping at the D1S80 locus with PAGE visualized by silver staining. Although no epithelial carryover was detected in the genotype, this outcome does not compare either to the level of sensitivity in current STR capillary electrophoresis analysis or the ability PCR multiplexing has in detecting a DNA mixture. This filtration method may be more suitable for samples containing large amounts of sperm with only trace contamination by epithelial cells.

Efforts are currently underway to develop a new cell separation technique using magnetic beads coated with antibodies [Herr, 2004]. This approach requires the identification and isolation of specific monoclonal antibodies that target the surface of the human sperm cell. A second antibody conjugated to a magnetic bead specifically binds to the first antibody and is then used to draw away the sperm cells from unwanted cells in a mixture. One of the challenges with any antibody-based method is the stability of cell surface antigens in environmentally compromised forensic samples. The magnetic bead technology itself is a proven technology and the forensic community awaits reports on the outcome of this research.

Y-chromosome STR analysis takes a non-separation approach to identifying a male's DNA from a female's DNA in a forensic mixture [Kauser et al., 1997]. The identification of the Y chromosome haplotype of a perpetrator can be determined in a mixed male/female specimen in which the female cells are in overwhelming quantity [Prinz el al., 1997] while maintaining the high level of sensitivity seen in autosomal STR analysis. In addition, Y-STR analysis can clarify the numbers of semen contributors when multiple males are involved and useful in rare

incidences of azoospermic perpetrators. Y-STR analysis does not discriminate sexual assault mixtures involving the same sex, such as sodomy of a male and does not specifically target sperm cells. Therefore, if multiple assailants are involved in a mixture, tying the origin of the genotype to the semen deposited or cell type can be unclear. Cell source attribution can be important in defining the type of sexual assault crime committed in court.

The Y-chromosome lacks recombination from father to son and the statistical product rule cannot be applied as many markers are required to achieve a practical degree of discrimination [Butler, 2001]. However, even with a multiplex assay, Y-chromosome analysis does not reach the degree of statistical power that the 13 core STR loci provide, and it has yet to be incorporated into the FBI's Combined DNA Index System (CODIS). Furthermore, when a Y-STR database is formed, the State and Federal costs of Y-typing archived and newly convicted offender blood samples in addition to the 13 core loci will have to be addressed.

The methods described above have weaknesses in efficient cell separation, yield, time effectiveness, cost, ability to work with minute amounts of starting material, identifying genotype to a specific cell type, and ability to discriminate multiple perpetrators. This research project proposes Laser Microdissection as a new sample separation method to address some of these weaknesses. Laser microdissection (LMD) technology has been increasingly used in biomedical research applications to harvest selected cells from histological sections of complex tissues [Emmert- Buck et al., 1996; Simone et al., 1998; Luizzi et al., 2001, Rook et al., 2004]. LMD has several potential advantages for forensic science over previous separation methods. It requires low-manipulation of sample and works by direct microscopic visualization, making it suitable for single-cell analysis. This resolution makes LMD ideal for forensic samples of minute

quantities. LMD directly separates and collects the target cells without contamination from a mixed cell population. This separation can be verified visually by the post-dissection image of the slide and also the post-collection inspection mode in the software allows the user to microscopically inspect the contents of tube. Thus LMD addresses the question about cell source type of the genetic profile obtained.

Laser microdissection technology was first introduced as "laser capture microdissection" or LCM (Arcturus). Recently LCM was reported to improve recovery of DNA from sperm on microscope slides [Elliott et al., 2003]. This technology involves the use of a laser to microscopically melt a thermoplastic film onto a target cell embedding and lifting the cell from the slide. Although this technology allows the capture of an enriched sperm fraction, female carryover is relatively common. This problem can be due to female DNA from lysed cells adhering to the sperm but it may also be due to the non-specific attachment of surrounding foreign cells to the plastic membrane. Despite contamination of female DNA in the male fraction LCM performs significantly better than the preferential lysis method in its ability to separate sperm from vaginal epithelial cells and can detect STR alleles from even a few sperm [Elliott et al., 2003].

Since the initial use of LCM, laser microdissection (LMD) engineering have evolved and other instruments developed. In this study the third generation Leica AS LMD instrument (Leica Microsystems) was evaluated and a method developed to separate pure populations of sperm cells from semen/epithelial cell mixtures compatible with forensic DNA analysis. The aim of this research was to develop a method to simultaneously identify and separate pure populations of sperm cells from an epithelial cell mixture suitable for forensic human identification testing.

The research questions addressed were: What histological staining method can be used with laser microdissection and downstream DNA analysis that can discriminate sperm cells from epithelial cells while both preserving DNA integrity and preventing PCR inhibition? What DNA isolation method can be used for LMD collected cells? Finally, can LMD separate sperm cells from a two donor semen and buccal swab mixtures without epithelial cell carryover for forensic STR analysis?

Development of laser microdissection could assist forensic scientists in analyzing sexual assault mixtures that previously could not be effectively separated using conventional means. LMD could also be the tool of choice for collection of minute numbers of sperm in mixtures for Low Copy Number Analysis. With proper development of software and integration of robotics, LMD could become more automated and has the potential of processing at a higher throughput for backlog reduction.

CHAPTER 2. MATERIALS AND METHODS

2.1 Specimen Collection

The Rosalind Franklin University of Medicine and Science Institutional Review Board approved collections of all human tissue specimens. Liquid semen samples containing sperm were obtained from the Los Angeles Police Department, Scientific Investigation Division laboratory and the Los Angeles Sheriffs Department Crime Laboratory. These laboratories retain frozen stock specimens of biological fluids for positive controls and standards used in forensic serology and DNA analysis. These stock semen samples were reported to be self-collected by anonymous male volunteer employees then transported to the crime laboratory for storage. 200-1000µl from five donors were shipped overnight on cold packs then stored at -20°C at the University laboratory. A 1:10 working solution of each semen sample was prepared with sterile water.

Buccal swabs from twelve female subjects were obtained from employees at the Los Angeles Police Department using one or two sterile dry cotton swabs at a time, the subjects self-swabbed the inside of their cheek for up to one minute. The swabs were air dried overnight at room temperature, shipped by express mail to the University laboratory then stored at -20°C until ready to use. Whole cotton tips were cut from the swab with a sterile blade and placed in 1ml of sterile water then incubated at room temperature with agitation for 15 minutes. Each swab was removed and placed in a spin basket microcentrifuge tube then centrifuged for 2min. Liquid from both tubes were consolidated and spun for 2-3 minutes. The supernatant was removed

leaving an epithelial cell sample recovered in a 50µl working solution. No information on age of subject or date of collection was obtained for any of the semen or buccal samples.

2.2 Hemacytometry

A hemacytometer was used on some samples to determine the concentration of cells in the working solutions. A Bright Line counting chamber (Hausser Scientific) was used with 5µl of epithelial cell working solutions mixed with 5µl of nuclear fast red or 5µl of semen working solutions mixed with 4.5µl of nuclear fast red and 0.5µl of picroindigocarmine. Average values of four 1mm square frames were used to estimate cell concentrations of the working solutions using the following formula: 1µl = 1 cubic mm = (# cells counted per square mm) X (dilution) X (10). The dilution factor for all samples was 2x to account for the staining solution.

2.3 LMD Slide Preparation

Glass microscope slides covered with a polyethylene napthalate, PEN, foil (Lecia Microsystems) were modified by adhering 1/4" diameter Avery hole reinforcement labels to the PEN surface. The slide fits three labels in a row and the internal open diameter is consistent with typical spot sizes used in forensic sperm identification. The labels also produced a dam to hold liquid when processed with histological stains. 2µl of the samples were spotted over the interior circle area in triplicate and dried at room temperature.

2.4 Histological Staining

Five histological staining methods were used: hematoxylin/eosin (H&E), nuclear fast red/picroindigocarmine (CTS), methyl green (MG), Wright's stain (WRT) and acridine orange (AO) were used for staining of cells. All solutions were sterile filtered before use. The staining protocols are as follows with all washes consisting of approximately 5 seconds in sterile water: H&E - QS modified Mayer's hematoxylin (Vector) for 5 minutes, Wash, 1% Acidic alcohol (70% EtOH) for 5-10 seconds, Wash, 1% w/v Eosin Yellowish Solution (non-alcoholic) for 5 minutes, Wash, Rinsed with 95% ethanol for 5 seconds; CTS - nuclear fast red (Vector Laboratories) for 5 minutes, Wash, picroindigocarmine (1.3% w/v picric acid, 7mM indigocarmine) for 30 seconds, Rinse with 95% ethanol for 5 seconds; WRT - Wright's blood stain solution(0.255%) for 5 minutes; spiked Wright's buffer (0.25% w/v Wright's stain in Wright's buffer), Rinse in Giordano buffer (pH 6.4) for 20 seconds; AO - Remel 0.01% acridine orange (1% w/v in AO Buffer) for 4 minutes, AO buffer (33mM Potassium dihydrogen phosphate, 33mM di-Sodium hydrogen phosphate ,pH 6) for 1 minute, 1M Calcium Chloride solution for 3 minutes, Rinse with AO Buffer 5-10 seconds; and MG - Sterile water for 10-15 seconds, methyl green (Vector Laboratories) for 5 minutes, Wash. Unstained control smears were rinsed with 95% ethanol for 5-10 seconds. Slides were vacuum desiccated and stored at -20°C until ready for LMD. H&E staining was modified in the DNA isolation and mixture studies to Mayer's hematoxylin for 1 minute, Wash, 0.5% Acidic Alcohol for 5-10 seconds, Wash, 0.1M Sodium Bicarbonate solution (pH8) for 1 minute, Eosin 5-10 seconds, Rinsed with 95% ethanol.

2.5 Laser Microdissection

Several laser microdissections systems were considered for this project. Three systems were further investigated for their suitability in this research. Arcturus Pixcell developed the first laser microdissection system, which was patented as "laser capture microdissection" (LCM). The LCM technology uses an IR laser to microscopically melt a thermoplastic film onto the cells of interest. This process embeds the cells in plastic followed by pulling the cells by force from the slide. Some concerns arose regarding the nature of this type of collection. The plastic film is held in a cap holder than comes into physical contact with a large area of the slide. This instrument was initially designed for tissue cryosections where the integrity of the tissue section can withstand the contact. However, it was suspected that with a slide smear this could result in a simple contact transfer of unwanted cells from the slide to the surface of the plastic thus contaminating the cap. With the sensitivity of PCR and the increasing popularity of Low Copy Number Analysis contact transfer can now be detected [Ladd et al., 1999]. Similarly, since the method does not employ cutting there were concerns that cells adjacent to or overlapping the target cell - such as a sperm head on the non-nuclear body of an epithelial cell - may lift the whole contaminating cell onto the cap. Finally, the operation of the instrument is performed in real-time somewhat like a video game where the operator controls a joystick type device directing the stage and firing the laser to collect cells. This appeared to be labor intensive and allowed little room for operator error. The Acturus system did have one advantage in that specimens could be recovered from a standard glass microscope slide. This could be useful

when faced with archival samples not specifically prepared for laser microdissection. However, old mounted specimens could still prove challenging as specimens on aged slides have a propensity to be more permanently adhered to the glass slide.

The second instrument considered was the P.A.L.M MicroBeam laser microdissection and pressure catapulting (LMPC). This system has the improvement of non-contact collection. It uses a laser to cut the specimen, however, since the slide sits upright the target material must be catapulted up against gravity using a pressure pulse. According to the manufacturer it is "like a ball that is kicked into the goal". The "goal" is an inverted microtube cap. The sample is held onto the underside of the cap by either tension or an adhesive cap. The engineering of this type of collection seemed counterproductive in recovering and retaining all cells given it is working against gravity. In addition, the most basic of PALM systems started at a cost well above the other systems considered.

It was determined that the Leica Microsystems AS LMD would be best suited for this projects application. The Leica system is based on a computer automated laboratory microscope integrated with a 337nm UV laser. The laser beam is directed through the objective lens and passes through the inverted glass microscope slide to the plastic PEN film on which the sample resides. Laser ablation occurs around the cell(s) of interest and the material is collected by a push from a charged laser pulse and gravity into a PCR tube cap below the stage. Figure 1 diagrams the instrument while Figure 2 illustrates the laser microdissection technique. This instrument offers the advantages of a non-contact collection and secures recovery of cells.

In facilitating accurate cell counting for this study, laser microdissection collection was limited such that clusters of sperm cells were avoided. This was done by limiting single software

tracings to a range of 1 to 10 cells at any one time. Slides were vacuum desiccated and brought to room temperature immediately before LMD. The LMD parameters used at the 40x objective are listed in Table 1.

An analog hand counter was used while 75, 150 or 300 sperm and epithelial cells were dissected by LMD from the prepared smears. Cells were automatically collected into the cap of 0.2ml thin walled PCR tubes containing 20µl of buffer (described in section "DNA Isolation" below). After collection cells were centrifuged down from the cap for 10 seconds.

2.6 DNA Isolation

2.6.1 Qiagen QIAamp

LMD cells collected in 20µl of TE buffer (10mM Tris-HCl, 0.1mM EDTA, pH8) were extracted using the QIAamp DNA Micro Kit (Qiagen). Lysis was performed by adding Buffer ATL to a final volume of 50µl, 10µl of Proteinase K, 30mM dithiothreitol (DTT), then incubated at 56°C for 3 hours with occasional agitation. 50µl of Buffer AL with 1 µg kit carrier RNA was added and mixed for 15 seconds. 50µl of 100% ethanol was added and mixed for 15 seconds. The entire lysate was added to the QIAamp MinElute Column and centrifuged for 1 minute for DNA binding to column. The column was washed with 500µl Buffer AW1 and centrifuged for 1 minute, then washed with 500µl of buffer AW2 and centrifuged for 1 minute. The column was dried by centrifugation for 3 minutes. Elution of DNA from the column was performed by adding 25µl of Buffer AE, incubating for 5 minutes at room temperature, and centrifuged for 1 minute to recover DNA extract.

2.6.2 MicroLYSIS

LMD cells collected in microLYSIS reagent were extracted using 20µl of reagent with the addition of DTT (30mM) and incubated in the thermal cycler according to the manufacturer's recommendations as follows: 65°C for 5 minutes, 96°C for 2 minutes, 65°C for 4 minutes, 96°C for 1 minute, 65°C for 1 minute, 96°C for 30 seconds, 20°C hold. LMD sample collection, lysis and PCR were all performed in the same 0.2ml thin-walled tube.

2.6.3 Lyse-N-Go

LMD cells collected in Lyse-N-Go reagent were extracted using 20µl of reagent with the addition of DTT (30mM) and incubated in the thermal cycler according to the manufacturer's recommendations as follows: 65°C for 30 seconds, 8°C for 30 seconds, 65°C for 90 seconds, 97°C for 180 seconds, 8°C for 60 seconds, 65°C for 180 seconds, 97°C for 60 seconds, 65°C for 60 seconds, 80°C for 5 minutes. LMD sample collection, lysis and PCR were all performed in the same 0.2ml thin-walled tube.

2.6.4 Chelex

LMD cells collected in a 5% Chelex-100 (Bio-Rad) solution (pH 10) were extracted in 20µl of the bead suspension with the addition of DTT (30mM) and 1µl of proteinase K (10mg/mL) then incubated in a heat block at 56°C for 45 minutes. Samples were vortexed for 10 seconds and incubated at 100°C for 8 minutes followed by another 10-second vortex and

centrifuged for 2-3 minutes. The DNA extract was removed from the resin by a capillary pipette tip to separate from the beads. All of the ~ 15µl of DNA extract recovered was used for PCR.

2.7 PCR and Human Identification

Short tandem repeat (STR) markers are polymorphic DNA loci that contain a repeated nucleotide sequence. The STR repeat unit can be from two to seven nucleotides in length but most commercial kits used in forensic science applications are composed of tetra-nucleotide repeats. Alleles of different lengths are possible because the number of repeat units at an STR locus may vary. STR loci can be amplified and fluorescently labeled using the polymerase chain reaction (PCR). PCR products were separated by electrophoresis to distinguish the alleles according to size, then collected data was analyzed by software to size the DNA fragments and genotype the alleles. The use of several STR loci provides a high discrimination power useful for human identification purposes [LaFountain et al., 2001].

In this study DNA amplification was performed using Applied Biosystems AmpF*l*STR® Profiler Plus™ Kit for nine short tandem repeat loci (Blue: FAM-labeled D351358, vWA, FGA; Green: JOE-labeled D8S1179, D21S11, D18S51, and Yellow: NED-labeled D5S818, D13S317, D7S820) plus amelogenin. This kit was chosen due to its reliability in human identification shown through many validation studies [LaFountain et al., 2001; Frank et. al 2001, Leclair et al. 2004, Moretti et al. 2001]. PCR was performed on a Bio-Rad iCycler according to manufacturer recommendations using a 50µl reaction volume per sample under standard conditions. Standard PCR conditions were utilized as follows: 21µl AmpFlSTR PCR Reaction Mix, 1µl AmpliTaq

Gold DNA Polymerase, 11 µl AmpFlSTR Profiler Plus Primer Set, 20 µl sample DNA; Thermal cycling conditions - Incubate 95°C for 11 minutes (polymerase activation); 94°C for 1 minute (denaturation), 59°C for 1 minute (annealing), 72°C for 1 minute (extension) for 28 cycles; then 60°C for 45 minutes (final extension). In addition, extended cycles analysis was used and amplified as follows: 25µl of PCR product amplified under the standard conditions was removed and added to a new tube with 0.25µl of AmpliTaq Gold DNA Polymerase, and then PCR was continued as described above for six additional cycles.

2.8 Electrophoresis Conditions

1.5µl of each PCR product was added to 24µl of HI-DI formamide with 1µl of ROX-500 size standard (Applied Biosystems) and denatured at 95°C for 3 minutes then snap cooled on ice for 3 minutes. Denatured samples were shipped overnight on ice pack to either the Los Angeles Police Department Crime Laboratory or ReliaGene Technologies for electrophoresis and data collection. Capillary electrophoresis was performed using POP-4 polymer through a 36cm capillary with a 60°C block temperature at a run voltage of 15kV. The histology study samples were injected for 5 seconds on an ABI Prism 310 Genetic Analyzer. The DNA isolation and mixture study samples were injected for 11 seconds using an ABI Prism 3100 Genetic Analyzer.

2.9 Data Analysis

GeneScan 3.1.2 and Genotyper 2.5.2 (Profiler Plus template 4.1) software were utilized to analyze the electrophoresis data at Rosalind Franklin University of Medicine and Science. Baselining, matrix correction and light smoothing were applied to all samples. The minimum peak height threshold was set at 50 RFU to allow for detection of all peaks clearly above background. Peaks were examined to determine if they qualified as true alleles excluding fluorescent signal that was identified as stutter, spectral overlap, incomplete nucleotide extension, electronic noise, dye blobs or raised baseline. The peak height, in relative fluorescent units (RFU), of all true alleles was used for statistical analysis. Amplified product quantity was measured using peak height as a parameter. Data compilation and statistical calculations were performed in Microsoft Excel 2001and GraphPad Prism 4.0 including mean, standard error, unpaired t-test, Wilcoxon test, and ANOVA analysis with Bonferroni's post-tests.

CHAPTER 3. RESULTS AND DISCUSSION

3.1 Technical Discussion

The Leica AS LMD is designed primarily to cut mounted tissue cryosections. Since cell smears were used in this study, some time was spent on determining the optimal sample preparation and cutting conditions for this study. The instrument has several available cutting parameters what can be manipulated as show in Table 1. The UV laser operates at 30Hz and up to 60 Hz in burst mode. The intensity of the laser can be adjusted using a "less" to "more" sliding scale. It was observed that determining the minimum necessary energy to cut the material in question was important because using excessive laser strength caused leakage of laser light to a nearby area of the slide. Shadows of the cutting pattern were occasionally observed about 100-200μm from the target cut when the laser was operated at maximum power. Although the intensity of the deflected light was not strong enough to cut through the material there was obvious ablation damage to the surface. It was conceivable that this leakage from the UV laser could degrade or destroy nuclear material from nearby cells if not controlled.

The cutting speed parameter can be adjusted on a slow to fast sliding scale. By slowing down the laser speed, a deeper cut could be achieved and laser intensity reduced. The drawback although to reducing speed was an increase in collection time. The balance between laser intensity and speed were the two parameters that had the most dramatic effect on cutting performance but once the desired settings were determined for a particular type of specimen the

specification could be saved and quickly restored by the computer software for future cutting sessions. This allowed the instrument to be used for a variety of other applications such as pathology samples, hair roots or other tissue without re-optimizing for each different tissue type or user prior to each session.

Laser aperture can also be adjusted on a sliding scale. A narrow aperture gave more precision and control in excising specific cells without the ablation of adjacent cells while a wider aperture better facilitated the drop of the specimen into the collection tube. It was found however that the larger apertures were only necessary when large, odd-shaped, free-drawn cuts were performed. It is analogous to a jigsaw puzzle where an elaborate piece would be more difficult to punch out than the circular piece. In this study most of the specimens involved collection of only one or two cells no larger than a circle with a 30-micron diameter and throughout the experiments performed, a narrow aperture was found to be sufficient for dissection of these small pieces.

Toward the end of each cut, the instrument pauses at a short distance from completion of the closed loop. A larger and wider burst of laser power is then pulsed to push the cell(s) into the collection tube. Both the gap (small, medium, large) during the pause and the amount of increased laser intensity and aperture can be controlled. In general, longer bridge gaps allowed more controlled sample drops. The shorter the gap the more frequent the PEN membrane would prematurely peal away before the final burst potentially causing a hanging sample which has been named "hanging chad" after the 2000 presidential election controversy. The sample would hang below the slide attached only by a small bridge. This premature movement could cause the focal plane to be offset from the remaining membrane to be cut. Because the effectiveness of the

laser is in part dependent on the microscope objective's focus on the specimen, the final burst occasionally did not complete the final cut when hanging chads occurred. This problem may be prevented in two ways. 1) Longer gap settings 2) Wider more intense final laser bursts. However, once a hanging chad occurs it was found that the best method effective in completing the cut and collection was to refocus and retarget the bridge using the line cut feature. Optimal LMD parameters were determined for sperm and epithelial cells as described in the methods section. In general, slower more intense laser settings were requires to collect epithelial cells.

The 4x, 10x and 20x objectives were insufficient to identify the small sperm cells of 5µm. The 63x objective was helpful in identifying or verifying sperm morphology and could also be used for dissection of spermatozoa. Although, the 63x objective created a smaller field of view reducing the potential number of sperm cells that could be cut at any one time thereby slowing the recovery process. The 40x objective was the best balance for identifying and dissecting both sperm and epithelial cells simultaneously.

Images of sperm/epithelial cell mixtures cut by LMD are show in Figure 3. Circles were found to be the most effective in targeting sperm cells using the circle and reuse tool option, which replicates the previously used cutting shape and size. Moreover, epithelial cells could also be recovered in a separate collection tube either simultaneously or after sperm collection. Changing to more aggressive cutting parameters (Table 1) could collect sperm cells overlapping with epithelial cells. If a sperm cell resided very close to an epithelial cell nucleus ablation of the nucleus could destroy epithelial cell DNA before collection of the sperm cell to avoid contamination.

Sample preparation requires some unique challenges when performing laser microdissection. Two different types of slides were evaluated: polyethylene terephthalate (PET) and the polyethylene napthalate (PEN) slides. Leica Microsystems first developed the PEN slide followed by the production of PET slides in response for the need for an inert material for mass spectrophotometry applications. No cover slip is used in laser microdissection, therefore, the quality of the image is reduced compared to traditional microscopy. One of the characteristics explored in this study was the plastic clarity of the PET over the PEN slides. The PEN slides contain what appear to be minute bubbles in the membrane that are nearly absent in the PET slide. Several samples were prepared and examined on the PET slides finding some distinct disadvantages over the PEN slides. The PET membrane is stretched over a metal frame such that it hangs open with no contact against a solid surface. Preparing smears onto this fragile membrane was difficult as even if the smear was gently applied and supported against a solid surface deformities frequently occurred on the membrane. This exasperated the problem of continuous focal adjustment from field to field on the microscope. The membrane was also more difficult to cut requiring increased laser intensity and sometimes repeat cuts. Samples that contain very few sperm heads, which are difficult to identify, could benefit from using PET slides, however, it was determined that the drawbacks outweighed the optical advantages for routine analysis.

Histological techniques to improve the optical quality of sectioned tissue specimens are worth exploring but were beyond the scope of this project. Clearing agents have been used to de-fat tissues and used routinely for laser microdissection followed by nucleic acid analysis [Ehrig et al., 2001]. However this would involve more toxic chemicals and its effects on forensic STR

analysis would have to be investigated. Also, a recent development using a thin aqueous mounting solution with an adhesive gum base has been reported to greatly improve tissue morphology for laser microdissection. No detrimental effects were detected on DNA isolation and efficiency of PCR amplification including only a minimal reduction of LMD cutting efficiency [van Dijk et al., 2003]. Another practice of simply dehydrating the slides by rinsing through a gradient of low to high percentage ethanols is a technique that would not be expected to affect any downstream analysis. This could improve clarity and would also better dehydrate the specimen, an important component described in more detail below.

The cell concentration of the slide smear is also a consideration. In preliminary studies 2-5µl of the cell pellet were tested over quarter inch diameter circles on the slide. In general the smaller aliquot smeared over the spot area allowed a wider distribution of cells reducing the occurrence of sperm cells overlapping with epithelial cells. Most of the samples tested did not contain high densities of epithelial cells, which are frequently encountered in postcoital vaginal swabs. It was anticipated that with increasing numbers of epithelial cells the smears may have to be adjusted such that a larger area is utilized on the slide or the cell pellet is diluted before slide preparation.

Forensic laboratories are familiar with the need to control a facility's environmental conditions within the laboratory to maintain consistent operation of sensitive analytical equipment, and integrity of evidence samples. For example some DNA sequencers are sensitive to room temperature changes effecting electrophoresis mobility. In addition, excessive moisture and/or heat can compromise biological specimens if not stored properly. Control of environmental conditions was found to be critical for successful laser microdissection. A slide

specimen had to be well dehydrated before dissection. Water will dissipate the laser energy resulting in poor ablation. If moisture remained on the slide after a cut have been made on the plastic membrane the water could migrate between the glass slide and the membrane, making cutting nearly impossible. It was important when removing slides from the freezer to immediately vacuum desiccate to prevent the accumulation of condensation on the surface. However, in contrast excessively dry conditions created static electricity which sometimes caused a cut piece of PEN film to be attracted to the underside of the slide instead of dropping into the PCR tube. This problem was discovered as changes in cutting performance were observed through the cold, dry winters and hot, humid summers. The humidity was controlled inexpensively by using a simple room dehumidifier in the summer and a humidifier in the winter to reach the ideal humidity of 35-45 percent for LMD cutting. This avoided static electricity while preventing the absorption of water by the sample during the collection period. By controlling the humidity there was also a reduction in the effect of another dilemma with LMD collection, evaporation of buffer from the collection cap.

The collection cap has an effective working volume of 20-30µl using the 0.2ml PCR tube option (0.5ml tube option available). The brightfield illumination originates from below the stage and travels through the cap to the slide above. The heat from this light and the open exposure of the buffer to the environment can quickly cause evaporation of this small amount of buffer from the collection cap. Steps were initiated to address this problem. First, a filter was used to reduce heat exposure to the cap, and the bulb intensity was dimmed to a lower level. The digital video camera was able to compensate for the lower light level. Second, the collection cap can be put into a standby position where light is not transmitted through the cap and moved into

position just before each cutting was initiated. The second method although effective in reducing the heat on the cap was time consuming as each time a new field of view was changed the tube had to be directed out of position then back into position. However, this bottleneck is expected to improve with pending software improvements better automating the process. In the meantime the first method was effective in minimizing volume loss. In this study an acceptable ~ 5µl volume loss during a 45-minute cutting session was experienced. Volume could be compensated by the addition of 5µl of either buffer or water to the collection cap prior to LMD. Given the evaporation loss there should be minimal dilution effect.

3.2 Histology Comparison

To compare the staining effectiveness and downstream effect of various histological chemistries, samples from 6 donors (3 semen and 3 oral swabs) were examined. Slides were prepared with the working solutions described in the specimen collection section. Six slides were prepared for each single source donor specimen and stained as described in the histology methods. Cell identification was performed using the 40x and 63x objectives without a coverslip under brightfield and fluorescent microscopy on the Leica AS LMD microscope. Scores were assigned to describe the stain's ability to facilitate cell identification as follows:
double minus (- -) = cannot ID or highly challenging; minus (-) = poor; plus/minus (+/-) = satisfactory; plus (+) = good; double plus (+ +) = excellent. Collections of 300 sperm cells and 150 epithelial cells were recovered by LMD while duration of collection was recorded. The DNA was isolated using the Qiagen QIAamp DNA purification method followed by STR

analysis. Accuracy and completeness of genotypes were verified by analyzing samples from the untreated donor specimens. Total RFU values were tabulated for each sample at each locus and normalized to values of the unstained specimen to determine relative yields. Spreadsheet data are included in Appendix B.

Table 2 shows the identification scores assigned to the specimens. Both hematoxylin/eosin and Christmas tree stain readily provided morphological discrimination of spermatozoa and epithelial cells. This is consistent with previous reports [Allery et al., 2001]. Both Wright's stain and methyl green staining resulted in poor visualization of sperm cells making identification difficult. The penetration of methyl green was difficult to control and the Wright's staining method appeared to cause some deformation either in the epithelial cells or the PEN membrane hindering identification. Acridine orange performed well for identification of sperm, although, it appeared that differentiation amongst a concentrated field of epithelial cells might be challenging as the larger epithelial cells brightly fluoresced, potentially masking hidden sperm cells. Examples of H&E, CTS, AO, and unstained cells are shown in Figure 4.

Collection times were monitored in all the histology experiment samples. Table 3 summarizes the times required to collect 300 sperm cells from UNSTN, CTS, H&E and AO specimens. No significant difference was observed in the collection times between the four types of staining ($p>0.05$, Wilcoxon test). The average time to collect 300 sperm cells was 43 ± 2.9 minutes. The similarities in collection times could be attributed to a consistent cutting speed, as the same cutting parameters were uses as in this study. Differences in cell identification time may have not been observed as they were incorporated into the longer dissection times. A

record of the two time periods were not monitored, therefore, more samples would be required to further evaluate the effect of staining method on identification speed.

STR data from H&E, CTS, AO and unstained samples were compared. MG and WRT stains were excluded in this comparison due to the poor performance in the visualization of cells and also a suspected inaccuracy of cell numbers directly resulting from the high difficulty of identification. Figure 5 illustrates data in the blue dye which shows a decrease of RFU peak height exhibited by stained cells. Total RFU values of all Profiler Plus loci from the stained sperm and epithelial cells were compared to that of the unstained control to determine relative percent PCR yield. ANOVA analysis followed by Bonferroni's post-tests were applied excluding one semen donor sample due to the amplification failure of five loci from an unstained control. H&E samples exhibited RFU values 62.4% $\pm$6.6% of that observed by the unstained control ($p < 0.01$). CTS samples exhibited RFU values 42.6% $\pm$ 5.5% of that observed by the unstained control ($p< 0.001$). Cells stained with AO produced no amplified product in all samples tested.

It should be noted that due to the increased difficultly of identifying unstained specimens, error associated with underestimation of the number of unstained cells collected could exist but was not measured. In unstained specimens, collection was limited to sperm cells defined by their morphology, which included intact tails. Sperm heads alone were more difficult to identify without a stain. It is suspected that some sperm heads adjacent to targeted spermatozoa may have been recovered but not accounted for. Therefore, there may be an overestimate in the loss of RFU observed in stained specimens. In addition, the loss in PCR product may be due to either DNA degradation or PCR inhibition. Although not performed in this study, Human DNA

quantification by real-time PCR assay could provide data on quantity and quality of extracted DNA, information necessary to determine if these mechanisms are responsible for the reduced yield.

The separation and recovery of sperm cells by laser microdissection for DNA analysis differs from the preferential lysis method in that LMD is best performed when the material is stained for a more accurate and efficient microscopic identification of the cells of interest. It is important that histological dyes chosen do not interfere with downstream analysis of the sample DNA material. PCR inhibition of genomic DNA by dyes and fixatives has been observed with gross, stained tissue samples [Murase et al., 2000; Serth el al., 2000]. However, negative effects on DNA analysis from histological dyes can be reduced when the tissue is recovered using laser microdissection instead of manually dissecting tissue [Ehrig et al., 2001. This contrast is most likely attributable to the amount of tissue sampled as the cellular material collected by LMD is microscopically small and the instrument's ability to precisely excise the area of interest results in a low contamination of dye substances into further downstream analysis.

The objective of this part of the study was to investigate routinely used dye chemistry for their risk of degradative or inhibitory properties and their ability to provide good visual identification of the target cells. Five common stains were chosen to investigate their effect on downstream analysis of LMD collected material.

Hematoxylin/eosin is conventionally used as a nuclear stain in pathology and forensic laboratories and has been successfully used to recover LMD tissue for nucleic acid analysis [Ehrig et al., 2001]. Hematoxylin is a multicomponent dye with hematein as the active staining ingredient binding to acidic components. The blue to purple color depends on the mordant used

to make the hematoxylin dye bind to the molecules in the tissue [Kiernan et al., 1990]. Of the histological stains evaluated in this study, H&E performed best. It readily provided morphological discrimination of spermatozoa and epithelial cells which is consistent with findings reported by Allery et al. The use of H&E however resulted in lower RFU values compared to unstained specimens. This supports reports indicating that hematoxylin produces less PCR product than unstained controls in laser microdissected tissue sections [Ehrig et al., 2001, Murase el al., 2000] but it continues to be a popular choice in laser microdissection. Although the mechanism responsible for the reduced yield is not completely understood, hematoxylin-bound DNA seems resistant to complete digestion which may make the DNA less available for enzymatic replication [Burton et al., 1998]. In addition, while Eosin Y has shown no effect on PCR yield in laser microdissected tissues [Murase el al., 2000] it is an acidic dye that could be responsible for DNA damage. Despite the observed reduction in PCR product, the use of H&E did not prevent the acquisition of sufficient PCR product for successful STR genotyping. Shortened exposure times of H&E staining were used as a simple tactic to reduce the uptake of dyes by the cells and lessen the negative effect of these chemicals in the subsequent studies.

The Christmas tree stain consisting of nuclear fast red and picroindicocarmine is the forensic standard used to differentiate sperm cells from epithelial cells in stain identification [Oppitz et al., 1969]. Although the Christmas tree stain provided excellent morphological discrimination of spermatozoa and epithelial cells, its use produced significantly lower RFU values than H&E specimens ($p < 0.05$, paired t-test). This loss may be due to the picric acid component as highly acidic solutions will depurinate nucleic acids [Moore et al., 2002]

damaging DNA. In addition, indigo carmine, used in the textile industry for dyeing denim, is a known inhibitor of PCR [Larkin et al., 1999] further causing low yields. Use of nuclear fast red stained paraffin-embedded tissues prior to laser microdissection has produced superior yield over other histological stains [Burton et al., 1998] and used alone may be an approach to increasing yield if found sufficient for morphological identification.

Methyl green with an active ingredient of ethyl green, is a one-component nuclear dye believed to bind to the negatively charged DNA in nuclei, and has shown no adverse effect on laser microdissected tissue producing consistent amplification [Murase et al. 2000]. It has also been suggested that methyl green may increase amplification efficiency in manually dissected tissues [Ehrig el al., 2001]. However in this study methyl green did not provide adequate staining to visualize and identify sperm and epithelial cells.

Wright's Stain, consisting of azure blue and eosin, is commonly used to differentiate blood cells, which can potentially be used in cases where a mixture with blood is involved. It is a neutral stain produced by the interaction of methylene blue, an acidic dye and eosin, a basic dye, producing a large salt molecule with a colored dye in both its parts. It however yields less PCR product than methyl green and nuclear fast red [Burton et al. 1998]. In this study Wright's stain did not provide adequate staining to visualize and identify sperm and epithelial cells.

Acridine orange is a nucleic acid stain used to visualize sperm from vaginal swabs particularly from samples with dense epithelial cell populations [Mercurio et al., 2001]. However, a marked decrement in DNA yield is observed in brain tissue stained with AO [Ginsberg et at., 2004]. The only florescent stain used in this study, AO provided good visual identification of sperm but differentiation from epithelial cells became more difficult amongst

high concentrations of epithelial cells and/or sperm without tails. It however proved to not be compatible with downstream analysis. AO intercalates with double stranded DNA and binds electrostatically to the phosphate backbone [Lerman 1961] which may hinder primer access to the template.

These histology study results support the previous reports of problems with PCR yield associated with hematoxylin, acidic solutions, indigo carmine and acridine orange. STR analysis of the H&E and CTS treated cells can produce sufficient PCR product for genotyping, however, further work is necessary to increase yield. Given the successful history of nuclear fast red compatibility with downstream molecular analysis, it is suggested that further investigation into using this alone for forensic sperm identification and LMD collection should be examined.

3.3 DNA Isolation Comparison

To determine what isolation method would be appropriate for LMD cells three isolation methods were compared. Samples from 10 donors (5 semen and 5 oral swabs) were examined. Slides were prepared with the working solutions described in the specimen collection section and stained with the modified H&E method. Collections of 300 sperm cells and 150 epithelial cells were collected by LMD in triplicate to compare QIAamp, microLYSIS and Lyse-N-Go DNA isolation methods. All samples were typed for STRs using both standard PCR conditions and extended cycles analysis. Accuracy and completeness of genotypes were verified by analyzing aliquots from the untreated donor specimens. Total RFU values were tabulated for each sample

at each locus and normalized to corresponding values of the Qiagen QIAamp samples to determine relative yield. Spreadsheet data are included in Appendix C.

All DNA extraction protocols were modified to include dithiothreitol (DTT) to facilitate the rupture of sperm cells. DTT in high concentrations can be a PCR inhibitor, therefore, some experimental tests on DTT concentration were performed using all three extraction methods. The tests indicated that 5mM DTT was insufficient in completely reducing the disulfide bonds and releasing the DNA but 10-30mM of DTT sufficiently ruptured the sperm without having a negative in effect on the PCR reaction (data not shown). The use of 30mM of DTT in the lysis step is consistent with analysis protocols from the Los Angeles Police Department [LAPD manual] and AmpFlSTR kit recommendations [Applied Biosystems] of ~30-40mM.

Figure 6 shows mean values of the number of loci detected from samples processed with the three isolation methods. MicroLYSIS performed poorly with both sperm and epithelial cells samples displaying a high degree of allelic drop-out and resulting on average less than 50% of loci detected. Both Lyse-N-Go and QIAamp methods successfully isolated sperm DNA such that all loci were detected in 100% of the samples ("300 cell" count), however, results from epithelial cell extractions varied ("150 cell" count). On average 74$\pm$6.8% of loci were detected using Lyse-N-Go on epithelial cells while the QIAamp method produced on average 90$\pm$5.4% of the female donors' loci. However, within this study population the increase could not be deemed significant after ANOVA analysis ($p = 0.10$).

RFU values from Lyse-N-Go and QIAamp samples were also compared to evaluate quantity of PCR product produced. Relative differences were determined by normalizing RFU values of each donor sample at each locus to the corresponding QIAamp RFU values. Figure 7

shows the differences in amount of Lyse-N-Go PCR product detected from sperm and epithelial cells. QIAamp® extractions resulted in observed RFU values approximately 75% higher than the Lyse-N-Go™ method, a significant increase ($p < 0.05$, paired t-test), when used to extract epithelial cells. However, when applied to sperm cells the Lyse-N-Go method produced higher RFU values in 5 out of the 6 samples compared to the QIAamp® method.

The LMD collection cap has a working volume of 20-30µl. The goal of this study was to develop a method that can work in this small volume format and ideally in a single-tube format to minimize sample loss and be potentially automatable. The use of Chelex™ is a rapid inexpensive method widely used in forensic casework [Walsh et. al, 1991]. Chelex™ resin binds with Mg^{++}, rendering DNAases inoperable. However, in this study preliminary data (not shown) indicated that the use of Chelex™ resin was a poor method for the extraction of DNA from LMD cells. It was difficult to use in such a small volume format, challenging to remove all the liquid from the resin beads and resulted in little or no interpretable STR results when 400 and fewer cells were analyzed.

QIAamp spin columns have been successfully used to purify DNA from LCM samples [Martino et al. 2004] and forensic casework [Greenspoon et al., 1998]. This method uses a column containing a silica-based membrane that selectively binds nucleic acids as the lysate is drawn through by centrifugation. Through a series of washes, Salt and pH conditions ensure that proteins and other contaminants, which can inhibit PCR and other downstream enzymatic reactions, are not retained on the membrane. The DNA is then eluted off the membrane recovering a pure sample.

MicroLYSIS reagent and Lyse-N-Go reagent are one-step proprietary commercial reagents designed to lyse cells ready for PCR in one tube but do nothing to remove impurities. These solutions allow the release of DNA through a series of heating and cooling, which causes the cells and their organelles to lyse open [Burr et al., 2001]. The heating step also promotes the inactivation of endogenous nucleases. These methods offer the advantages of low-manipulation of forensic samples, a rapid incubation, and economical in the small volume required of LMD. However, the drawback of these methods is impurities often found in forensic samples would not be removed and may cause PCR inhibition if introduced in effective concentrations.

The nucleus of a human cheek cell is 5 microns in diameter, about the same length of a human sperm head. In this experiment, the nuclear material of the buccal cell was collected by recovering the whole epithelial cell body which is several times larger than the sperm cell. The amount of biological material including bound histological chemicals is therefore expected to be greater from the epithelial cells than the much smaller collected sperm heads. The QIAamp® kit is designed to remove proteins and possibly other contaminants that can inhibit PCR improving DNA yield from the epithelial cell samples. No such purification is done using the Lyse-N-Go™ method leaving effective contaminants in the PCR reaction. In contrast the sperm cell samples most likely contributed a smaller concentration of inhibitory histological dyes into the PCR reaction than the LMD collected epithelial cells. This may have allowed the sperm cell samples to benefit from the Lyse-N-Go™ method's ability to conserve sample.

The technique of diluting a DNA extract to reduce inhibitors and facilitate amplification, though with reduced sensitivity, is well documented [Yoshida el al., 1995]. A similar approach of dissecting only the nuclei of the larger epithelial cells may reduce the contribution of

inhibitory or degradative dyes into downstream analysis while maintaining the same concentration of DNA. In addition, it can be anticipated that as fewer amounts of cells are collected by LMD the concentration of inhibitors would decrease making collection of minute numbers of cells for Low Copy Number analysis more amenable to non-purification, one-step lysis buffers such as Lyse-N-Go™. Therefore consideration of cell type and number should be a factor in the choice of DNA extraction method to address the presence of potentially inhibitory histological dyes when using LMD.

A variety of types of biological samples are encountered in forensic evidence. Not only is there an assortment of different types of samples such as blood, semen, saliva, tissue and hair but due to the nature of criminal investigations specimens will have degrees of available material, degradation, and contamination with foreign substances or bacteria. As a result there are many different DNA isolation methods routinely used in crime laboratories to address the basic steps of lysing the cells and recovering a relatively pure DNA sample for PCR analysis.

Some unique challenges emerge when developing and determining what DNA isolation method is appropriate for LMD cells. When faced with performing molecular DNA analysis of stained cells, a method that removes the histological chemicals from the DNA would be preferred to avoid polymerase inhibition. In addition, choosing a DNA isolation method that conserves the DNA and provides a concentrated DNA extract would be desirable for LMD cells. The time required to collect cells was approximately 15 minutes per 100 sperm cells using the Leica system software version 4.1.3. It was important then to minimize the number of cells collected and chose a DNA isolation method such that the entire extract can be used for PCR. This would be particularly important in the recovery and analysis of very minute evidence

sample and Low copy number (LCN) analysis. For the LMD collection of sperm cells the Lyse-N-Go extraction method conserved cells, had a rapid lysis and could provide complete STR typing.

3.4 Cell Separation Study

To evaluate the capability of LMD to separate and recover sperm cells from a semen/epithelial cell mixture, five mixed cell samples from 10 donors (5 semen and 5 oral swabs) were examined. Mixtures were created by combining 25µl of the epithelial cell pellet working solution with 10µl of the 1:10 semen working solution and prepared on PEN slides as described in the methods section and stained with the modified H&E protocol. Collections of 75, 150 and 300 sperm cells were separated by LMD from the mixtures. A serial dilution of the AmpF*l*STR positive control standard was included to simulate 2ng, 1ng, 0.5ng, 0.25ng and 0.125ng DNA amplifications to compare to collected cell amounts. STR typing was performed on all samples under both standard and extended cycles analysis. Accuracy and completeness of genotypes were verified by analyzing samples from the untreated donor specimens. Total RFU values and peak height ratios were tabulated at each locus. Spreadsheet data is included in Appendix D.

Using standard PCR conditions all 10 loci of the sperm donors in all samples containing 300 LMD sperm were detected. Samples containing 150 sperm exhibited on average 96$\pm$3% of the male donors' alleles and samples containing 75 sperm cells displayed on average 72$\pm$12% of the male donors' alleles. STR plots of the LMD sperm fractions from three collection amounts

are illustrated in Figure 8 and the donor genotypes of the original mixture are listed in Table 4. In all samples tested, no true allele peaks were detected in non-overlapping positions from the female buccal cell donors.

Extended cycles analysis was used for two purposes. First, increased amplifications can detect even minute amounts of carryover female DNA that may be contaminating the sperm fraction. Second, increasing cycle number could be useful in detecting male donor alleles not detected under standard PCR conditions. Using extended cycle analysis 100% of samples containing 75 and 150 sperm cells displayed all of the sperm donor alleles. Non-overlapping female alleles (alleles that are not the same for both donors) were not detected in any samples using a total of 34 PCR cycles demonstrating the collection of a pure population of sperm cells without female DNA contamination. This is illustrated in Figure 9 showing STR plots of the two collection amounts from the same mixture. Samples containing 300 sperm were not interpreted due to the preponderance of peak heights over the linear range and an abundance of PCR artifacts such as increased stutter and -A nucleotide products.

RFU (signal intensity) is a measure of PCR product quantity. Peak height data is summarized in Figure 10 showing the total fluorescence signal detected (the sum of two heterozygote peaks or the value of a single homozygote peak) at each locus for the three collection amounts. In general the total RFUs detected increased with an increase in the number of LMD collected sperm cells. Assuming one human haploid cell contains 3.3pg of genomic DNA, the examination of 75, 150, and 300 sperm cell amounts contains approximately 0.25, 0.5 and 1.0ng of DNA respectively prior to DNA isolation. The lower limit recommended by the AmpF*l*STR kit is 1ng although forensic casework is routinely performed below this limit. Signal

intensity was compared from the experimental samples with a dilution series of the AmpF*l*STR® DNA positive control under standard PCR conditions. Figure 11 shows a plot with regression line of the positive DNA control analyzed from 0.125 to 2ng. As expected the positive control showed a linear relationship between RFU value and quantity of DNA (r^2= 0.9961). Mean values of the LMD samples plotted on the same graph maintained a linear relationship (r^2= 0.9179) with RFU values 2.5 - 3.7 times less than the positive control values. This reduction likely reflects DNA recovery or PCR inefficiency but it is not possible to measure this precisely due to inherent inaccuracies with the quantitation of the positive control by the manufacturer.

Threshold RFU values for individual peak heights are typically determined by each laboratory based on internal validation of their protocols and interpretation guidelines. The Applied Biosystems User's Manual recommends a minimum threshold value of 150 RFU. However, in practice alleles are routinely identified below this level with special cautions in interpretation. Under standard PCR conditions, heterozygote and homozygote peak height ranged from [51, 217] for "75 sperm", [54, 479] for "150 sperm" and [56, 494] for "300 sperm" samples with loci of larger base pair sizes generally exhibiting lower peak heights. Under extended cycles analysis peak heights ranged from [306, 7786] for "75 sperm", and [657, 9413] for "150 sperm" samples.

Balanced allele peaks are important for accurate genotyping from DNA mixtures. Heterozygote peak height ratios were calculated, which is defined as the height of the lower peak divided by the height of the higher peak, of the sperm cell fractions. Samples that displayed only one allele at a locus where the donor was heterozygous were excluded in the calculations. Peak height ratios calculated and at each locus are shown in Figure 12. Mean peak height ratios over

all loci under standard conditions were 76.3±3.3% for "75 sperm", 81.1±1.3% for "150 sperm and 82.0±1.4% for "300 sperm" samples. Mean peak height ratios decreased with extended cycles analysis to 67.0%±4.2% for "75 sperm" but did not significantly change for "150 sperm" samples at 85.2±2.1%, however, variance increased in peak height ratio values when extended cycle analysis was used.

Under ideal conditions a PCR reaction will contain an equivalent number of copies of both alleles. In theory amplification should occur resulting in equal amplicon yields from each target sequence, although in practice various factors affect PCR efficiency creating disproportionate products. Reactant depletion, accumulation of pyrophosphate and other contributing factors reduce PCR efficiency in later cycles resulting in fewer large amplicons become targets in subsequent cycles [Leclair et al., 2004]. Therefore, even under ideal of conditions peak height ratios are less than 1. A validation study using the Profiler Plus kit calculated peak height ratios from 425 database and casework samples which reported mean values for single source specimens above 86% [Frank et al., 2001]. The AmpFlSTR kit is optimized to produce relatively balanced levels of PCR product both within and between loci and rarely produce height ratios less then 70% in a heterozygous individual. Ratios observed in single source samples less then 70% may indicate degraded DNA, presence of inhibitors, a rare sequence not efficiently amplified, or low amount of DNA or cells [AmpF*l*STR User's Manual].

A few factors were believed to be at work in this study's experimental samples resulting in mean peak height ratios below expected values. First, as indicated in the histology study, histological stains either acted as effective PCR inhibitors or degraded the DNA. Second, the amount of input DNA into the PCR reactions was lower than the recommended range. Third, the

samples involved the analysis of haploid cells. In abundant amounts of haploid cells one would expect to have equal amounts of each allele due to random sampling. However, as fewer cells were collected the probability of having a disproportionate representation of alleles increased. Finally, increasing PCR cycles above the optimized range could cause preferential amplification of one allele in late cycles as described earlier. Despite the apparent loss in PCR product yield, 75-300 sperm separated from an epithelial cell mixture produced clear single source genotypes with satisfactory peak signals and allelic balance.

The nature of forensic samples results in a variety of cell numbers on a vaginal swab. In this study mixtures were created to be equivalent to 1µl of semen and one half a buccal swab to get consistency in the mixture experiments. However, it was later determined that using this approach to create mixed specimens could still result in a wide variety of cell ratios and concentrations due to the individual variability of the donor samples. Hemacytometer analysis of 5 buccal swabs and 4 semen samples were performed to determine cell concentrations. Table 5 lists cell amounts estimated for 1/2 buccal swab and 1µl of semen. A large variability in both buccal swabs and semen samples was observed with standard deviations exceeding 50% of the mean values. This unintentional variability may actually closer represent postcoital samples. This indicates that controlled cell ratio studies are warranted in the future to determine the LMD separation power of sperm cells from increasing concentrations of epithelial cells.

To fully evaluate the LMD method's separation capability, real postcoital and forensic casework samples should be analyzed. A hurdle anticipated in more compromised samples is the premature lysis of vaginal epithelial cells prior to evidence collection. Previous physical separation methods discussed earlier reports carryover contamination of the female fraction, due

primarily to free-floating DNA. It is predicted that most free-floating DNA can be avoided using LMD due to the precise dissection capabilities of the instrument. However, of greater concern is the affinity of exogenous DNA to sperm heads.

Sperm cells from nearly all species are able to take up foreign DNA [Spadafpra et al., 1998]. Exogenous DNA binding is an ionic reversible interaction that occurs in the subacrosomal segment of the sperm head and interestingly seminal fluid strongly repels the binding. The glycoprotein, IF-1, has been identified as an inhibitory factor in seminal fluid that selectively binds to the same cell compartment targeted by exogenous DNA binding, protecting the sperm. It would be expected that as the postcoital interval increases there would be a reduction in seminal fluid concentration and degradation of seminal fluid proteins. This combined with the normal metabolic lysis of vaginal epithelial cells could be an environment encouraging the binding of the female's vaginal DNA with the semen depositor's sperm. Plasmid DNA has been found to detach from epididymal spermatozoa by excess of cold competitor DNA or by other polyanions as heparin and dextran sulphate [Lavitrano et al., 1992]. For future studies, an approach to separate exogenous DNA from sperm cells prior to LMD may lie in the exposure of the cell pellet to an appropriate negatively charged macromolecule solution to remove the vaginal DNA bound to the sperm head.

CHAPTER 4. CONCLUSIONS

These results demonstrate that laser microdissection is an effective technique for recovering spermatozoa from a sperm/epithelial cell mixture. LMD collects pure populations of sperm with no apparent cross contamination from epithelial cells. Hematoxylin/eosin and nuclear fast red/picroindigocarmine histological stains can be used for sperm identification in conjunction with LMD separation for STR genotyping. Used in combination with the Lyse-N-Go extraction procedure the LMD method is a simple, low-manipulation method that conserves collected sperm cells. This has the potential of facilitating analysis of low numbers of sperm.

The simplicity of the LMD method can be demonstrated in Figure 13 compared it to the traditional methods of processing a sexual assault mixture. Using preferential lysis with Chelex or organic phenol/chloroform DNA extraction requires about one and a half days from the time the laboratory begins processing of a single swab until the sample is ready for PCR. Alternatively, the LMD method can take a sample and be PCR ready within a couple of hours. It is also conceivable that hospital sexual assault evidence kits (SAK) could be prepared with PEN slides for preparation of smears further facilitating the LMD process.

LMD combines and eliminates other forensic DNA analysis steps making the laboratory process more efficient. The sperm identification step and separation step are done simultaneously using the LMD method. The direct human DNA quantification step is eliminated as cells can easily be counted during LMD collection and extrapolated into an estimate of DNA quantity added to the PCR reaction. DNA quantification is particularly important in samples with high DNA concentrations, as it is necessary to limit the amount of DNA entered into the PCR reaction

to avoid off-scale data, PCR inhibition and other amplification artifacts. The AmpF*l*STR kit recommends a maximum DNA input of 1-2.5ng of DNA. LMD collection is preferably done and can be limited to < 2.5ng or ~750 sperm cells. Adherence to SWGDAM validation guidelines [TWGDAM 1995] concerning the quantification of DNA could be achieved by validating a cell counting method with the DNA extraction yield to estimate quantity. Not only does LMD eliminate the need for a time consuming DNA quantification procedure but also conserves precious sample for genotyping.

A disadvantage of using the LMD method at this time is the separation step cannot be batched as in the preferential lysis method. Each sample slide is handled separately and the time it takes to recover the samples is dependant on the number of cells collected. However, relief at this bottleneck will most likely occur in the near future as the manufacturer has recently released software modifications to allow all target cells to be dissected from an entire slide using a single initiation of dissection rather than field by field. In addition, software is in development to perform auto-recognition of target cells based on identification parameters designated by the operator. These improvements could revolutionize this technology. Yet even now, when a few samples require an immediate and quick analysis LMD could be the critical tool of choice.

Other potential forensic application of the LMD could be the use of fluorescence in-situ hybridization (FISH) to target sex chromosomes on the slide. FISH has been used to identify male epithelial cells in cervicovaginal smears and found more sensitive than conventional cytolocical stains [Rao et al., 1995]. Integrating LMD with FISH could allow the separation of mixtures of identical cell types from two donors of different sex. DNA mixtures found in cases

involving forced oral copulation, sodomy and digital penetration could potentially be separated using LMD even when sperm is absent.

Products of conception may have evidentiary value in determining the identity of a rapist or even link a pregnancy to a motive for homicide. Occasionally products of conception become available in criminal cases due to elective abortion, miscarriage or autopsy and could be analyzed using LMD in its traditional capacity, dissections from tissue cryosections. Historically, manual dissection of products of conception has been the method used by trained pathologists to recover fetal tissue. However, frequently the fetal material is contaminated with maternal tissue especially in early pregnancies. A case study has been documented of paternity testing from residues of chorionic villi after pregnancy termination 5 weeks before the victim was murdered [Bauer et al., 2002]. Fetal cells were selectively isolated by laser-induced microdissection to avoid contamination with maternal DNA followed by STR genotyping.

Nucleic acid analysis from single cells has been documented [Findlay et al., 1997; Garvin et al., 1998] but challenging and difficult to reproduce. Much focus and research in the forensic community is turned to low copy number analysis in order to expand the current limits of detection [Gill et al., 2000; 2001]. It is in the foreseeable future that development of single-cell analysis will be utilized in forensic casework to allow a single sperm cell to be haplotyped when the proper molecular tools are in place and statistical models for identification determined. In sexual assaults with multiple perpetrators, single-cell analysis though recovery by LMD may one day differentiate individual donors.

This research will continue by testing semen and blood mixtures, mixtures with high concentrations of epithelial cells, and its application to recover minute numbers of sperm cells

for Low Copy Number analysis. Further studies are warranted to establish if testing of genuine casework specimens respond to LMD separation like the mock specimens in this study.

APPENDIX A
FIGURES AND TABLES

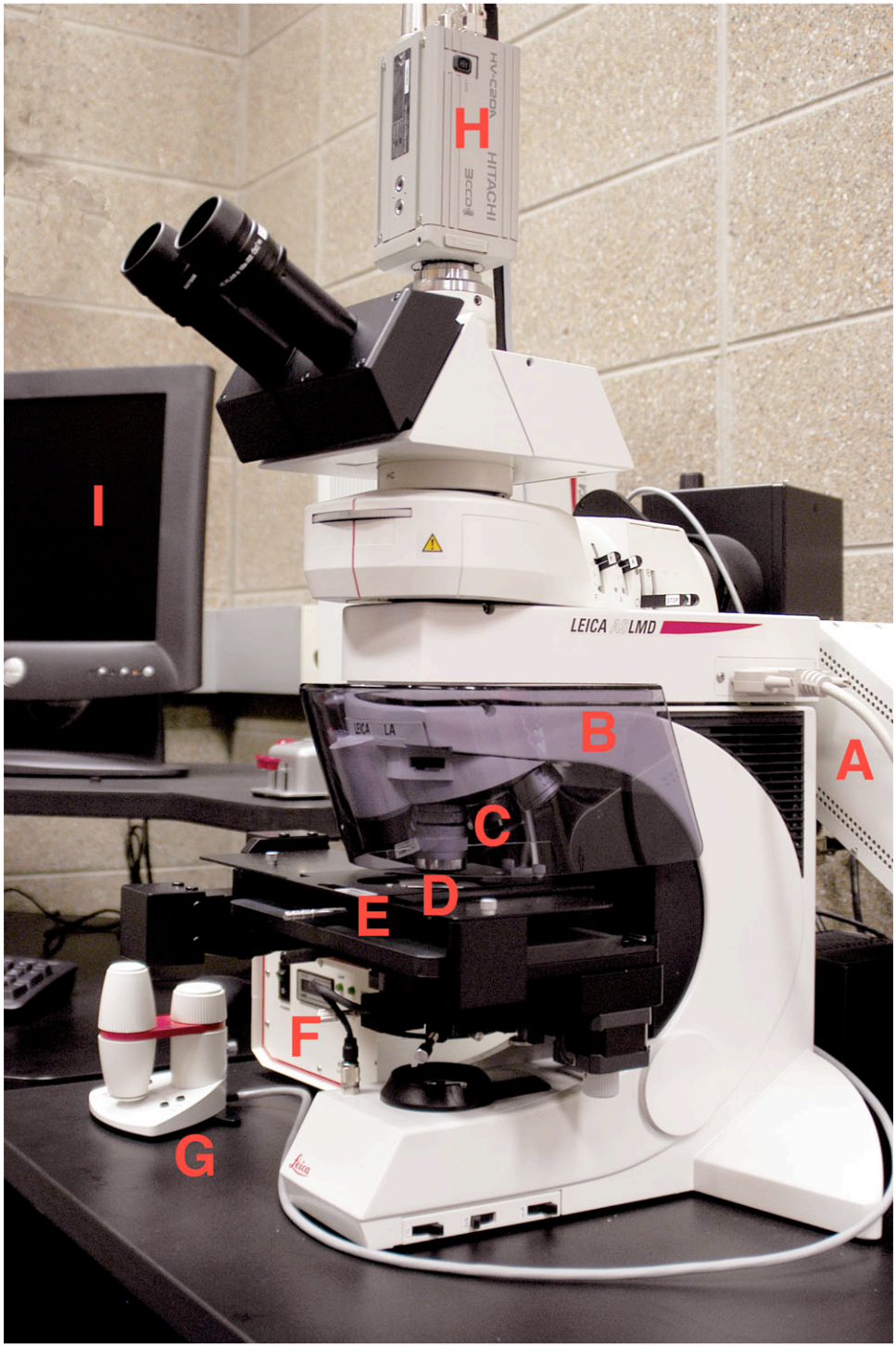


Figure 1. Leica AS LMD System A) Laser B) Laser shield C) Motorized objectives 4x, 10x, 20x, 40x & 63x D) Slide/specimen holder E) PCR tube holder F) Fluorescence option G) XYZ control mouse H) Live camera I) Computer controlled dissection

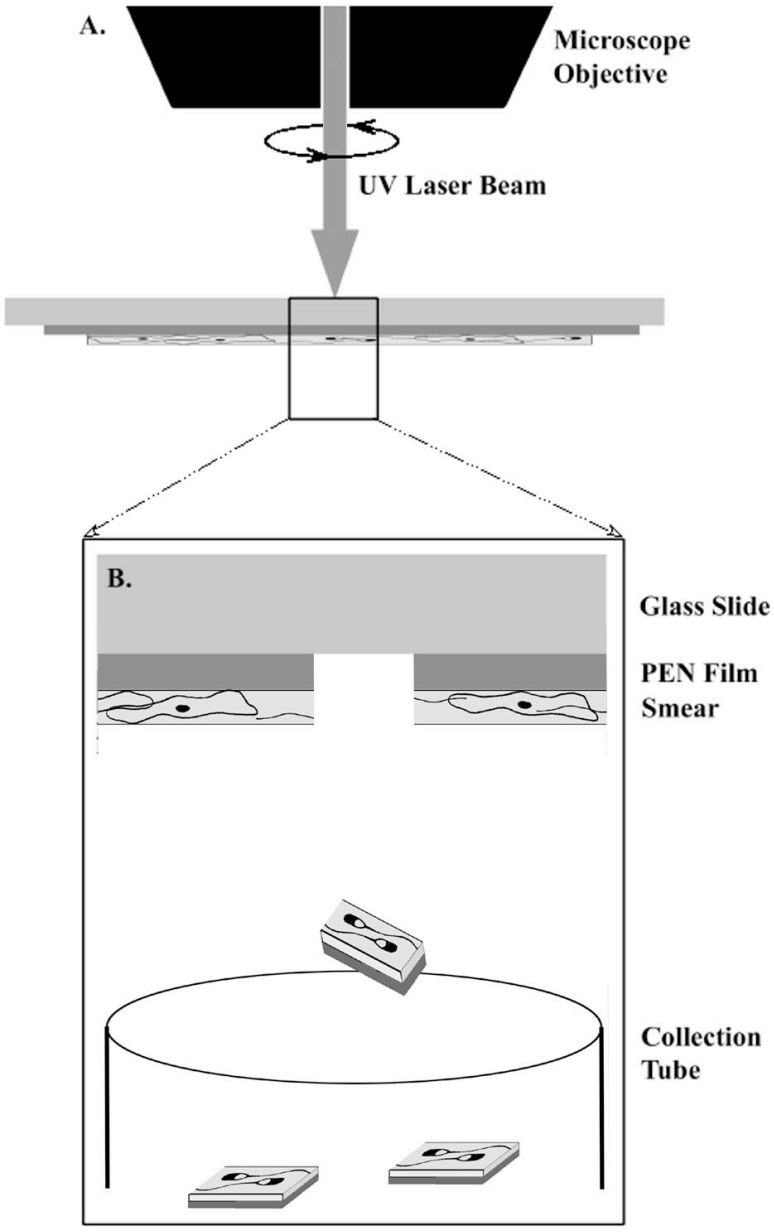
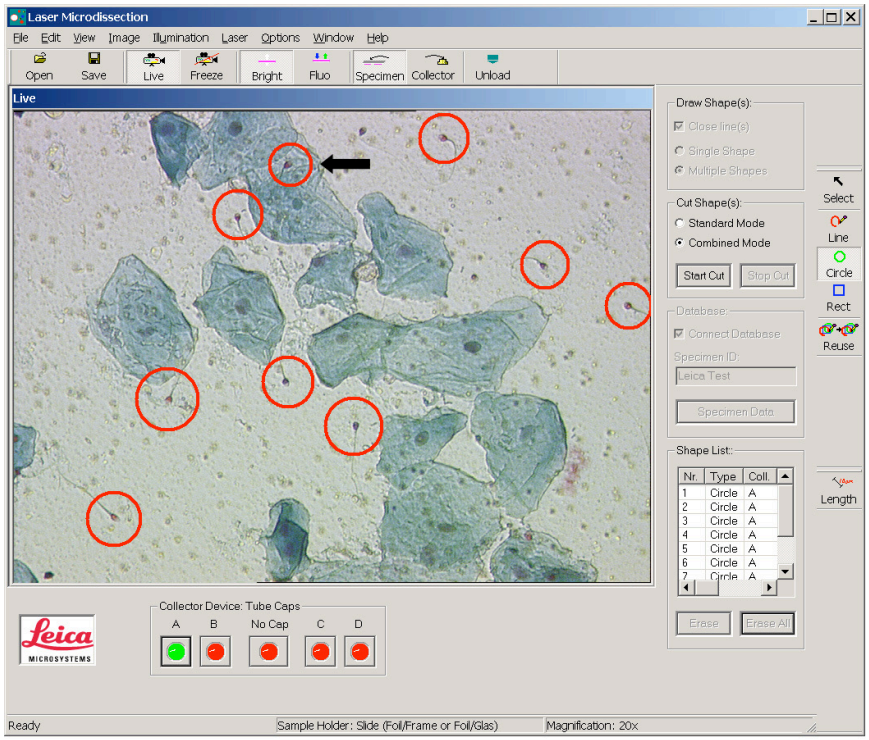


Figure 2. Laser Microdissection on the Leica AS LMD A) The slide rests inverted on the microscope stage with specimen facing down. The laser is focused through the objective to dissect around sperm cells cutting the PEN film. B) Magnified cross section of sperm cells adhered to PEN film dropping into collection tube below stage.

A



B

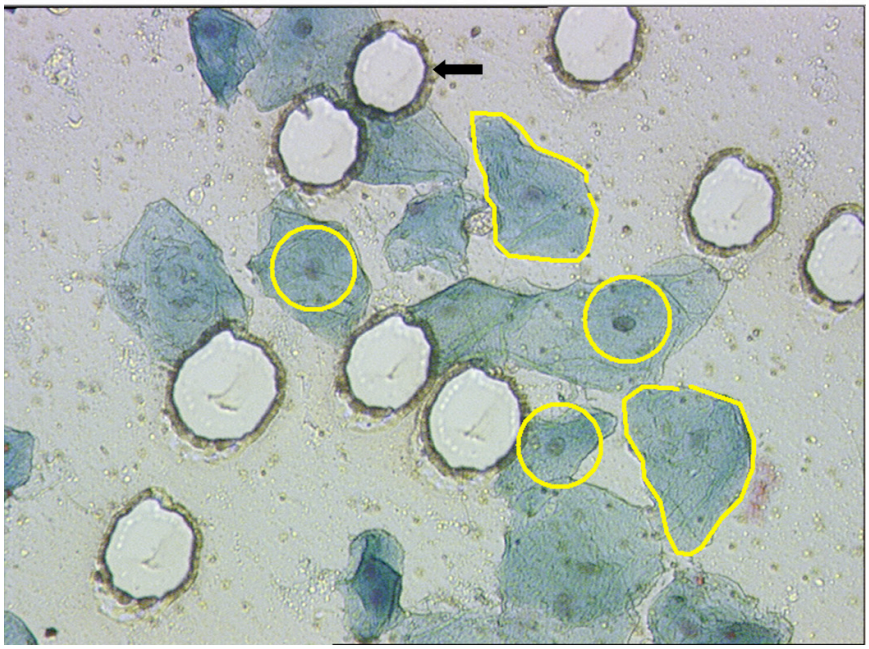


Figure 3. Computer Controlled Laser Microdissection on the Leica AS LMD. A) An epithelial and sperm cell mixture is displayed on the computer screen bordered by a control options window. Sperm cells are identified and targeted by circles drawn through the software control panel with tube "A" selected to the right of the "Leica" logo. B) After sperm dissection, epithelial cells are targeted by circles and drawn lines for a separate collection. Arrows specify a sperm cell commingled with two epithelial cell bodies.

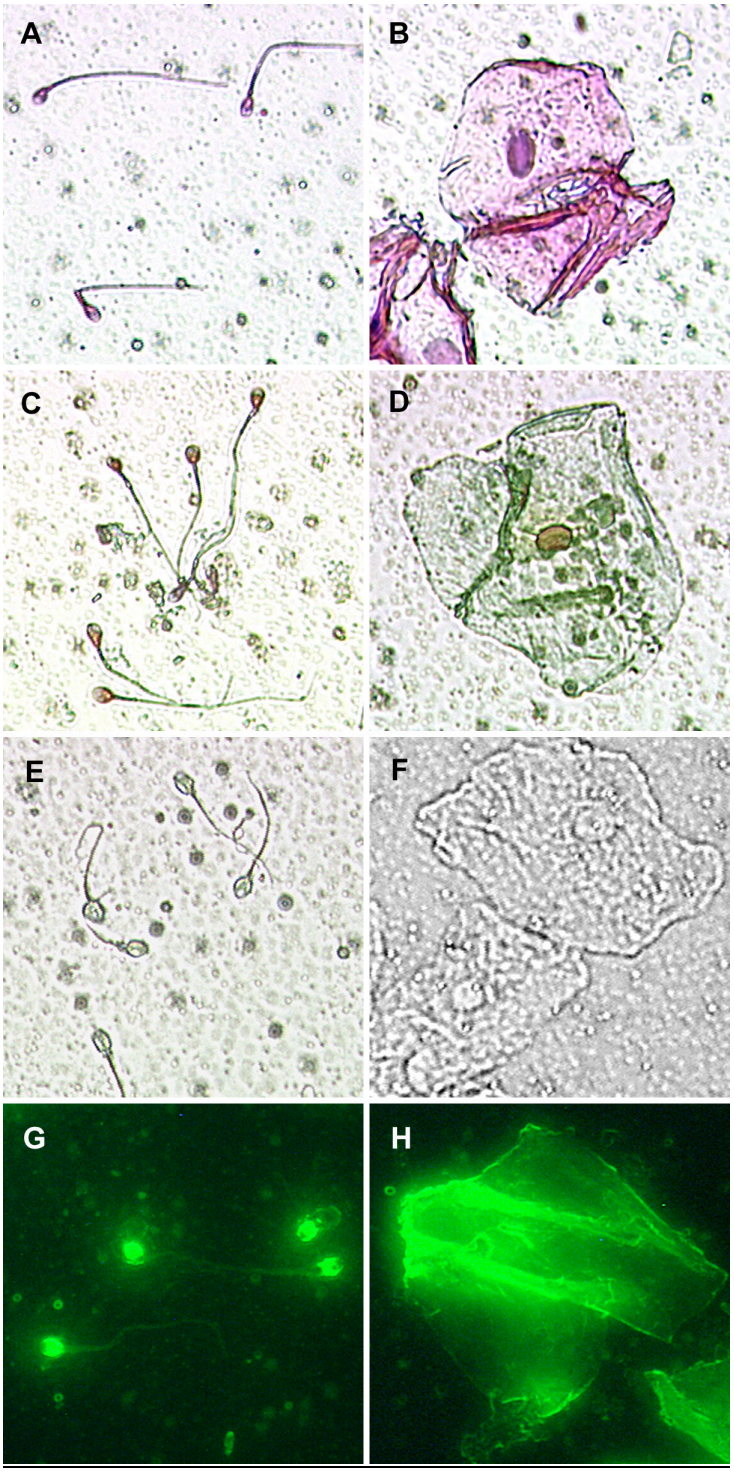


Figure 4. Images of Stained Sperm and Epithelial Cells. A) H&E sperm cells, B) H&E epithelial cells, C) CTS sperm cells, D) CTS epithelial cells, E) UNSTN sperm cells, F) UNSTN epithelial cells, G) AO sperm cells, H) AO epithelial cells

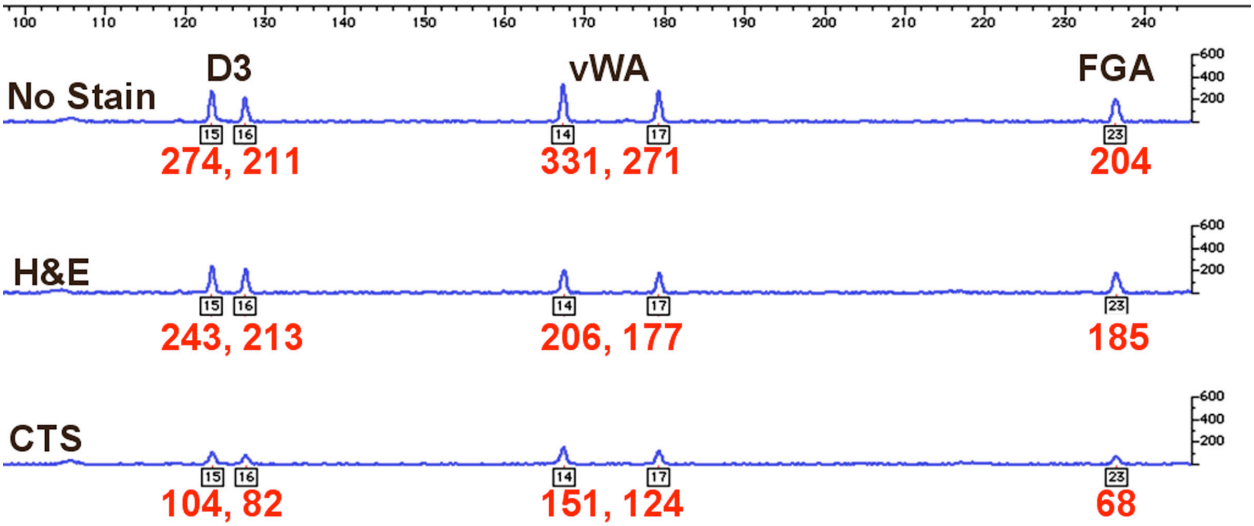


Figure 5. Peak Height Comparison of Stained Cells. STR Plots at the D351358, wWA and FGA loci of 150 oral epithelial cells with no stain, H&E and CTS. Red numbers below plots are RFU values of each peak.

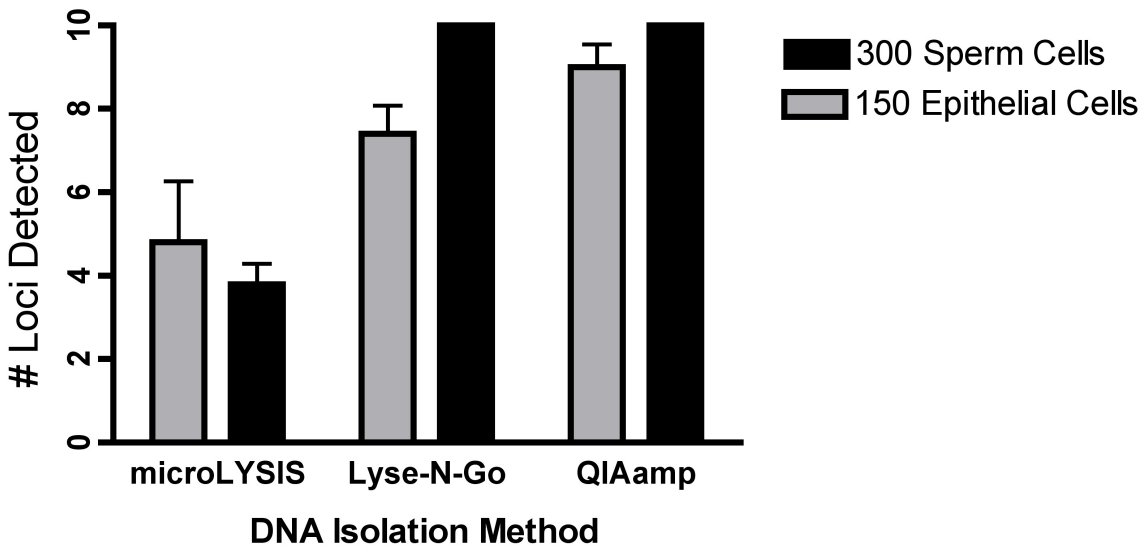


Figure 6. Detection of STR Loci Using Different Isolation Methods. Samples comprising of 300 sperm and 150 oral epithelial cells were subjected to three DNA isolation methods: microLYSIS, Lyse-N-Go and QIAamp. Mean number of loci (n=5) detected out of ten possible Profiler Plus markers is shown from each group.

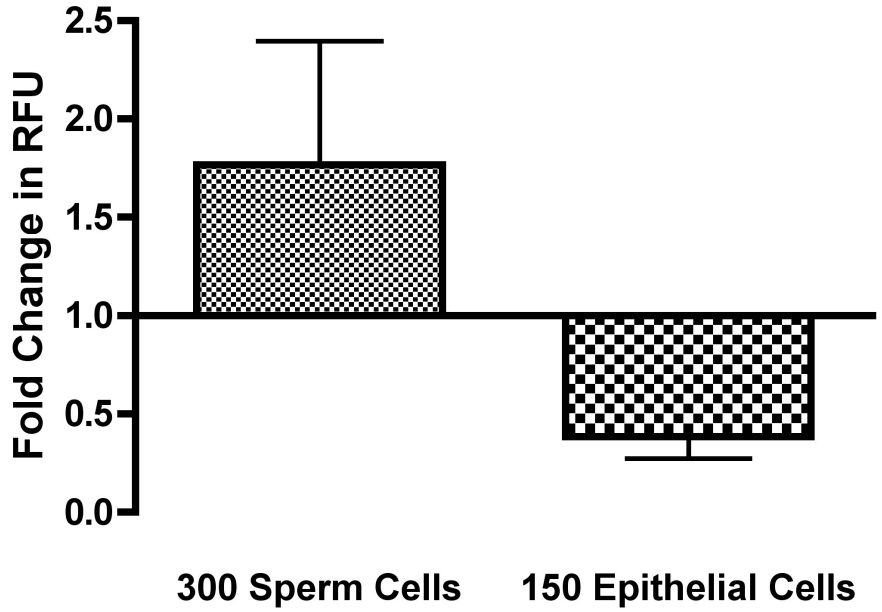


Figure 7. PCR Product Differences Using in Lyse-N-Go vs. QIAamp Extraction. RFU values of each donor sample was normalized at each locus to the corresponding QIAamp RFU values with mean fold changes shown for sperm samples (n=5) and epithelial cell samples (n=5).

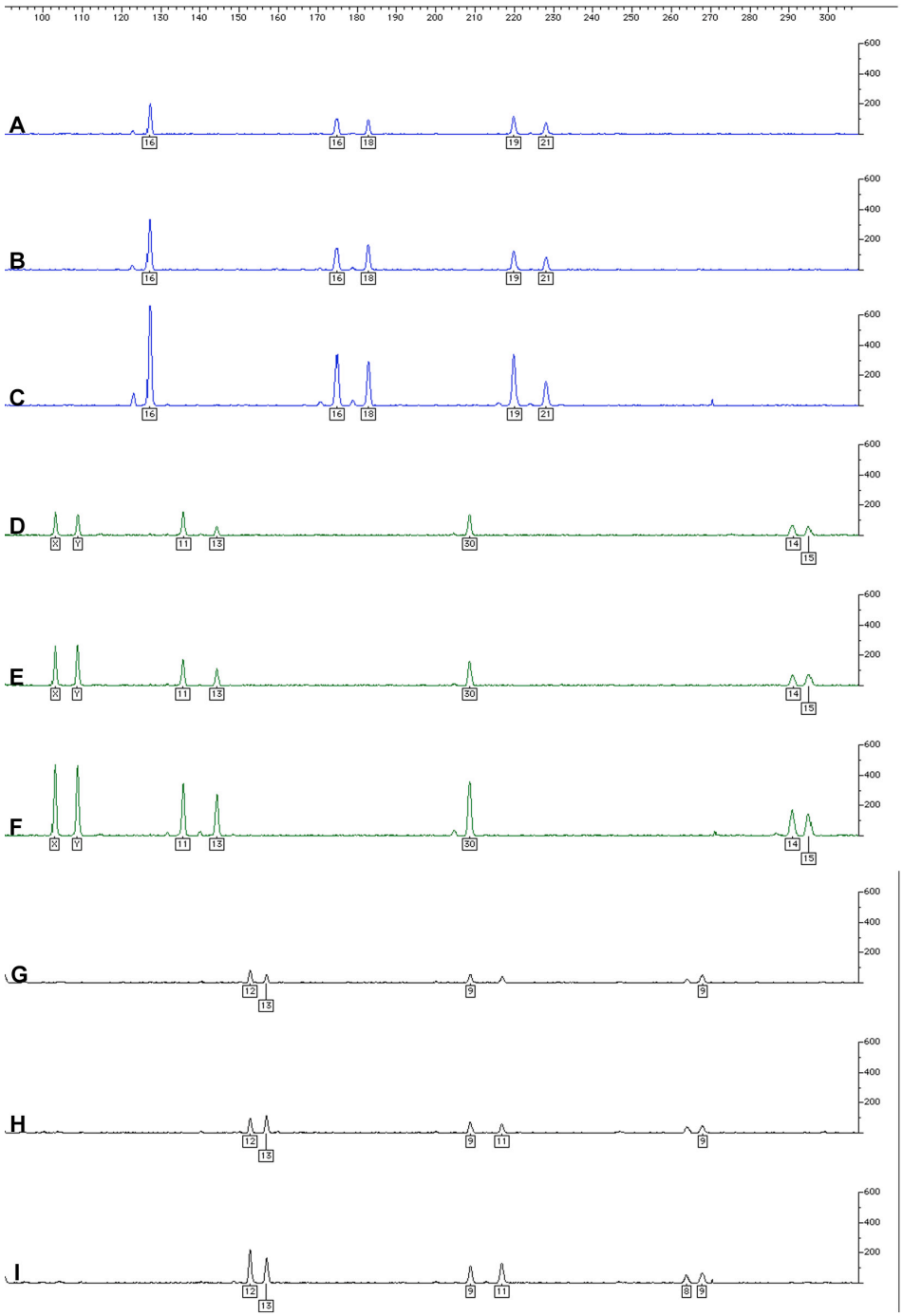


Figure 8. STR Plots of Sperm Cells from a Mixture. Profiler Plus alleles of LMD collected sperm cells from a sperm/epithelial cell mixture observed in the following: blue loci at A) 75 sperm B) 150 sperm and C) 300 sperm; green loci at D) 75 sperm E) 150 sperm and F) 300 sperm; yellow loci (shown in black) at G) 75 sperm H) 150 sperm and I) 300 sperm (y-axis scale at 600 RFU). Allelic drop-out observed in yellow loci for 75 and 150 cells.

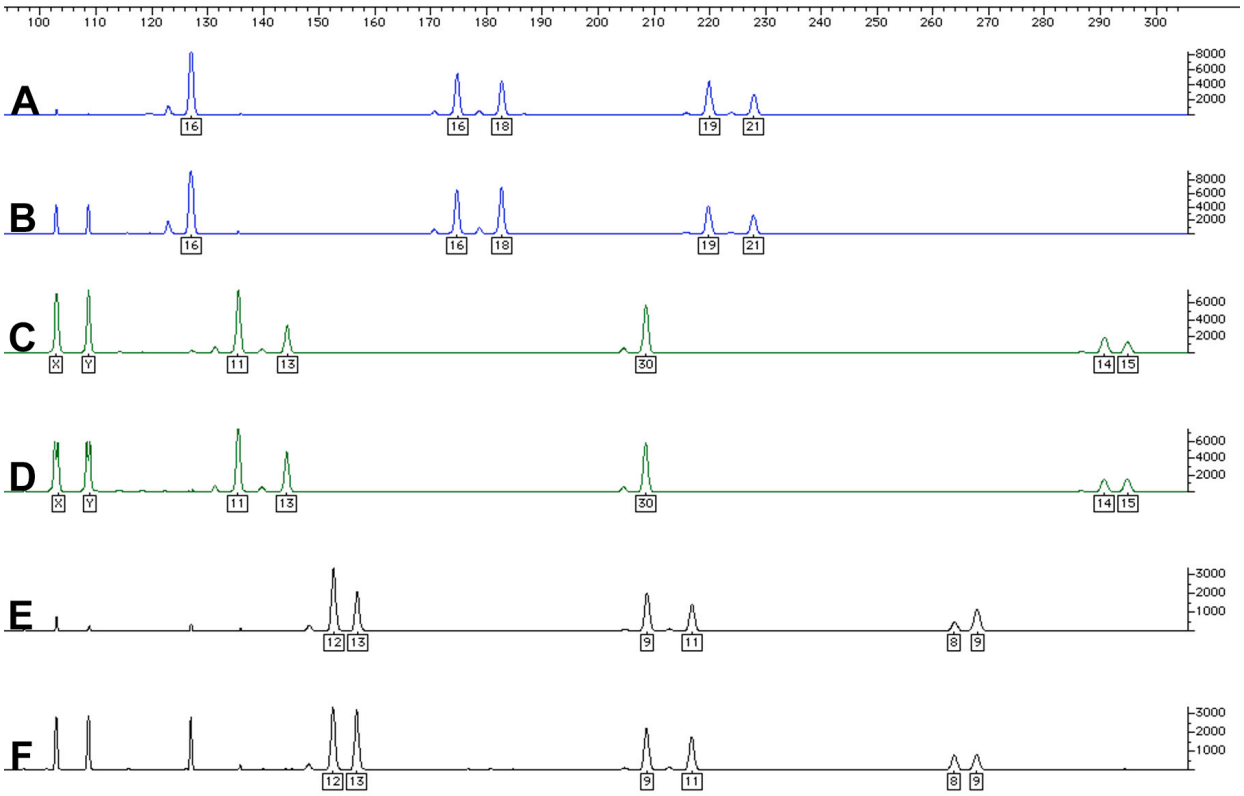
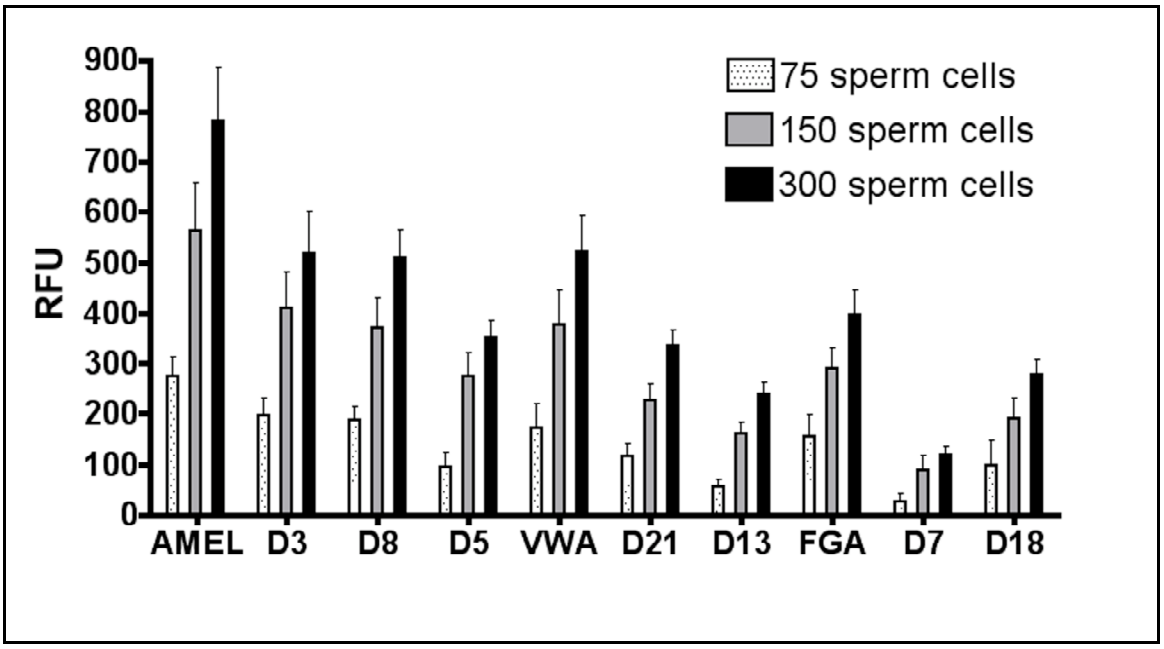


Figure 9. STR plots of Sperm Cells from a Mixture with Extended Cycles Analysis. Profiler Plus alleles of LMD collected sperm cells from a sperm/epithelial cell mixture using extended cycles analysis observed in the following: blue loci A) 75 sperm B) 150 sperm; green loci C) 75 sperm D) 150 sperm; and yellow loci E) 75 sperm F) 150 sperm. All alleles were detected from male donor without female carryover (chart in Table 3).

A



B

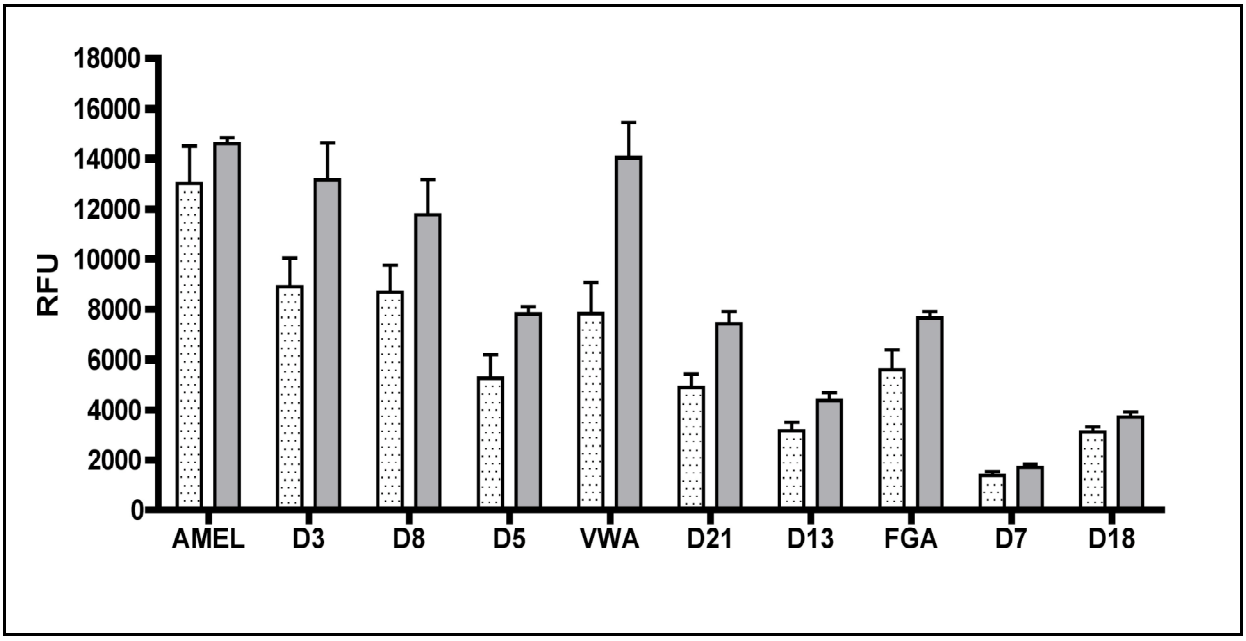


Figure 10. Level of PCR Product Detected in Standard and Extended Cycle Analysis. Total RFU peak values at each loci were averaged for A) 75, 150, and 300 sperm cell specimens using standard PCR conditions and B) 75 and 150 sperm cell specimens using extended cycles analysis.

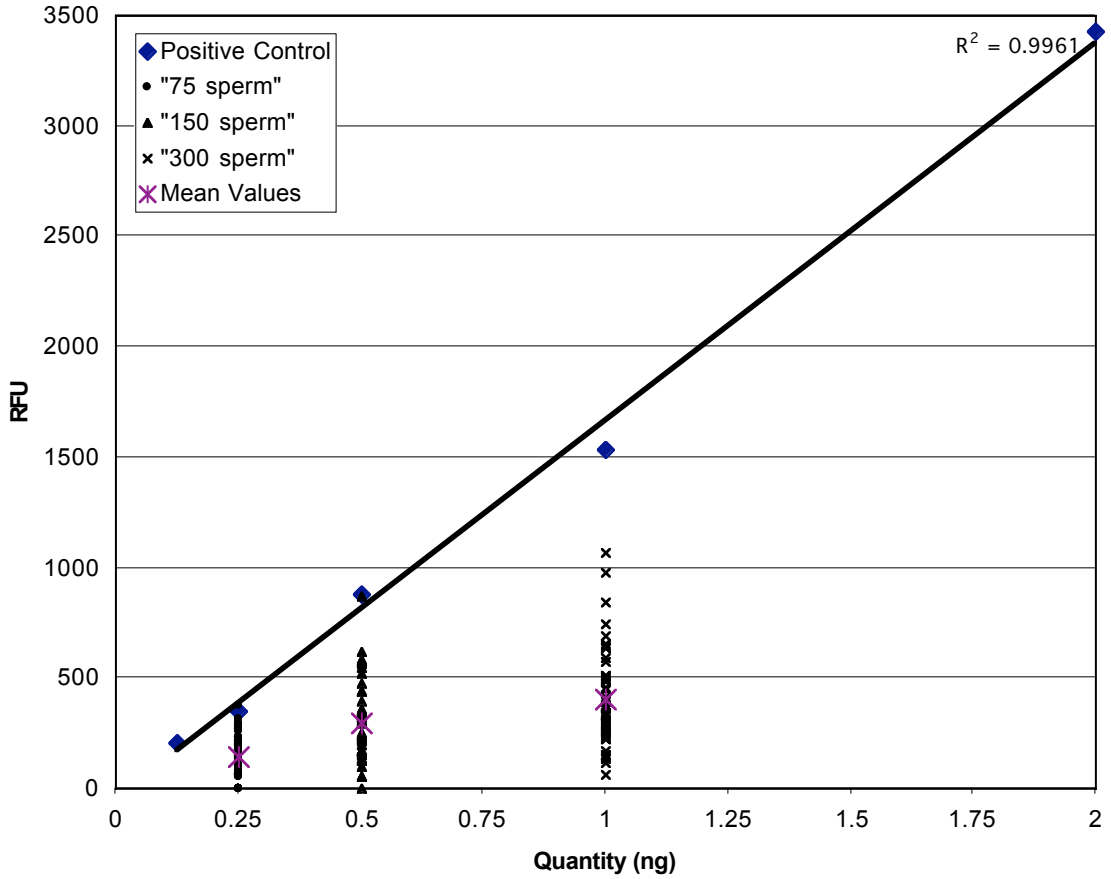
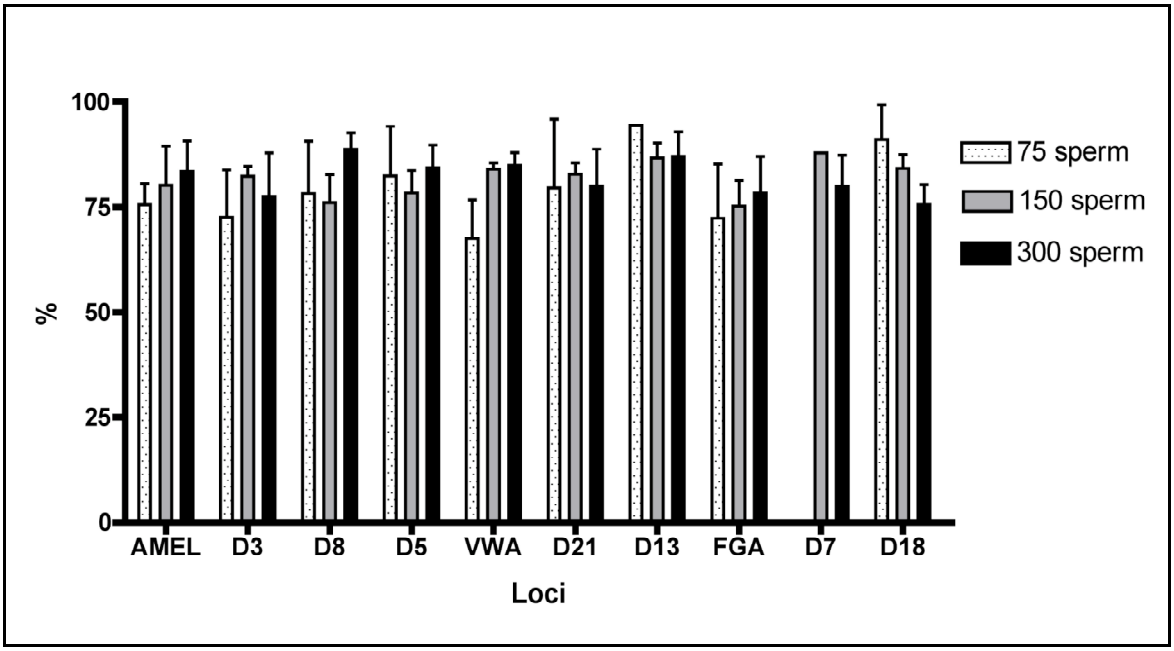


Figure 11. PCR Product Level of 75, 150, and 300 Cells. Five positive control dilutions of 0.125, 0.25, 0.5, 1 & 2ng were plotted against observed RFU values. RFU values for 75, 150 & 300 sperm samples were plotted at their maximum theoretical DNA quantity.

A



B

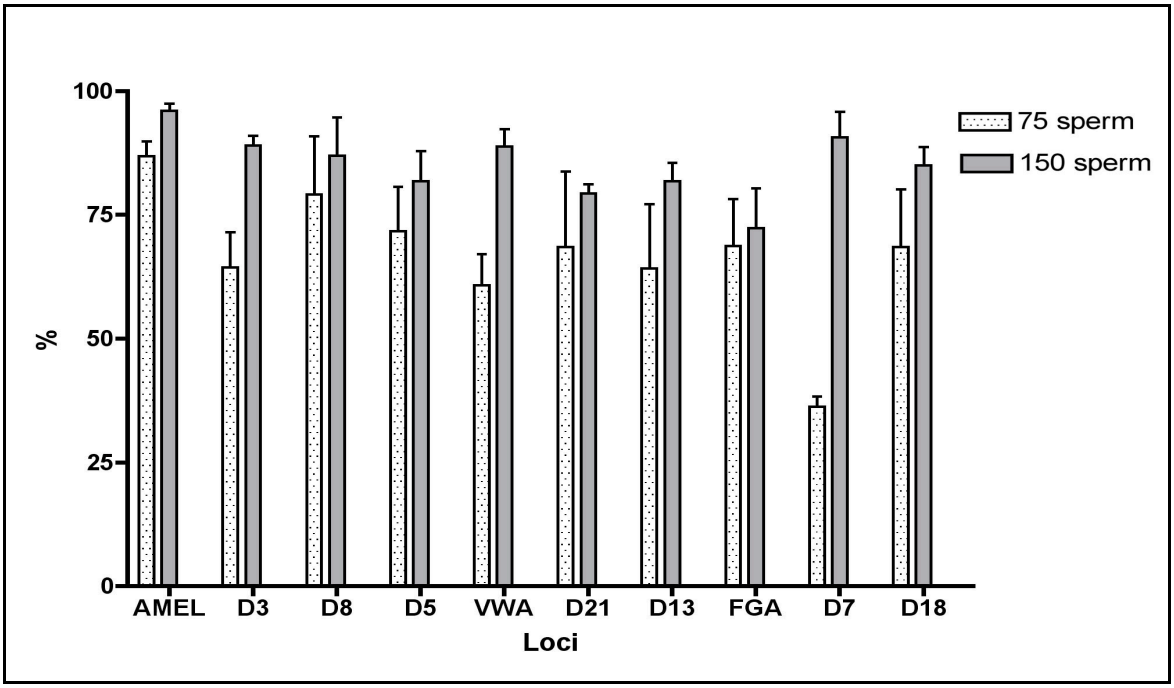


Figure 12. Peak Height Ratios at Each Loci for Standard and Extended Cycles Analysis. Peak height ratios of heterozygous loci were averaged for A) 75, 150, and 300 sperm cell specimens using standard PCR conditions and B) 75 and 150 sperm cell specimens using extended cycles analysis.

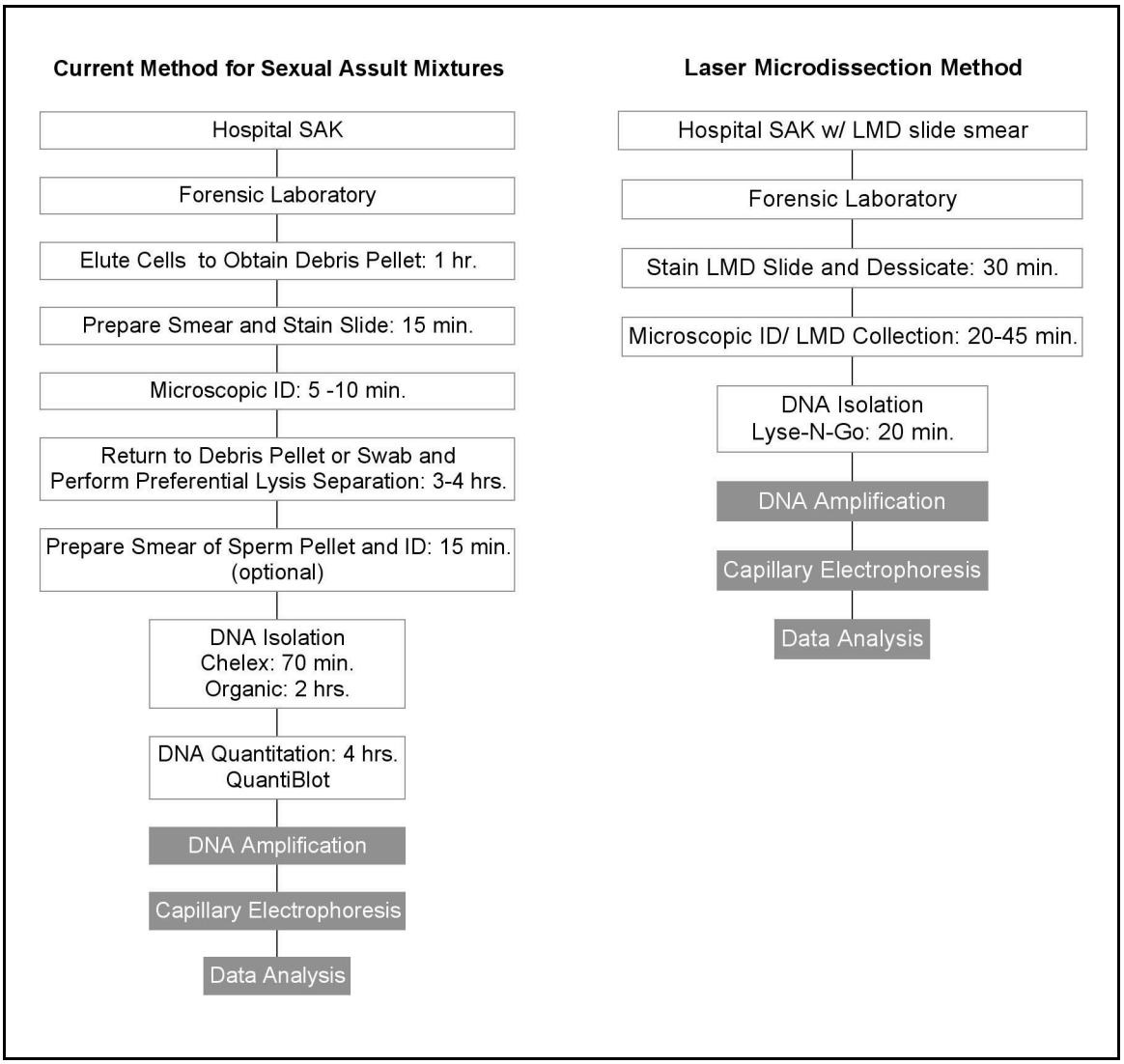


Figure 13. Protocol Flow Chart for Processing A Sexual Assault Kit. Laboratory steps and analysis times for the current traditional method are compared to the LMD method for a sexual assault evidence sample.

Table 1. LMD Parameters for Sperm and Epithelial Cells using 40x Objective

Parameter	Sperm cells with low epithelial cell density	Epithelial cells or Sperm cells with high epithelial cell density
Aperture	2/16	2/16
Intensity	39/46	44/46
Speed	6/20	3/20
Bridge (Gap)	Medium	Medium
Aperture diff	6	6
Off set	40	40

Denominators denote maximum setting allowed. Numerators denote setting used for collection

Table 2. Microscopic Identification Scores of Cells for Each Histology Stain.

		Histological Stain					
	Sample	UNSTN	H&E	CTS	MG	WRT	AO
Spermatozoa	1	+ / -	+	+	- -	-	+
	2	+ / -	+	+ +	-	- -	+
	3	+ / -	+	+ +	- -	-	+
Buccal Cells	1	+ / -	+	+ +	-	- -	+ / -
	2	-	+	+ +	-	- -	-
	3	+ / -	+	+ +	- -	- -	+

UNSTN = not stained.
H&E = hematoxylin/eosin.
CTS = nuclear fast red/picroindigocarmine.
MG = methyl green.
WRT = Wright's stain.
AO = acridine orange.
- - : cannot ID or highly challenging
- : poor
+ / - : satisfactory
+ : good
+ + : excellent

Table 3. LMD Collection Times for "300 sperm" Populations.

Stain	Semen Donor	Time (minutes)	Mean ± SEM (minutes)
UNSTN			
	Donor 1	30	
	Donor 2	57	
	Donor 3	53	
			46.7±8.4
CTS			
	Donor 1	37	
	Donor 2	49	
	Donor 3	42	
			42.7±3.5
H&E			
	Donor 1	37	
	Donor 2	33	
	Donor 3	45	
			38.3±3.5
AO			
	Donor 1	36	
	Donor 2	61	
	Donor 3	37	
			44.7±8.2

Unstained = UNSTN; Christmas Tree = CTS; Hematoxylin/Eosin = H&E; Acridine Orange = AO

Table 4. Profiler Plus Genotypes of Donors Used in Cell Mixture from Fig. 8 & Fig. 9.

Donor	Amel.	D3S1358	Vwa	FGA	D8S1179	D21S11	D18S51	D5S818	D13S317	D7S820
Male Semen	XY	16	16, 18	19, 21	11, 13	30	14, 15	12, 13	9, 11	8, 9
Female Buccal Cells	X	15, 17	14, 20	22, 25	10, 14	29	15, 18	12	11	9, 10

Table 5. Estimated Cell Concentrations of Donor Specimens

Female Buccal Swab	# Epithelial cells / half swab	Male Semen	# Sperm cells / 1µl semen
Donor A	36,000	Donor F	48,950
Donor B	49,000	Donor G	90,750
Donor C	85,250	Donor H	138,900
Donor D	90,250	Donor I	31,350
Donor E	14,500		

APPENDIX B
HISTOLOGY COMPARISON DATA

HISTOLOGY COMPARISON: Cell Identification and Collection Times

	ID Grade	Time (minutes)
UNSTN Samples		
S-67	3	30
S-2	3	57
S-94	3	53
O-06	2	50
O-04	3	48
O-30	3	42
CTS Samples		
S-67	5	37
S-2	4	49
S-94	5	42
O-06	5	28
O-04	5	20
O-30	5	25
H&E Samples		
S-67	4	37
S-2	4	33
S-94	4	45
O-06	4	45
O-04	4	30
O-30	4	47
WRT Samples		
S-67	1	47
S-2	2	64
S-94	2	58
O-06	1	56
O-04	1	29
O-30	1	fail
AO Samples		
S-67	4	36
S-2	4	61
S-94	4	37
O-06	2	40
O-04	3	fail
O-30	4	51
MG Samples		
S-67	2	48
S-2	1	fail
S-94	1	fail
O-06	2	50
O-04	2	36
O-30	1	fail

RFU Values for Unstained, Hematoxylin/Eosin, and Christmas Tree Stained Sperm and Epithelial Cells

UNSTN	o-4	o-6	o-30	s-2	s-67	s-94
amel	553	627	758	593	371	173
D3	557	485	707	539	358	152
vwa	415	283	573	655	354	199
fgf	245	120	438	278	239	0
D8	456	891	781	590	354	159
D21	324	219	487	339	263	0
D18	139	137	291	244	187	0
D5	362	533	505	530	306	179
D13	297	163	334	316	238	0
D7	0	0	154	85	94	0

H&E	o-4	o-6	o-30	s-2	s-67	s-94
amel	371	495	416	233	329	358
D3	218	456	261	0	263	150
vwa	136	385	245	62	415	305
fgf	0	185	118	0	0	0
D8	292	459	311	148	499	575
D21	146	249	146	0	79	59
D18	52	61	0	0	0	0
D5	145	315	251	51	279	218
D13	0	202	109	0	0	0
D7	0	0	0	0	0	0

CTS	o-4	o-6	o-30	s-2	s-67	s-94
amel	88	270	173	204	295	546
D3	0	186	139	131	239	359
vwa	0	275	155	54	216	507
fgf	0	68	83	0	0	192
D8	0	299	205	0	347	855
D21	0	109	74	0	115	240
D18	0	0	0	0	0	158
D5	0	182	144	0	243	535
D13	0	0	0	0	0	146
D7	0	0	0	0	0	0

APPENDIX C
DNA ISOLATION COMPARISON DATA

DNA Isolation Comparison: STR Data

		Alleles		RFU			
Sample Info	**Category**	**Peak 1**	**Peak 2**	**Peak 1**	**Peak 2**	**Peak Ht. Ratio**	**Total RFU**
LNG OS_07_150	AMEL	X		403			403
LNG OS_07_150	D13S317	11		69			69
LNG OS_07_150	D18S51						
LNG OS_07_150	D21S11	29		200			200
LNG OS_07_150	D3S1358	15	17	76	92	82.6%	168
LNG OS_07_150	D5S818	12		208			208
LNG OS_07_150	D7S820						
LNG OS_07_150	D8S1179	10	14	105	111	82.6%	216
LNG OS_07_150	FGA	22	25	77	92	82.6%	169
LNG OS_07_150	vWA	14	20	163	89	54.6%	252
LNG OS_07_150							
M OS_07_150	AMEL	X		294			294
M OS_07_150	D13S317						
M OS_07_150	D18S51						
M OS_07_150	D21S11	29		50			50
M OS_07_150	D3S1358						
M OS_07_150	D5S818	12		110			110
M OS_07_150	D7S820						
M OS_07_150	D8S1179	10	14	68	56	82.4%	124
M OS_07_150	FGA						
M OS_07_150	vWA	14	20	95	64	67.4%	159
M OS_07_150							
Q OS_07_150	AMEL	X		927			927
Q OS_07_150	D13S317	11		52			52
Q OS_07_150	D18S51						
Q OS_07_150	D21S11	29		253			253
Q OS_07_150	D3S1358	15	17	162	150	92.6%	312
Q OS_07_150	D5S818	12		237			237
Q OS_07_150	D7S820						
Q OS_07_150	D8S1179	10	14	629	467	74.2%	1096
Q OS_07_150	FGA						
Q OS_07_150	vWA	14	20	499	287	57.5%	786
Q OS_07_150							

		Alleles		RFU			
Sample Info	**Category**	**Peak 1**	**Peak 2**	**Peak 1**	**Peak 2**	**Peak Ht. Ratio**	**Total RFU**
LNG OS_08_150	AMEL	X		258			258
LNG OS_08_150	D13S317						
LNG OS_08_150	D18S51						
LNG OS_08_150	D21S11	30.2		50			50
LNG OS_08_150	D3S1358	15	17	84	72	85.7%	156
LNG OS_08_150	D5S818						
LNG OS_08_150	D7S820						
LNG OS_08_150	D8S1179	12		69			69
LNG OS_08_150	FGA						
LNG OS_08_150	vWA	17		80			80
LNG OS_08_150							
M OS_08_150	AMEL	X		173			173
M OS_08_150	D13S317						
M OS_08_150	D18S51						
M OS_08_150	D21S11						
M OS_08_150	D3S1358						
M OS_08_150	D5S818						
M OS_08_150	D7S820						
M OS_08_150	D8S1179						
M OS_08_150	FGA						
M OS_08_150	vWA						
M OS_08_150							
Q OS_08_150	AMEL	X		946			946
Q OS_08_150	D13S317	9	12	149	118	79.2%	267
Q OS_08_150	D18S51	14	16	169	147	87.0%	316
Q OS_08_150	D21S11	30.2	32.2	206	172	83.5%	378
Q OS_08_150	D3S1358	15	17	350	293	83.7%	643
Q OS_08_150	D5S818	11	12	360	267	74.2%	627
Q OS_08_150	D7S820						
Q OS_08_150	D8S1179	12	16	512	531	96.4%	1043
Q OS_08_150	FGA	24	25	179	188	95.2%	367
Q OS_08_150	vWA	17	18	505	406	80.4%	911
Q OS_08_150							

		Alleles		RFU			
Sample Info	**Category**	**Peak 1**	**Peak 2**	**Peak 1**	**Peak 2**	**Peak Ht. Ratio**	**Total RFU**
LNG OS_09_150	AMEL	X		337			337
LNG OS_09_150	D13S317						
LNG OS_09_150	D18S51						
LNG OS_09_150	D21S11	30		80			80
LNG OS_09_150	D3S1358	14	18	141	146	96.6%	287
LNG OS_09_150	D5S818	12		128			128
LNG OS_09_150	D7S820						
LNG OS_09_150	D8S1179	11	15	118	99	83.9%	217
LNG OS_09_150	FGA	22		84			84
LNG OS_09_150	vWA	17	18	133	93	69.9%	226
LNG OS_09_150							
M OS_09_150	AMEL	X		295			295
M OS_09_150	D13S317						
M OS_09_150	D18S51						
M OS_09_150	D21S11						
M OS_09_150	D3S1358						
M OS_09_150	D5S818	12		110			110
M OS_09_150	D7S820						
M OS_09_150	D8S1179	11	15	54	79	68.4%	133
M OS_09_150	FGA						
M OS_09_150	vWA	17	18	91	70	76.9%	161
M OS_09_150							
Q OS_09_150	AMEL	X		718			718
Q OS_09_150	D13S317	11	12	175	180	97.2%	355
Q OS_09_150	D18S51	12	13	171	135	78.9%	306
Q OS_09_150	D21S11	30	33.2	184	187	98.4%	371
Q OS_09_150	D3S1358	14	18	309	282	91.3%	591
Q OS_09_150	D5S818	12		704			704
Q OS_09_150	D7S820	8	9	119	95	79.8%	214
Q OS_09_150	D8S1179	11	15	415	272	65.5%	687
Q OS_09_150	FGA	22	23	233	241	96.7%	474
Q OS_09_150	vWA	17	18	363	307	84.6%	670
Q OS_09_150							

		Alleles		RFU			
Sample Info	**Category**	**Peak 1**	**Peak 2**	**Peak 1**	**Peak 2**	**Peak Ht. Ratio**	**Total RFU**
LNG OS_10_150	AMEL	X		400			400
LNG OS_10_150	D13S317	12	13	54	58	93.1%	112
LNG OS_10_150	D18S51						
LNG OS_10_150	D21S11	32.2		152			152
LNG OS_10_150	D3S1358	17	OL	70	84	83.3%	154
LNG OS_10_150	D5S818	11		85			85
LNG OS_10_150	D7S820						
LNG OS_10_150	D8S1179	13		182			182
LNG OS_10_150	FGA	20	23	122	70	57.4%	192
LNG OS_10_150	vWA	16	17	96	82	85.4%	178
LNG OS_10_150							
M OS_10_150	AMEL	X		338			338
M OS_10_150	D13S317						
M OS_10_150	D18S51						
M OS_10_150	D21S11						
M OS_10_150	D3S1358						
M OS_10_150	D5S818	12		50			50
M OS_10_150	D7S820						
M OS_10_150	D8S1179	13		231			231
M OS_10_150	FGA						
M OS_10_150	vWA	16	17	124	138	89.9%	262
M OS_10_150							
Q OS_10_150	AMEL	X		1418			1418
Q OS_10_150	D13S317	12	13	290	283	97.6%	573
Q OS_10_150	D18S51	14	OL	223	151	67.7%	374
Q OS_10_150	D21S11	32.2		696			696
Q OS_10_150	D3S1358	17	OL	530	430	81.1%	960
Q OS_10_150	D5S818	11	12	453	470	96.4%	923
Q OS_10_150	D7S820	8	12	72	76	94.7%	148
Q OS_10_150	D8S1179	13		1292			1292
Q OS_10_150	FGA	20	23	455	340	74.7%	795
Q OS_10_150	vWA	16	17	695	720	96.5%	1415
Q OS_10_150							

		Alleles		RFU			
Sample Info	**Category**	**Peak 1**	**Peak 2**	**Peak 1**	**Peak 2**	**Peak Ht. Ratio**	**Total RFU**
LNG_OS_30_150	AMEL	X		316			316
LNG_OS_30_150	D13S317						
LNG_OS_30_150	D18S51	13		50			50
LNG_OS_30_150	D21S11	29	30	83	57	68.7%	140
LNG_OS_30_150	D3S1358	15	18	110	67	60.9%	177
LNG_OS_30_150	D5S818	11	12	70	84	83.3%	154
LNG_OS_30_150	D7S820	9		57			57
LNG_OS_30_150	D8S1179	10	12	56	66	84.8%	122
LNG_OS_30_150	FGA	22		143			143
LNG_OS_30_150	vWA	18		174			174
LNG_OS_30_150							
M_OS_30_150	AMEL	X		1756			1756
M_OS_30_150	D13S317	11	12	365	308	84.4%	673
M_OS_30_150	D18S51	13	17	230	186	80.9%	416
M_OS_30_150	D21S11	29	30	534	412	77.2%	946
M_OS_30_150	D3S1358	15	18	597	594	99.5%	1191
M_OS_30_150	D5S818	11	12	579	586	98.8%	1165
M_OS_30_150	D7S820	9		383			383
M_OS_30_150	D8S1179	10	12	618	586	68.70%	1204
M_OS_30_150	FGA	22		918			918
M_OS_30_150	vWA	18		1234			1234
M_OS_30_150							
Q_OS_30_150	AMEL	X		454			454
Q_OS_30_150	D13S317	11		51			51
Q_OS_30_150	D18S51	13	17	92	78	84.8%	170
Q_OS_30_150	D21S11	29	30	82	86	95.3%	168
Q_OS_30_150	D3S1358	15	18	131	113	86.3%	244
Q_OS_30_150	D5S818	11	12	114	117	97.4%	231
Q_OS_30_150	D7S820						
Q_OS_30_150	D8S1179	10	12	416	359	86.3%	775
Q_OS_30_150	FGA	22		105			105
Q_OS_30_150	vWA	18		357			357

		Alleles		RFU			
Sample Info	**Category**	**Peak 1**	**Peak 2**	**Peak 1**	**Peak 2**	**Peak Ht. Ratio**	**Total RFU**
LNG_S_1_300	AMEL	X	Y	772	758	98.2%	1530
LNG_S_1_300	D13S317	10	11	281	258	91.8%	539
LNG_S_1_300	D18S51	13	14	305	297	97.4%	602
LNG_S_1_300	D21S11	30	30.2	322	375	85.9%	697
LNG_S_1_300	D3S1358	15	16	733	590	80.5%	1323
LNG_S_1_300	D5S818	11	12	309	313	98.7%	622
LNG_S_1_300	D7S820	10		259		0.0%	259
LNG_S_1_300	D8S1179	11	14	418	599	69.8%	1017
LNG_S_1_300	FGA	20	24	408	410	99.5%	818
LNG_S_1_300	vWA	14	17	506	551	91.8%	1057
LNG_S_1_300							
M_S_1_300	AMEL	X	Y	631	614	97.3%	1245
M_S_1_300	D13S317						
M_S_1_300	D18S51						
M_S_1_300	D21S11						
M_S_1_300	D3S1358	15	16	82	62	75.6%	144
M_S_1_300	D5S818	11	12	125	100	80.0%	225
M_S_1_300	D7S820						
M_S_1_300	D8S1179	11	14	337	314	93.2%	651
M_S_1_300	FGA						
M_S_1_300	vWA	14	17	286	334	85.6%	620
M_S_1_300							
Q_S_1_300	AMEL	X	Y	210	171	81.4%	381
Q_S_1_300	D13S317	10	11	77	59	76.6%	136
Q_S_1_300	D18S51	13	14	92	78	84.8%	170
Q_S_1_300	D21S11	30	30.2	57	62	91.9%	119
Q_S_1_300	D3S1358	15	16	136	105	77.2%	241
Q_S_1_300	D5S818	11	12	144	108	75.0%	252
Q_S_1_300	D7S820	10		55			55
Q_S_1_300	D8S1179	11	14	209	132	63.2%	341
Q_S_1_300	FGA	20	24	63	92	68.5%	155
Q_S_1_300	vWA	14	17	182	189	96.3%	371
Q_S_1_300							

		Alleles		RFU			
Sample Info	**Category**	**Peak 1**	**Peak 2**	**Peak 1**	**Peak 2**	**Peak Ht. Ratio**	**Total RFU**
LNG S_2_300	AMEL	X	Y	372	464	80.2%	836
LNG S_2_300	D13S317	10	11	143	184	77.7%	327
LNG S_2_300	D18S51	13	14	147	104	70.7%	251
LNG S_2_300	D21S11	30	30.2	154	207	74.4%	361
LNG S_2_300	D3S1358	15	16	316	361	87.5%	677
LNG S_2_300	D5S818	11	12	207	230	90.0%	437
LNG S_2_300	D7S820	10		68		0.0%	68
LNG S_2_300	D8S1179	11	14	261	269	97.0%	530
LNG S_2_300	FGA	20	24	323	175	54.2%	498
LNG S_2_300	vWA	14	17	338	326	96.4%	664
LNG S_2_300							
M S_2_300	AMEL	X	Y	842	648	77.0%	1490
M S_2_300	D13S317						
M S_2_300	D18S51						
M S_2_300	D21S11						
M S_2_300	D3S1358	15	16	89	95	93.7%	184
M S_2_300	D5S818	11	12	242	131	54.1%	373
M S_2_300	D7S820						
M S_2_300	D8S1179	11	14	465	332	71.4%	797
M S_2_300	FGA						
M S_2_300	vWA	14	17	451	280	62.1%	731
M S_2_300							
Q S_2_300	AMEL	X	Y	684	739	92.6%	1423
Q S_2_300	D13S317	10	11	448	439	98.0%	887
Q S_2_300	D18S51	13	14	361	368	98.1%	729
Q S_2_300	D21S11	30	30.2	475	438	92.2%	913
Q S_2_300	D3S1358	15	16	684	545	79.7%	1229
Q S_2_300	D5S818	11	12	522	518	99.2%	1040
Q S_2_300	D7S820	10		498			498
Q S_2_300	D8S1179	11	14	524	456	87.0%	980
Q S_2_300	FGA	20	24	540	514	95.2%	1054
Q S_2_300	vWA	14	17	638	507	79.5%	1145
Q S_2_300							

		Alleles		RFU			
Sample Info	**Category**	**Peak 1**	**Peak 2**	**Peak 1**	**Peak 2**	**Peak Ht. Ratio**	**Total RFU**
LNG S_60_300	AMEL	X	Y	737	840	87.7%	1577
LNG S_60_300	D13S317	12		504			504
LNG S_60_300	D18S51	15	16	296	291	98.3%	587
LNG S_60_300	D21S11	28	31	424	386	91.0%	810
LNG S_60_300	D3S1358	15	17	642	512	79.8%	1154
LNG S_60_300	D5S818	11	12	420	359	85.5%	779
LNG S_60_300	D7S820	10	11	187	107	57.2%	294
LNG S_60_300	D8S1179	12	13	501	523	95.8%	1024
LNG S_60_300	FGA	21	24	459	470	97.7%	929
LNG S_60_300	vWA	16	18	486	538	90.3%	1024
LNG S_60_300							
M S_60_300	AMEL	X	Y	253	318	125.7%	571
M S_60_300	D13S317						
M S_60_300	D18S51						
M S_60_300	D21S11						
M S_60_300	D3S1358						
M S_60_300	D5S818						
M S_60_300	D7S820						
M S_60_300	D8S1179	12	13	144	128	88.9%	272
M S_60_300	FGA						
M S_60_300	vWA	16	18	119	101	84.9%	220
M S_60_300							
Q S_60_300	AMEL	X	Y	445	520	85.6%	965
Q S_60_300	D13S317	12		665			665
Q S_60_300	D18S51	15	16	383	272	71.0%	655
Q S_60_300	D21S11	28	31	377	435	86.7%	812
Q S_60_300	D3S1358	15	17	409	556	73.6%	965
Q S_60_300	D5S818	11	12	480	398	82.9%	878
Q S_60_300	D7S820	10	11	254	196	77.2%	450
Q S_60_300	D8S1179	12	13	524	588	89.1%	1112
Q S_60_300	FGA	21	24	452	444	98.2%	896
Q S_60_300	vWA	16	18	388	521	74.5%	909
Q S_60_300							

		Alleles		**RFU**			
Sample Info	**Category**	**Peak 1**	**Peak 2**	**Peak 1**	**Peak 2**	**Peak Ht. Ratio**	**Total RFU**
LNG S_67_300	AMEL	X	Y	885	734	82.9%	1619
LNG S_67_300	D13S317	9	10	326	279	85.6%	605
LNG S_67_300	D18S51	12	15	353	346	98.0%	699
LNG S_67_300	D21S11	30		944			944
LNG S_67_300	D3S1358	15	16	794	707	89.0%	1501
LNG S_67_300	D5S818	10	11	442	383	86.7%	825
LNG S_67_300	D7S820	10		261			261
LNG S_67_300	D8S1179	13		1140			1140
LNG S_67_300	FGA	21	22	583	623	93.6%	1206
LNG S_67_300	vWA	17	18	725	547	75.4%	1272
LNG S_67_300							
M S_67_300	AMEL	X	Y	425	561	75.8%	986
M S_67_300	D13S317						
M S_67_300	D18S51						
M S_67_300	D21S11						
M S_67_300	D3S1358						
M S_67_300	D5S818						
M S_67_300	D7S820						
M S_67_300	D8S1179	13		440			440
M S_67_300	FGA						
M S_67_300	vWA	17	18	106	149	71.1%	255
M S_67_300							
Q S_67_300	AMEL	X	Y	496	520	95.4%	1016
Q S_67_300	D13S317	9	10	195	221	88.2%	416
Q S_67_300	D18S51	12	15	263	195	74.1%	458
Q S_67_300	D21S11	30		455			455
Q S_67_300	D3S1358	15	16	386	349	90.4%	735
Q S_67_300	D5S818	10	11	320	352	90.9%	672
Q S_67_300	D7S820	10		129			129
Q S_67_300	D8S1179	13		915			915
Q S_67_300	FGA	21	22	265	294	90.1%	559
Q S_67_300	vWA	17	18	463	379	81.9%	842
Q S_67_300							

		Alleles		RFU			
Sample Info	**Category**	**Peak 1**	**Peak 2**	**Peak 1**	**Peak 2**	**Peak Ht. Ratio**	**Total RFU**
LNG S_94_300	AMEL	X	Y	700	667	95.3%	1367
LNG S_94_300	D13S317	8	12	250	187	74.8%	437
LNG S_94_300	D18S51	15	16	232	223	96.1%	455
LNG S_94_300	D21S11	28	30	420	385	91.7%	805
LNG S_94_300	D3S1358	15	18	399	528	75.6%	927
LNG S_94_300	D5S818	10	12	361	269	74.5%	630
LNG S_94_300	D7S820	8	11	114	135	84.4%	249
LNG S_94_300	D8S1179	11	12	590	353	59.8%	943
LNG S_94_300	FGA	22	25	438	325	74.2%	763
LNG S_94_300	vWA	17	18	540	519	96.1%	1059
LNG S_94_300							
M S_94_300	AMEL	X	Y	232	222	95.7%	454
M S_94_300	D13S317						
M S_94_300	D18S51						
M S_94_300	D21S11						
M S_94_300	D3S1358						
M S_94_300	D5S818						
M S_94_300	D7S820						
M S_94_300	D8S1179	11	12	188	167	88.8%	355
M S_94_300	FGA						
M S_94_300	vWA	17	18	113	93	82.3%	206
M S_94_300							
Q S_94_300	AMEL	X	Y	339	399	85.0%	738
Q S_94_300	D13S317	8	12	185	128	69.2%	313
Q S_94_300	D18S51	15	16	147	142	96.6%	289
Q S_94_300	D21S11	28	30	206	210	98.1%	416
Q S_94_300	D3S1358	15	18	307	287	93.5%	594
Q S_94_300	D5S818	10	12	262	255	97.3%	517
Q S_94_300	D7S820	8	11	85	62	72.9%	147
Q S_94_300	D8S1179	11	12	441	383	86.8%	824
Q S_94_300	FGA	22	25	189	203	93.1%	392
Q S_94_300	vWA	17	18	348	355	98.0%	703

Naming nomenclature = Isolation Method _Cell Type_Donor_# Cells
LNG = Lyse-N-Go
M = microLYSIS
Q = QIAamp
S= Semen
OS =Buccal Cells

APPENDIX D
CELL SEPARATION STUDY DATA

CELL SEPARATION: STR DATA USING STANDARD ANALYSIS

S107		Alleles		RFU				
# sperm Cells	Category	Peak 1	Peak 2	Peak 1	Peak 2	Peak Ht. Ratio		Total RFU
75	AMEL	X	Y	167	146	87.4%		313
150	AMEL	X	Y	281	286	98.3%		567
300	AMEL	X	Y	494	480	97.2%		974
75	D13S317	9		59				59
150	D13S317	9	11	75	63	84.0%		138
300	D13S317	9	11	120	132	90.9%		252
75	D18S51	14	15	77	64	83.1%		141
150	D18S51	14	15	72	77	93.5%		149
300	D18S51	14	15	178	152	85.4%		330
75	D21S11	30		143				143
150	D21S11	30		169				169
300	D21S11	30		361				361
75	D3S1358	16		209				209
150	D3S1358	16		353				353
300	D3S1358	15	16	85	760	11.2%		845
75	D5S818	12	13	88	62	70.5%		150
150	D5S818	12	13	102	119	85.7%		221
300	D5S818	12	13	232	169	72.8%		401
75	D7S820	9		55				55
150	D7S820	9		53				53
300	D7S820	8	9	59	75	78.7%		134
75	D8S1179	11	13	162	67	41.4%		229
150	D8S1179	11	13	176	116	65.9%		292
300	D8S1179	11	13	355	280	78.9%		635
75	FGA	19	21	122	79	64.8%		201
150	FGA	19	21	138	91	65.9%		229
300	FGA	19	21	344	169	49.1%		513
75	vWA	16	18	108	102	94.4%		210
150	vWA	16	18	152	173	87.9%		325
300	vWA	16	18	353	297	84.1%		650

S208								
# sperm Cells	Category	Peak 1	Peak 2	Peak 1	Peak 2	Peak Ht. Ratios		
75	AMEL	X	Y	157	217	72.4%		374
150	AMEL	X	Y	263	352	74.7%		615
300	AMEL	X	Y	338	317	93.8%		655
75	D13S317	10		61				61
150	D13S317	10	11	83	85	97.6%		168
300	D13S317	10	11	127	130	97.7%		257
75	D18S51	13	14	138	137	99.3%		275
150	D18S51	13	14	137	109	79.6%		246
300	D18S51	13	14	122	171	71.3%		293
75	D21S11	30	30.2	99	95	96.0%		194
150	D21S11	30	30.2	108	134	80.6%		242
300	D21S11	30	30.2	126	160	78.8%		286
75	D3S1358	15	16	137	130	94.9%		267
150	D3S1358	15	16	330	254	77.0%		584
300	D3S1358	15	16	296	298	99.3%		594
75	D5S818	11	12	86	81	94.2%		167
150	D5S818	11	12	165	138	83.6%		303
300	D5S818	11	12	172	166	96.5%		338
75	D7S820	10		83				83
150	D7S820	10		124				124
300	D7S820	10		153				153
75	D8S1179	11	14	112	125	89.6%		237
150	D8S1179	11	14	233	209	89.7%		442
300	D8S1179	11	14	236	213	90.3%		449
75	FGA	20	24	138	126	91.3%		264
150	FGA	20	24	172	187	92.0%		359
300	FGA	20	24	188	148	78.7%		336
75	vWA	14	17	182	114	62.6%		296
150	vWA	14	17	243	278	87.4%		521
300	vWA	14	17	254	233	91.7%		487

S6009								
# sperm Cells	Category	Peak 1	Peak 2	Peak 1	Peak 2	Peak Ht. Ratios		
75	AMEL	X	Y	173	100	57.8%		273
150	AMEL	X	Y	479	395	82.5%		874
300	AMEL	X	Y	568	496	87.3%		1064
75	D13S317	12		57				57
150	D13S317	12		247				247
300	D13S317	12		312				312
75	D18S51							
150	D18S51	15	16	135	164	82.3%		299
300	D18S51	15	16	194	142	73.2%		336
75	D21S11	28		67				67
150	D21S11	28	31	181	160	88.4%		341
300	D21S11	28	31	238	156	65.5%		394
75	D3S1358	15	17	86	133	64.7%		219
150	D3S1358	15	17	317	254	80.1%		571
300	D3S1358	15	17	252	325	77.5%		577
75	D5S818	11		62				62
150	D5S818	11	12	196	248	79.0%		444
300	D5S818	11	12	248	199	80.2%		447
75	D7S820							
150	D7S820	10	11	91	80	87.9%		171
300	D7S820	10	11	56	60	93.3%		116
75	D8S1179	12	13	78	92	84.8%		170
150	D8S1179	12	13	349	221	63.3%		570
300	D8S1179	12	13	322	315	97.8%		637
75	FGA	21	24	67	71	94.4%		138
150	FGA	21	24	237	156	65.8%		393
300	FGA	21	24	269	247	91.8%		516
75	vWA	16	18	113	62	54.9%		175
150	vWA	16	18	306	245	80.1%		551
300	vWA	16	18	317	373	85.0%		690

S6710								
# sperm Cells	Category	Peak 1	Peak 2	Peak 1	Peak 2	Peak Ht. Ratios		
75	AMEL	X	Y	73	58	79.5%		131
150	AMEL	X	Y	154	323	47.7%		477
300	AMEL	X	Y	166	295	56.3%		461
75	D13S317							
150	D13S317	9	10	74	61	82.4%		135
300	D13S317	9	10	83	74	89.2%		157
75	D18S51							
150	D18S51	12	15	120	98	81.7%		218
300	D18S51	12	15	108	66	61.1%		174
75	D21S11	30		60				60
150	D21S11	30		231				231
300	D21S11	30		227				227
75	D3S1358	16		65				65
150	D3S1358	15	16	161	192	83.9%		353
300	D3S1358	15	16	183	91	49.7%		274
75	D5S818							
150	D5S818	10	11	112	130	86.2%		242
300	D5S818	10	11	98	132	74.2%		230
75	D7S820							
150	D7S820	10		101				101
300	D7S820	10		59				59
75	D8S1179	13		100				100
150	D8S1179	13		327				327
300	D8S1179	13		334				334
75	FGA							
150	FGA	21	22	145	164	88.4%		309
300	FGA	21	22	140	103	73.6%		243
75	vWA							
150	vWA	17	18	149	178	83.7%		327
300	vWA	17	18	154	138	89.6%		292

S9411								
# sperm Cells	Category	Peak 1	Peak 2	Peak 1	Peak 2	Peak Ht. Ratios		
75	AMEL	X	Y	161	130	80.7%		291
150	AMEL	X	Y	148	145	98.0%		293
300	AMEL	X	Y	339	407	83.3%		746
75	D13S317	8	12	54	51	94.4%		105
150	D13S317	8	12	58	70	82.9%		128
300	D13S317	8	12	131	92	70.2%		223
75	D18S51	15		75				75
150	D18S51	15		54				54
300	D18S51	15	16	126	145	86.9%		271
75	D21S11	28	30	50	79	63.3%		129
150	D21S11	28	30	92	73	79.3%		165
300	D21S11	28	30	200	209	95.7%		409
75	D3S1358	15	18	148	86	58.1%		234
150	D3S1358	15	18	103	91	88.3%		194
300	D3S1358	15	18	183	219	83.6%		402
75	D5S818	10		100				100
150	D5S818	10	12	109	63	57.8%		172
300	D5S818	10	12	173	170	98.3%		343
75	D7S820							
150	D7S820							
300	D7S820	8	11	84	57	67.9%		141
75	D8S1179	11	12	109	106	97.2%		215
150	D8S1179	11	12	122	104	85.2%		226
300	D8S1179	11	12	268	236	88.1%		504
75	FGA	22	25	132	51	38.6%		183
150	FGA	22	25	106	68	64.2%		174
300	FGA	22	25	187	189	98.9%		376
75	vWA	17	18	118	69	58.5%		187
150	vWA	17	18	91	74	81.3%		165
300	vWA	17	18	285	212	74.4%		497

Mixture sample nomenclature = Last two numbers designate epithelial cell donor; First two or three characters designate semen donor

CELL SEPARATION STUDY: STR DATA USING EXTENDED CYCLES ANALYSIS

S107		Alleles		RFU			
#sperm cells	Category	Peak 1	Peak 2	Peak 1	Peak 2	Peak Ht. Ratio	Total RFU
75	AMEL	X	Y	7574	7761	97.6%	15335
150	AMEL	X	Y	6785	7072	95.9%	13857
75	D13S317	9	11	2057	1453	70.6%	3510
150	D13S317	9	11	2324	1776	76.4%	4100
75	D18S51	14	15	1916	1331	69.5%	3247
150	D18S51	14	15	1472	1561	94.3%	3033
75	D21S11	30		5894			5894
150	D21S11	30		6005			6005
75	D3S1358	16		8466			8466
150	D3S1358	16		9413			9413
75	D5S818	12	13	3457	2172	62.8%	5629
150	D5S818	12	13	3466	3363	97.0%	6829
75	D7S820	8	9	472	1179	40.0%	1651
150	D7S820	8	9	794	832	95.4%	1626
75	D8S1179	11	13	7756	3396	43.8%	11152
150	D8S1179	11	13	7755	5000	64.5%	12755
75	FGA	19	21	4488	2763	61.6%	7251
150	FGA	19	21	4304	2780	64.6%	7084
75	vWA	16	18	5534	4586	82.9%	10120
150	vWA	16	18	6620	7133	92.8%	13753

S208							
#sperm cells	Category	Peak 1	Peak 2	Peak 1	Peak 2		
75	AMEL	X	Y	6410	7786	82.3%	14196
150	AMEL	X	Y	7452	7398	99.3%	14850
75	D13S317	10	11	1645	1155	70.2%	2800
150	D13S317	10	11	2334	2140	91.7%	4474
75	D18S51	13	14	1864	1811	97.2%	3675
150	D18S51	13	14	2412	1884	78.1%	4296
75	D21S11	30	30.2	2282	2319	98.4%	4601
150	D21S11	30	30.2	3457	4198	82.3%	7655
75	D3S1358	15	16	5225	4364	83.5%	9589
150	D3S1358	15	16	9067	7654	84.4%	16721
75	D5S818	11	12	3112	2637	84.7%	5749
150	D5S818	11	12	4781	3797	79.4%	8578
75	D7S820	10		1158			1158
150	D7S820	10		1887			1887
75	D8S1179	11	14	4017	4392	91.5%	8409
150	D8S1179	11	14	7501	7181	95.7%	14682
75	FGA	20	24	3097	2562	82.7%	5659
150	FGA	20	24	4390	3957	90.1%	8347
75	vWA	14	17	5365	2959	55.2%	8324
150	vWA	14	17	8901	8832	99.2%	17733

S6009							
#sperm cells	Category	Peak 1	Peak 2	Peak 1	Peak 2		
75	AMEL	X	Y	7778	6291	80.9%	14069
150	AMEL	X	Y	7274	7029	96.6%	14303
75	D13S317	12		2773			2773
150	D13S317	12		4078			4078
75	D18S51	15	16	1369	1663	82.3%	3032
150	D18S51	15	16	1837	1967	93.4%	3804
75	D21S11	28	31	3607	1734	48.1%	5341
150	D21S11	28	31	4770	3627	76.0%	8397
75	D3S1358	15	17	4934	7331	67.3%	12265
150	D3S1358	15	17	8805	7679	87.2%	16484
75	D5S818	11	12	3546	2835	79.9%	6381
150	D5S818	11	12	3691	4336	85.1%	8027
75	D7S820	10	11	934	306	32.8%	1240
150	D7S820	10	11	818	657	80.3%	1475
75	D8S1179	12	13	4542	5007	90.7%	9549
150	D8S1179	12	13	7501	6737	89.8%	14238
75	FGA	21	24	2946	3305	89.1%	6251
150	FGA	21	24	4955	2790	56.3%	7745
75	vWA	16	18	6403	3336	52.1%	9739
150	vWA	16	18	8826	7796	88.3%	16622

S6710							
#sperm cells	Category	Peak 1	Peak 2	Peak 1	Peak 2		
75	AMEL	X	Y	3845	3277	85.2%	7122
150	AMEL	X	Y	7712	7035	91.2%	14747
75	D13S317	9	10	1783	495	27.8%	2278
150	D13S317	9	10	2100	1594	75.9%	3694
75	D18S51	12	15	1820	501	27.5%	2321
150	D18S51	12	15	2080	1609	77.4%	3689
75	D21S11	30		2983			2983
150	D21S11	30		6660			6660
75	D3S1358	15	16	1850	3481	53.1%	5331
150	D3S1358	15	16	5766	6207	92.9%	11973
75	D5S818	10	11	778	860	90.5%	1638
150	D5S818	10	11	3403	3897	87.3%	7300
75	D7S820	10		957			957
150	D7S820	10		1387			1387
75	D8S1179	13		4802			4802
150	D8S1179	13		7226			7226
75	FGA	21	22	1531	1145	74.8%	2676
150	FGA	21	22	3424	3666	93.4%	7090
75	vWA	17	18	1801	1183	65.7%	2984
150	vWA	17	18	5492	6422	85.5%	11914

S9411							
#sperm cells	Category	Peak 1	Peak 2	Peak 1	Peak 2		
75	AMEL	X	Y	7621	6763	88.7%	14384
150	AMEL	X	Y	7517	7713	97.5%	15230
75	D13S317	8	12	2304	2041	88.6%	4345
150	D13S317	8	12	2513	3016	83.3%	5529
75	D18S51	15	16	1960	1299	66.3%	3259
150	D18S51	15	16	2096	1721	82.1%	3817
75	D21S11	28	30	2107	3560	59.2%	5667
150	D21S11	28	30	4713	3757	79.7%	8470
75	D3S1358	15	18	5867	3148	53.7%	9015
150	D3S1358	15	18	5345	5843	91.5%	11188
75	D5S818	10	12	4895	1993	40.7%	6888
150	D5S818	10	12	5131	3111	60.6%	8242
75	D7S820	8	11	1355	488	36.0%	1843
150	D7S820	8	11	1080	1040	96.3%	2120
75	D8S1179	11	12	4559	5032	90.6%	9591
150	D8S1179	11	12	5031	4925	97.9%	9956
75	FGA	22	25	4563	1615	35.4%	6178
150	FGA	22	25	5124	2941	57.4%	8065
75	vWA	17	18	5351	2592	48.4%	7943
150	vWA	17	18	5707	4481	78.5%	10188

Mixture Sample Nomenclature = Last two digits designate epithelial donor. First two to three characters designate semen donor

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