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In 1998, within the United States, it is estimated that a rape occurred every 2.3 minutes. In 1995, according to the Bureau of Justice Statistics, an estimated 350,000 rapes or sexual assaults (R/SA) were experienced by persons age 12 or older. Of the estimated 100,000 R/SA reported, there were only approximately 25,000 cases analyzed by crime labs nationwide. The majority of crime laboratories throughout the U.S., especially those in major metropolitan cities, have a significant backlog of unresolved R/SA cases. With the implementation of the Convicted Offender Database (CODIS), it is essential that all R/SA cases be analyzed, especially those lacking a known suspect. The comparison of the short tandem repeat (STR) profiles derived from sperm DNA recovered from evidentiary material with CODIS samples would provide the police with critical investigative leads resulting in the identification of the assailant.

The goal of this research is to develop a cellular sorting method for the isolation of sperm cells from sexual assault samples which will: 1) take advantage of differentiating features (extracellular antigenic sites) for complete separation of cell types, 2) provide a more efficient means of sperm recovery, increasing DNA yield from the male fraction, and 3) ensure the DNA isolation process is compatible with the amplification of the CODIS core STR loci. Overall, the proposed technique will increase the probability of success in the analysis of sexual assault case samples. (NIJGrant #: 2000-IJ-CX-K009).

OPTIMIZATION OF SPERMATOZOA CAPTURE DURING THE DIFFERENTIAL EXTRACTION PROCESS FOR STR TYPING WITH THE POTENTIAL FOR AUTOMATION

Pamela Marshall, B.S., M.S.

APPROVED:

Dr. Arthur Eisenberg, Major Professor

Dr. John Planz, Committee Member

Dr. Joe Warren, Committee Member

Dr. Michael Martin, University Member

Dr. Robert Wordinger, Division Head, Division of Cell Biology & Genetics

Dr. Thomas Yorio, Dean, Graduate School of Biomedical Sciences

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Pamela Marshall, B.S., M.S.

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INTRODUCTION

More than 100,000 sexual assaults are reported in the United States every year. The most powerful method of identifying a rapist is DNA testing. Sexual assault evidence is often composed of a mixture of both male and female components. These mixed stains are typically composed of a combination of cells (vaginal cells, buccal epithelial cells, white blood cells, red blood cells, and spermatozoa) and physiological fluids such as saliva, seminal plasma, and vaginal secretions [3]. The analysis of such evidence can yield vital information about the identity of the assailant(s). However, difficulties may arise in the identification of each cell contributor in these evidentiary samples. Currently, the only practical method for isolating DNA from mixtures of vaginal epithelial cells and spermatozoa is differential lysis [5]. This lysis method is based on differences in DNA packaging found in the sperm head versus epithelial cells or other cellular material [6,7]. DNA in sperm heads is packaged in tight disulfide linked cross-linked protamines [2]. Therefore, the differential extraction method relies on the ability to preferentially lyse one cell type over the other. Initially, epithelial cells are preferentially lysed in a buffer containing a protease and detergent. The sperm heads are resistant to lysis in the absence of a reducing agent. After a centrifugation step, the spermatozoa remain in a pellet along with cellular debris, while the vaginal epithelial DNA can be recovered in the supernatant. The sperm heads are repeatedly washed and then resuspended in a buffer containing dithiothreitol (DTT), a reducing agent. Although

effective, this method often does not yield complete separation of the male and female cellular material with crossover contamination being the result [9,10]. Incomplete or mixed DNA profiles can pose significant problems in the statistical analysis and the subsequent court trial of the assailant. The development and automation of a method capable of more completely separating spermatozoa from epithelial cells prior to DNA analysis would result in not only cleaner typing results but also higher throughput of evidence samples.

The purpose of this project was the development of sperm-specific antibody coated magnetic beads for the capture of spermatozoa from sexual assault/rape evidence. Three commercially available anti-sperm antibodies that have demonstrated specificity to human sperm were evaluated: MHS-10 (ATCC#HB-10039), NUH-2 (ATCC#HB-9762), and HS-21 (ATCC#HB-255) [1]. Two of the antibodies, MHS-10 and NUH-2, have received U.S. Patents [8,12]. Evaluation of the efficiency of this trio of antibodies was assessed through yield analyses using known concentrations of spermatozoa as well as through microscopy. Each antibody bead combination was evaluated independently, and in a mixture, to determine if any physical inhibitions or incompatibilities exist.

This research proposed to decorate magnetic beads with anti-human sperm antibodies and to attach chemical species to these antibodies, which formed covalent bonds between the antibody and the sperm after photoactivation. The binding of a sperm cell to this modified antibody should, after light activation, produce a permanent (i.e. covalent) antibody-sperm cell adduct. The magnetic capture of the beads allowed for the

complete separation of the sperm cells, and the DNA they carry, from all other cells and/or cellular debris present in the sample.

Physical capture of the spermatozoa was accomplished through the strong antibody-antigen reaction on the acrosome membranes of the sperm head as well as other morphological positions on the sperm cell surface. Published data regarding the antibodies under exploration suggests binding efficiencies between 80 and 95 percent to the spermatozoa, with the lack of cross-reactivity to other human cell types, namely vaginal epithelial cells [15,16].

The formation of a covalent bond between the spermatozoa and the antibody through photochemical activation should improve the retention of the spermatozoa to the magnetic beads. This enhanced binding would be advantageous during the rigorous wash cycles performed during the differential extraction procedure. Additionally, this would provide a mechanism to retain the cellular components of the spermatozoa following the final lysis of the sperm head, yielding an enriched male DNA fraction.

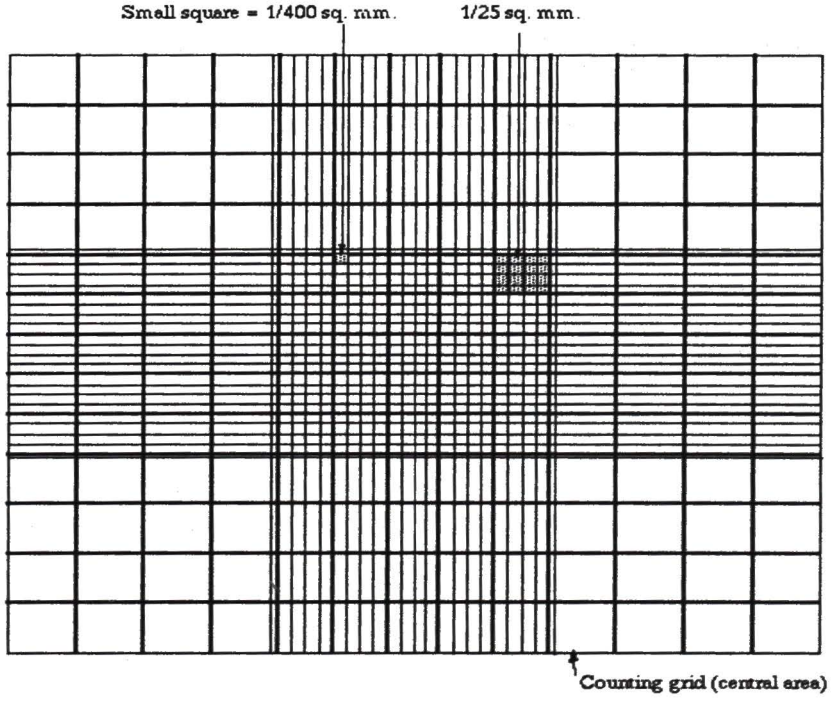
The primary objective of this research project was the development of sperm-specific antibody coated magnetic beads for the capture of spermatozoa from sexual assault evidence. This would provide a more efficient means of sperm recovery and increasing the DNA yield from the sperm fraction. The proposed method uses magnetic beads for both the cell separation and DNA purification steps. In addition, this method also sets the stage for the automation of the entire process, which could result in a more cost effective and more reliable process.

Overall, the proposed technique will increase the probability of success in the analysis of sexual assault case samples.

MATERIALS & METHODS

Cell Counting

A cover slip was positioned so that the glass covered both counting areas of the hemocytometer equally. A small portion of the sample was placed into the wells of the hemocytometer by letting it fill via capillary action (it will draw upwards automatically). The chamber was filled by gently setting the pipette tip on edge of chamber at etched cut. The area of counting on the hemocytometer is a 1-mm^2 ruled area. Five numbered squares, each corner and the center square, were counted. The number of cells counted in the five numbered squares multiplied by five is the number of cells in the total grid. This number was then multiplied by the dilution factor of the liquid and then by 10,000 (which incorporates the volume of fluid in the counting chamber) to give the number of cells/ml.



Christmas Tree Staining

Nuclear Fast Red Stain: 15g of Aluminum Sulfate (Fisher Catalog Number A613-500) was added to 600 ml of sterile water which had been heated to 100°C. 0.3g Nuclear Fast Red Stain (Sigma Chemical Company Catalog Number N-8002) was added immediately. The solution was stirred with a glass rod. The solution was then cooled to room temperature and filtered through 3mm Whatman paper.

Picro-Indigo Carmine Stain: 8.0g Picric Acid (Fisher Catalog Number A253-100) was added to 600 ml of sterile water in a 1 liter beaker. It was covered and left overnight to form a saturated solution. 2.0g Indigo Carmine stain (Fisher Catalog Number I166-25) was dissolved in the picric acid solution and filtered through 3mm Whatman paper.

Staining Slides for Presence of Spermatozoa For Smears on Microscope Slides

The slides were positioned on a slide staining rack and a sufficient amount of Nuclear Fast Red Stain was placed on each slide to cover the smear. The slides were then incubated for 15 min. Using a wash bottle with sterile water, the slides were then carefully washed to remove any stain solution. The smear portion of each slide was then covered with Picro-Indigo Carmine stain solution for 20 seconds. The remaining stain was washed off the slide using 95% ethanol in a wash bottle. The slides were then placed in a drying rack and allowed to air dry. Residual ethanol may be wiped from the slide margin using a KimWipe™.

Proposed Protocol for Spermatozoa Isolation using Antibody/Magnetic Beads (Figure 1)

1) Sperm and vaginal epithelial cells (VEC) were obtained and the concentration of the cell types was determined by counting aliquots using a hemocytometer. The theoretical yield of DNA per haploid cell is approximately 3.5 pg.
2) Mock sexual assault samples were prepared using a 4:1 dilution of VEC to sperm. Magnetic beads decorated with the antibodies were added to the solution of sperm and VEC so that the dilution was 4:1:4 (antibody beads: sperm: vaginal cells) and the solution incubated at 4°C for one hour.
3) The beads, with captured sperm cells attached, were separated from the mixture using a suitable magnet and resuspended in Phosphate Buffered Saline (PBS). The male bound fraction was then washed 2 times using 1ml PBS to remove any residual female epithelial cells.
4) Tubes containing the unbound female fraction and bound male fraction were labeled carefully. To the male fraction, 20µl DTT and 10µl Proteinase K were added. To the female fraction, 10µl Proteinase K was added. Tubes containing both fractions were incubated at 56°C for 2 hours. The tube containing the male fraction was then subjected to a magnet and the supernatant removed.
5) To each tube, an equal volume of buffered phenol-chloroform was then added and vortexed vigorously for 15 seconds. The tubes were then centrifuged at 13,000g for five minutes. The aqueous layer from each tube was then transferred to a new 1.5ml microcentrifuge tube. Genetic profiles for both cell types were then generated and analyzed.

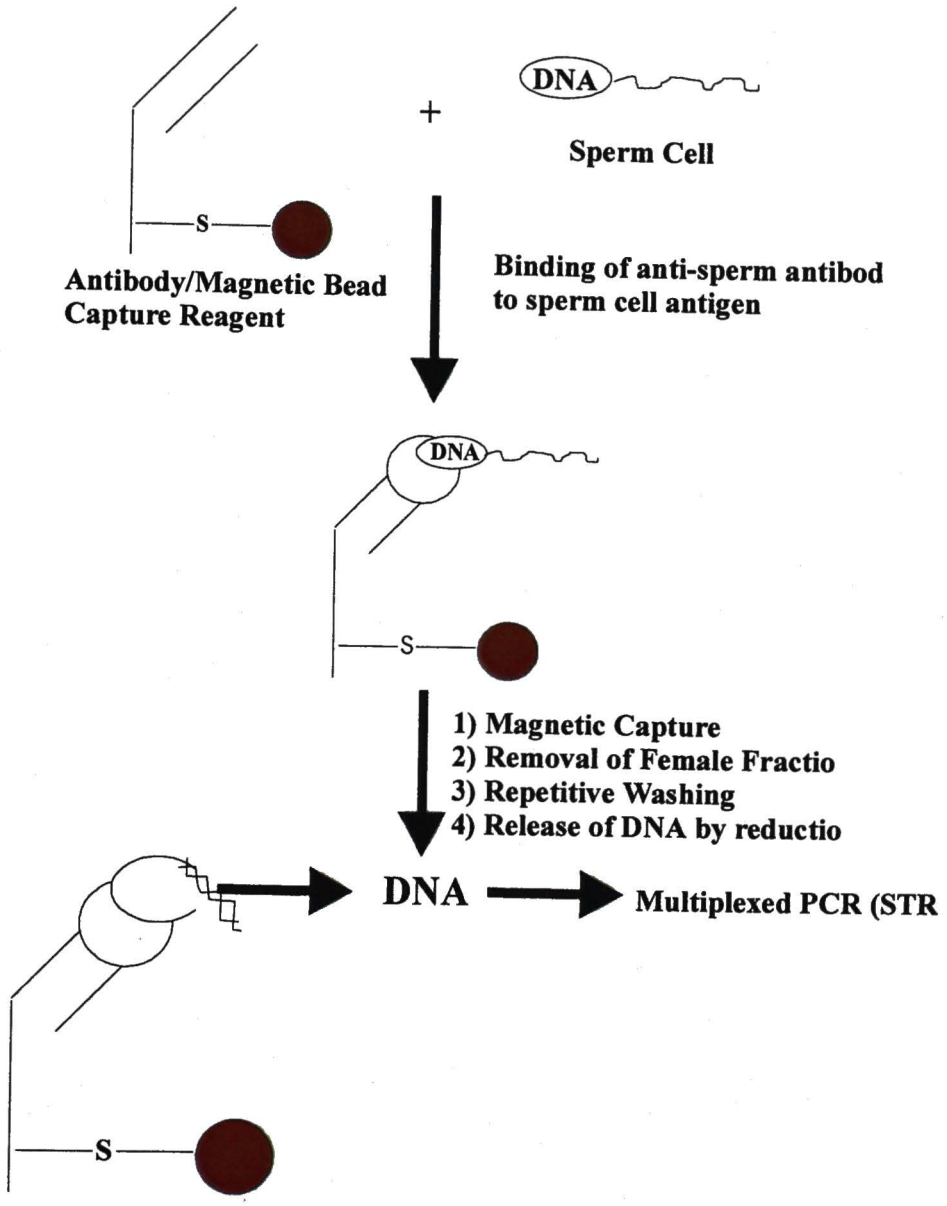


Figure 1. The use of the antibody/magnetic bead capture system to isolate human spermatozoal DNA.

Photoaffinity Labeling

Photoaffinity labeling is a popular technique for studying the binding interactions between biomolecules that accompany most biological events. The basic principles of the method are well understood; one of two biomolecules involved in a binding event is decorated with a chemical group, which will form covalent bonds with a second involved molecule during or after light activation (Figure 2). The important feature of the labeling method for this research is the permanent attachment of the two biomolecules. Through the photoaffinity labeling method, a decorated biomolecule, an antibody, was generated which became bound to a site(s) on spermatozoa and became permanently attached to the sperm during or after light activation. This permanent attachment step allowed the separation of spermatozoal cells from all other cellular debris. Specifically, the photoreactive group nitroarylazide (ANB-NOS) was examined.

Immunochemical Sperm Isolation

Monoclonal antibodies (MABs) that react with human spermatozoa have been generated. The antibodies used in this research were selected based upon the following characteristics: 1) no cross reactivity with cell types other than spermatozoa, 2) high binding efficiency to spermatozoa, and 3) specificity to the acrosome of the spermatozoa [4,11,13,15].

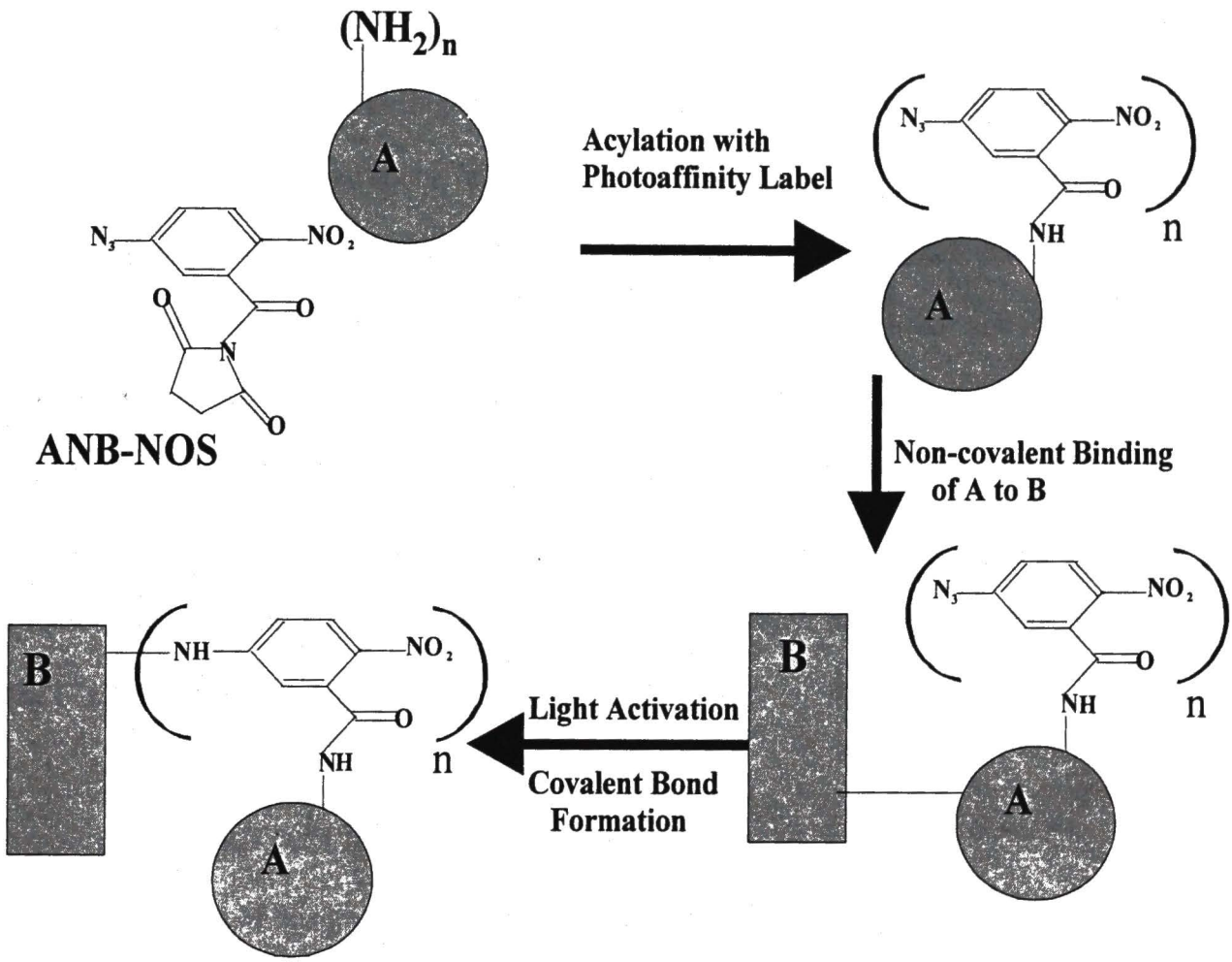


Figure 2. A schematic representation of the photoaffinity labeling method. The covalent binding of two biomolecules (A and B) using a nitroarylazide (ANB) and light activation.

Magnetic Beads for Sperm Cell Isolation

The current procedure for separation and isolation of the male cellular fraction (spermatozoa) requires a centrifugation step. This centrifugation step can be eliminated completely using decorated sperm antibodies attached to magnetic beads. The use of these magnetic beads will allow the isolation method to be completely automated, improving the overall reproducibility of this method. The magnetic beads and the techniques necessary for the preparation of the antibody coated magnetic beads are already available (DYNAL Inc., Bangs Laboratory, etc.) [14]. The proposed method for the synthesis of magnetic beads for the isolation of human sperm DNA is shown in Figure 3. It is also important to note that magnetic bead assays have been approved by the FDA for use by the clinical chemistry community.

Magnetic Beads for DNA Isolation

The goals of this project are to increase the throughput as well as the precision of PCR. DYNAL has claimed that their method can be used to isolate "PCR ready DNA" from many different sample types (e.g. buccal epithelial cells, blood, urine, etc.) in ten minutes using a single sample container. Additionally, the hardware for the proposed automation of the system is commercially available in conjunction with the BECKMAN Biomek 2000 robotic system. The sperm cell isolation protocol under development would be compatible with this robotic system.

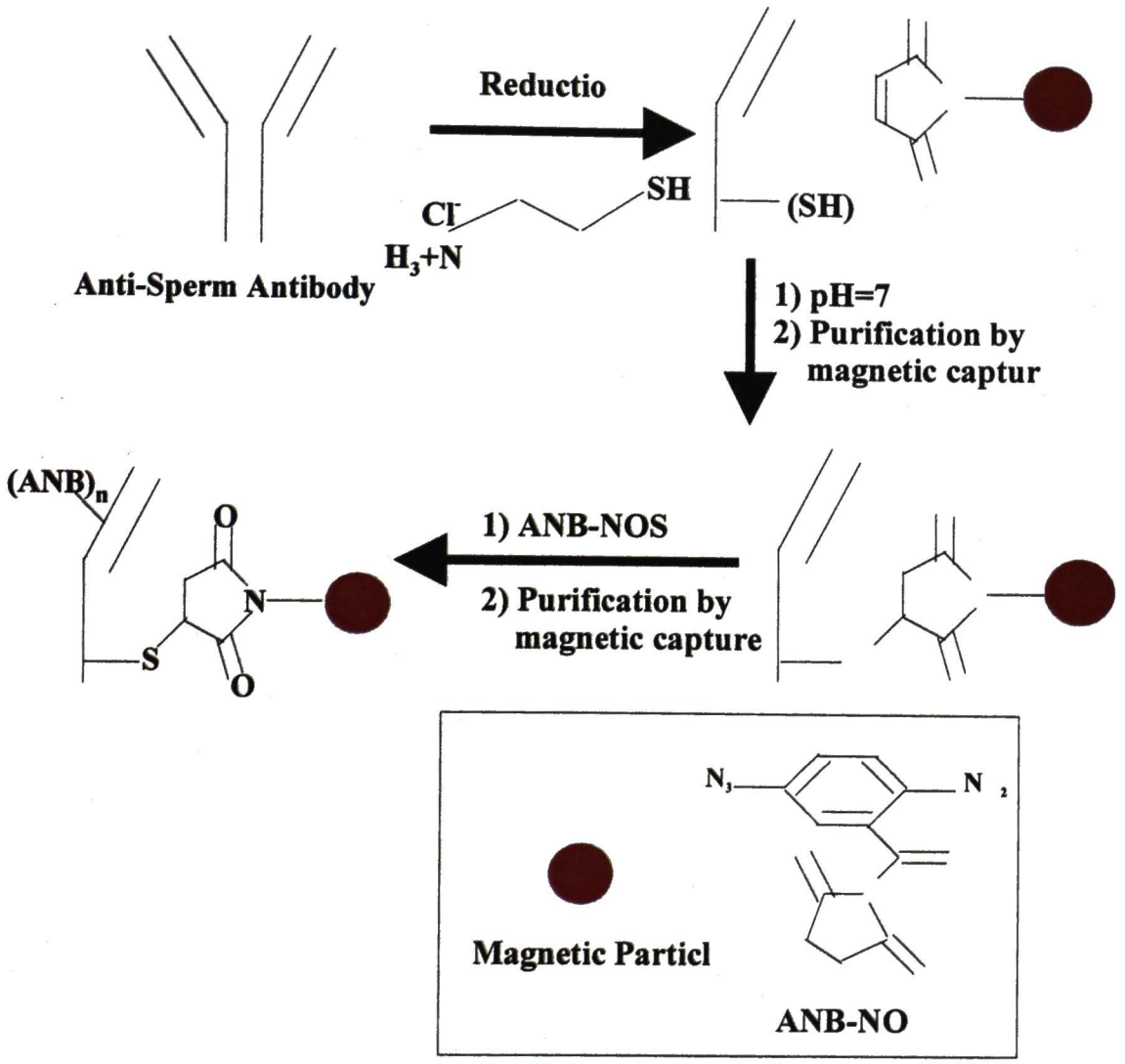


Figure 3. The approach to the synthesis of magnetic beads for the isolation of human sperm DNA.

Pico-Green Assay

For both standards and samples, 5µl DNA, 94µl Tris-EDTA (TE) Buffer, and 1ul of pico-green (PG) were placed into separate wells. Solutions of TE and PG were made in bulk for the appropriate number of standards and samples and then placed into the wells of a 96-well plate. The plate was allowed to sit in darkness for approximately 5 minutes in the scanner. The plate was then scanned at 500 volts using filter 530DF30.

Polymerase Chain Reaction

The amplification parameters for Profiler Plus and COfiler were as follows:

- 11 minute HOLD at 95°C
- 27 cycles [1 minute 94°C, 1 minute 59°C, 1 minute 72°C]
- 45 minutes at 60°C
- 15 minutes-infinity at 4°C

Profiler/COfiler Amplification

For reactions with up to 5µl of Template DNA, the following amplification mix was used: 5µl Buffer, 2.25µl Primer, 0.25µl Taq Gold. 7.5µl of this Master Mix was then added to 5µl Template DNA (x µl DNA + (5-x)µl water) for a final reaction volume of 12.5µl. For reactions with up to 5-10µl Template DNA, the following amplification mix was used: 10µl Buffer, 4.5µl Primer, 0.5µl Taq Gold. 15µl of this Master Mix was then added to 10µl Template DNA (x µl DNA + (10-x)µl water) for a final reaction volume of 25µl. 1 ng of Template DNA was used for the reaction mixtures. 1 ng of 9947A Control

DNA and sterile water was used for the positive and negative control tubes respectively. The amplification tubes were then placed in the thermocycler and the appropriate thermocycling method was chosen. Amplified products were stored at 4°C until electrophoresis.

Preparation of ABI 310 Genetic Analyzer

The instrument was first checked for proper capillary and polymer installment. Genescan POP-4 (Performance Optimized Polymer) and a 47cm capillary were used. The pump block was cleaned and inspected for bubbles, proper buffer level, and electrode-capillary alignment. Both a sample sheet and injection list were prepared. GS STR POP4 (1ml) F was the module chosen for each injection. The Profiler/COfiler Matrix was selected from the matrix files.

Preparation of Amplified Product for Electrophoresis

The necessary amounts of GeneScan-500 (ROX) Size Standard and sterile water were prepared in a microcentrifuge tube as follows: (number of samples + 2)*(1µl ROX) + (number of samples + 2)*(24µl deionized water). The number of samples included positive and negative controls as well as Profiler and CoFiler Allelic Ladders. 25µl of the ROX/water mix was placed into the appropriate number of micro-amp tubes. 1µl of amplified product or ladder was placed into the appropriate tubes. The tubes were then sealed and placed in the thermocycler to denature at 95°C for 4 minutes and then placed

on ice for 4 minutes. The tubes were placed into a sample tray, caps removed, and retained with septa and clip. The tray was placed into the 310 for electrophoresis.

STR Data Analysis using Genotyper

The Genescan files were imported into either the AmpFlSTR Profiler Plus or AmpFlSTR CoFiler Programs. Kazam macro was run to identify the alleles in each sample. The electropherograms were examined for alignment and peak assignment. The loci in each color category were visually inspected for stutter and artifacts by comparing the electropherogram for each sample with that of the appropriate ladder.

RESULTS

Optimization of Spermatozoa Capture

Various conditions for maximum spermatozoa capture were examined. These parameters included antibody efficiency, temperature, dilution ratio of antibody bead to spermatozoa, and incubation time. Both antibody beads and spermatozoa cells were counted using a hemocytometer. For each experiment, 10,000 sperm cells were used. Experiments were done in triplicate to determine reproducibility of the results as well as standard deviation values.

The primary experiment for this project was establishing which antibody-magnetic bead reagent was the most effective at capturing spermatozoa. Due to the fact that rape/sexual assault case samples often contain a limited amount of spermatozoa, it was necessary to find the most efficient antibody-bead capture system. As shown in Figure 4, MHS-10 captured 89% of the sperm in the sample while the other two antibodies captured significantly lower amounts (NUH-2: 82%; HS-21: 56%). Microscopy showed that the three antibodies bind different epitopes on the sperm surface (Figure 5). However, a mixture of the three antibodies failed to yield any significant difference in spermatozoa capture compared to the MHS-10 antibody-bead alone.

The effect of temperature on spermatozoa capture was also examined. As shown in Figure 6, sperm capture was slightly more efficient at 4°C than capture at room temperature (values were not significantly different). At 4°C, sperm capture was 91% for

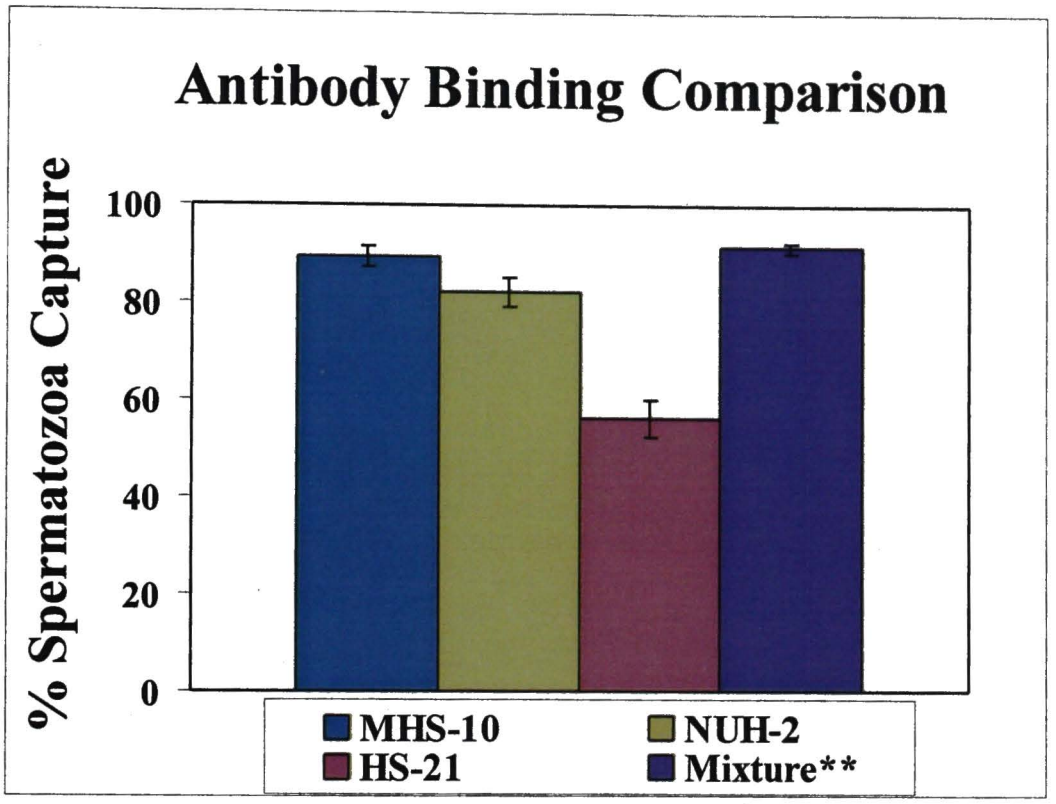


Figure 4. Optimal Antibody for Spermatozoa Capture System. Three different antibodies were examined for their efficacy in sperm capture. Using a 4:1 dilution of antibody/magnetic beads to sperm, the samples were placed on a rocker and incubated at 4°C for one hour. Magnetic capture was then performed and portions of the bound fraction were then resuspended in buffer and counted on a hemocytometer for the percent capture counts. Standard deviation error bars are shown to reflect the reproducibility of the results.

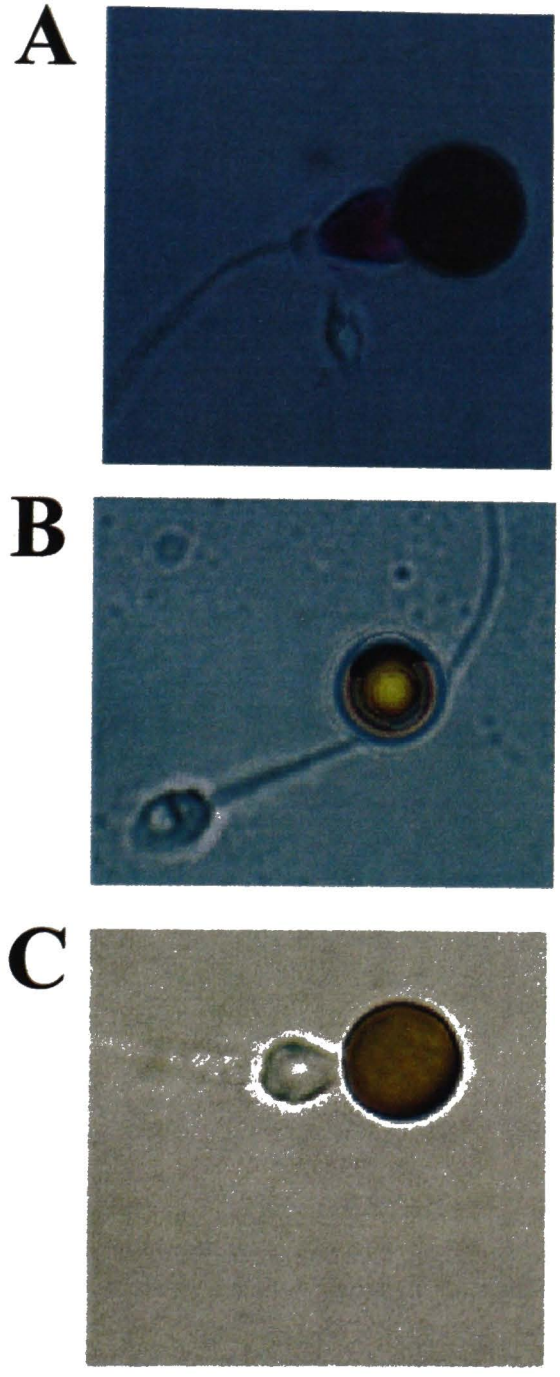


Figure 5. Photomicroscopy of Antibody/Magnetic Bead:Sperm Capture. Three different antibody/magnetic beads were tested. A) MHS-10 captured spermatozoa at the head of the sperm. B) NUH-2 captured sperm at the tail region. C) HS-21 captured sperm at the acrosomal cap.

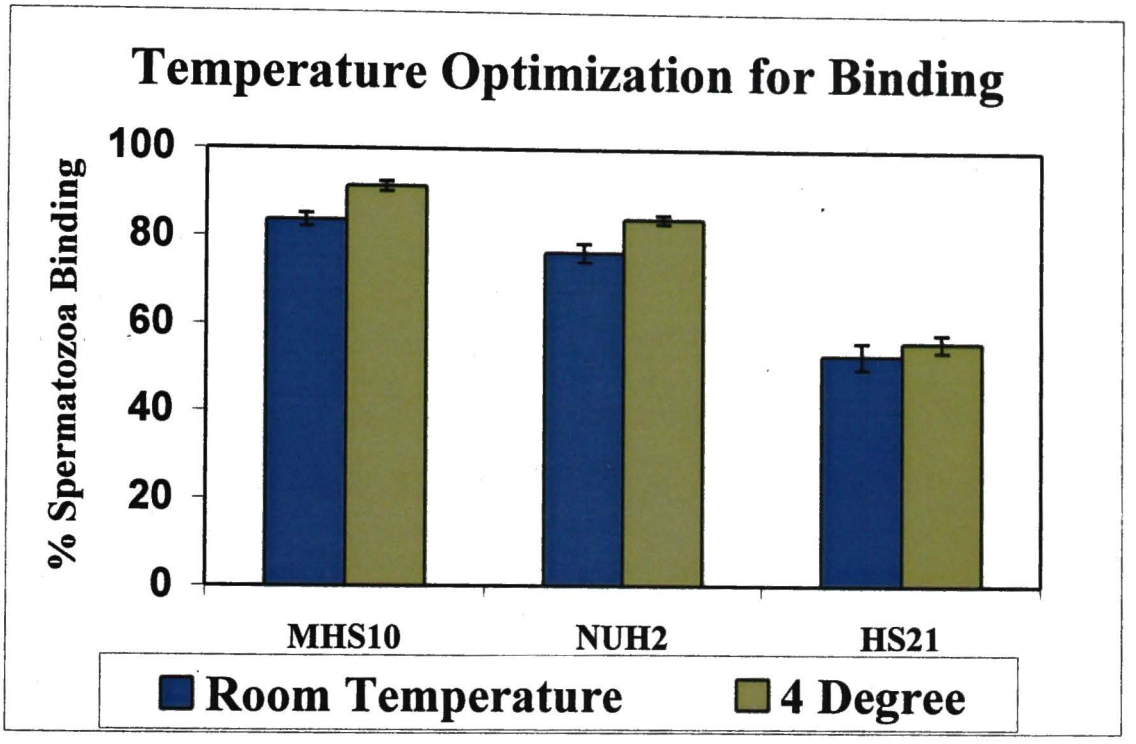


Figure 6. Optimal Temperature for Spermatozoa Capture System. Using a 4:1 dilution of antibody/magnetic beads to sperm, three different antibodies were analyzed for their ability to capture spermatozoa at two different temperatures – room temperature (RT) and 4°C. After exposure to the antibody/beads for one hour, magnetic capture was performed and a fraction of the bound portion was placed on a hemocytometer for binding counts.

MHS-10, 84% for NUH-2, and 56% for HS-21. At room temperature, sperm capture decreased with all three antibody systems (MHS-10: 84%; NUH-2: 76%; HS-21: 53%). In addition, the 4°C temperature may be advantageous in preventing any additional degradation of sperm DNA.

One limitation in the current differential extraction process is the amount of time spent by the analyst. This proposed method should be less time consuming for the DNA analyst. Several time periods were examined for magnetic capture of spermatozoa. As seen in Figure 7, sperm capture after a one-hour incubation period was slightly greater among antibody types. For MHS-10, percent capture did not increase either with a 90 or 120-minute capture. While the amount of sperm capture did increase after one hour using NUH-2 or HS-21 antibody beads, a longer incubation does not appear to provide any significant advantage.

The ratio of the MHS-10 antibody-beads to spermatozoa was also investigated. Four different dilutions were tested and the results are shown in Figure 8. The 4:1 dilution of antibody-bead: sperm proved the most effective, capturing 90% of the spermatozoa in the sample compared to 78% captured at a 1:1 dilution ratio. The 10:1 and 20:1 antibody bead-sperm ratio captured 51% and 43% respectively.

Based upon the results of these initial experiments, the conditions for the capture of the spermatozoa have been optimized. Thus, spermatozoa were captured in all future experiments using MHS-10 antibody-beads in a 4:1 dilution at 4°C for a period of one hour.

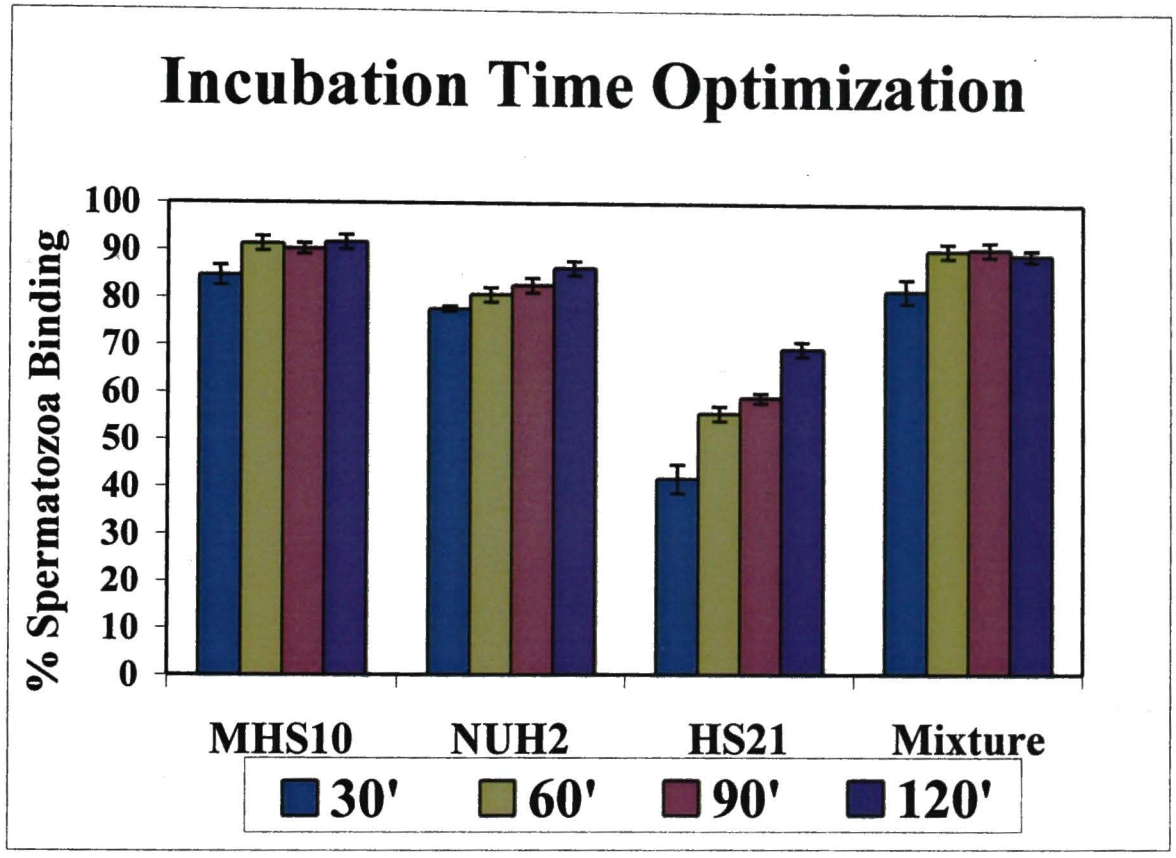


Figure 7. Optimal Incubation Time for Spermatozoa Capture System. Using a dilution of 4:1 antibody/magnetic beads to sperm, samples were incubated for four different time periods at 4°C. After incubation, magnetic capture was performed and a fraction of the bound portion was placed on a hemocytometer for binding counts.

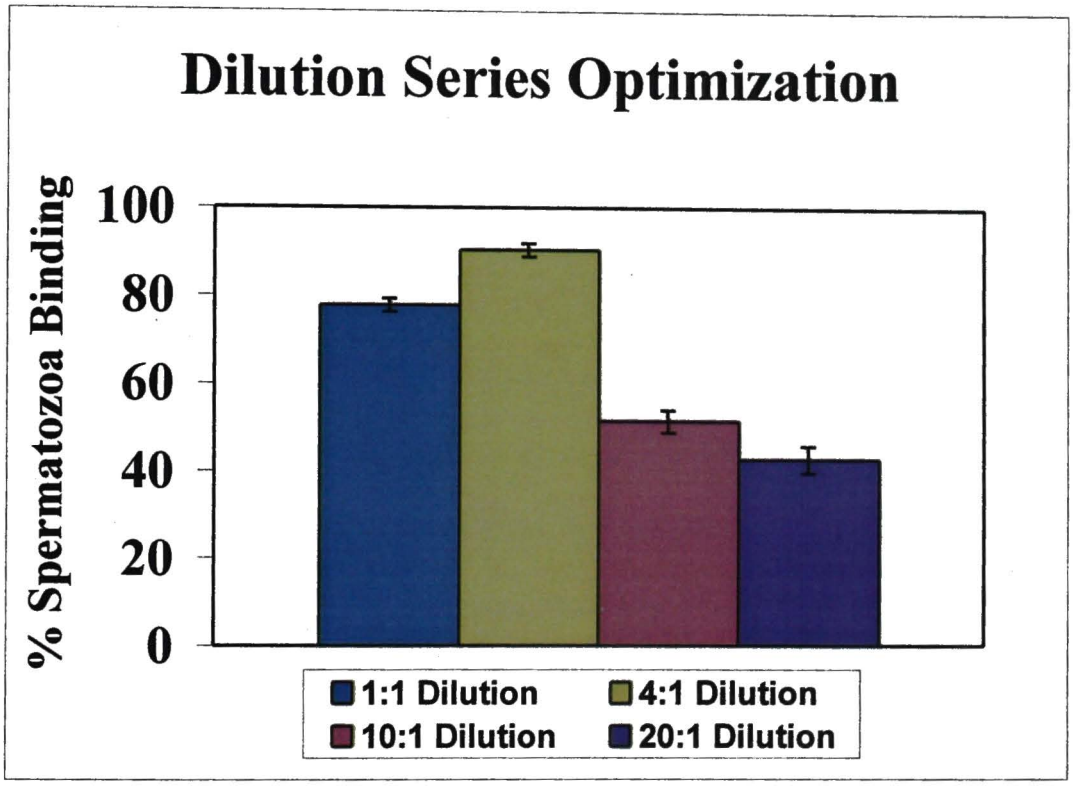


Figure 8. Optimal Dilution for Spermatozoa Capture System. Using MHS-10 antibody/magnetic beads, four different dilutions were tested at 4°C. After a one hour incubation period, magnetic capture was performed and the bound portions were placed on a hemocytometer for binding counts.

Strength of Antibody-Bead: Sperm Binding

Once magnetic capture of the sperm had been achieved, rigorous washing of the bound sperm is required. Washing is necessary to ensure the separation of the sperm from any other cells or cellular debris that may be present in the sample. To examine the strength of the magnetic binding, samples were exposed to a stress of vortexing or sonication for three time periods (30 seconds, one minute, and two minutes). After only 30 seconds of vortexing or sonication, greater than 95% of the sperm were released from the magnetic beads (Table 1).

In order to strengthen the bond, two different photoaffinity labels were evaluated for the covalent attachment of the sperm to the antibody/magnetic bead reagent. The initial experiments involved the attachment of the photoaffinity label ANB-NOS (N-5-azido-2-nitrobenzoyloxy-succinimide). As Table 2 illustrates, while several different concentrations were examined, the binding efficiency of the MHS-10 antibody/magnetic bead was dramatically reduced to only a few percent.

Photomicroscopy of ANB-NOS labeled beads was done both before and after photoactivation of the sample. As shown in Figure 9, spermatozoa were captured by the antibody-bead reagent system before the sample was exposed to UV light. After exposure, bead shape was noticeably different.

Additionally, the attachment of the photoaffinity label SFAD (sulfosuccinimidyl [perfluoroazido benzamido]-ethyl 1,3 dithiopropionate), was tested. After sperm binding and photo-activation, the captured sperm were then exposed to vortexing and sonication. Approximately 30% of the sperm appear to be covalently attached to the photoaffinity

labeled MHS-10 antibody/magnetic bead reagent (Table 3). Control experiments showed that using the same photoaffinity labeled MHS-10 antibody/magnetic bead reagent, without exposure to UV light, and subsequent vortexing or sonication, caused release of the bound sperm.

Antibody	Binding Efficiency (B.E.)	BE After Vortexing 30s	BE After Sonication 30s
MHS 10	93%	5%	2%
NUH-2	82%	4%	3%
HS-21	60%	5%	5%
Mixture	93%	4%	4%

Table 1. Nonlabeled Antibody/Magnetic Beads Exposed to Stress. Using a 4:1 dilution of nonlabeled antibody/magnetic beads to spermatozoa, samples were incubated for one hour at 4°C and then vortexed or sonicated for 30 seconds, one minute, and two minutes. Magnetic capture was performed and portions of the bound fractions were counted using a hemocytometer.

ANBNOS Labeled* Antibody	Binding Efficiency (B.E.)	BE After Vortexing 30s	BE After Sonication 30s
MHS 10	61%	4%	5%
NUH-2	44%	2%	0%
HS-21	31%	1%	1%

*1mM concentration of ANBNOS Label; Beads NOT exposed to UV

ANBNOS Labeled* Antibody	Binding Efficiency (B.E.)	BE After Vortexing 30s	BE After Sonication 30s
MHS 10	54%	6%	5%
NUH-2	41%	2%	4%
HS-21	29%	3%	2%

*1mM concentration of ANBNOS Label; Beads EXPOSED to UV

Table 2. ANB-NOS Labeled Antibody/Magnetic Beads Exposed to Stress. Using a 4:1 dilution of ANB-NOS labeled antibody/magnetic beads to spermatozoa, samples were incubated for one hour at 4°C and then either exposed to UV light or not exposed. Samples were then vortexed or sonicated for 30 seconds, one minute, and two minutes. Magnetic capture was performed and portions of the bound fractions were counted using a hemocytometer.

SFAD Labeled* Antibody	Binding Efficiency (B.E.)	BE After Vortexing 30s	BE After Sonication 30s
MHS 10	81%	18%	20%
NUH-2	69%	22%	19%
HS-21	36%	24%	16%

*10mM concentration of SFAD Label; Beads NOT exposed to UV

SFAD Labeled* Antibody	Binding Efficiency (B.E.)	BE After Vortexing 30s	BE After Sonication 30s
MHS 10	35%	32%	30%
NUH-2	30%	29%	30%
HS-21	39%	36%	32%

*10mM concentration of SFAD Label; Beads EXPOSED to UV

Table 3. SFAD Labeled Antibody/Magnetic Beads Exposed to Stress. Using a 4:1 dilution of SFAD labeled antibody/magnetic beads to spermatozoa, samples were incubated for one hour at 4°C and then either exposed to UV light or not exposed. Samples were then vortexed or sonicated for 30 seconds, one minute, and two minutes. Magnetic capture was performed and portions of the bound fractions were counted using a hemocytometer.

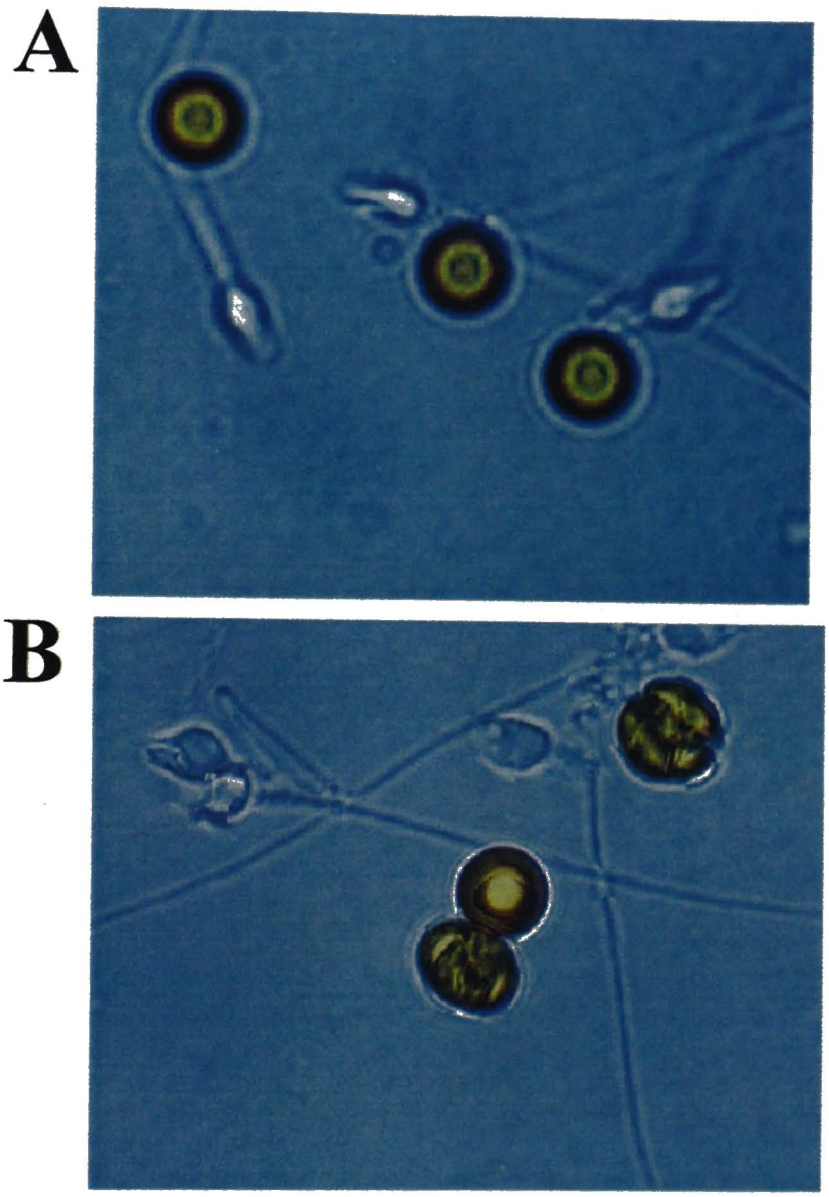


Figure 9. Photomicroscopy of Magnetic Bead: Spermatozoa Capture Before and After Photoactivation. A) ANB-NOS labeled MHS-10 antibody/magnetic beads captured spermatozoa before exposure to UV light. B) After exposure to UV light, the same beads show structural changes and show decreased spermatozoa capture.

DNA Profiling

To simulate sexual assault evidence, cultured vaginal epithelial cells (VEC) were obtained. In order to determine the reactivity of the antibody beads with VEC, sperm were mixed with vaginal cells along with antibody magnetic beads and followed by magnetic capture. As shown in Table 4, regardless of which antibody was used, the antibody magnetic bead reagent did not bind to the VEC. Cell counts indicated that 100% +/- 2 of the VEC were found in the "unbound" fraction. This was also demonstrated using photomicroscopy (Figure 10).

The genetic profiles for the vaginal epithelial cell line and the anonymous sperm donor were then determined for the 13 core STR loci. These are shown in Figures 11 and 12.

In an additional experiment, various amounts of sperm were mixed with vaginal cells in order to determine the minimum amount of sperm necessary to generate a DNA profile. Table 5 shows the numbers of cells that were analyzed. DNA profiles were generated for both male and female fractions (Figure 13). A genetic profile was obtained with as little as 300 sperm.

Beads: Sperm: VECs	Captured Sperm	Unbound VECs
1:1:1	90%	100%
4:1:1	91%	100%
20:1:1	45%	100%
4:1:4	89%	100%
20:1:4	53%	100%

Table 4. Unbound Vaginal Epithelial Cells Exposed to Antibody/Magnetic Beads. Using various dilutions of antibody/magnetic beads, spermatozoa, and vaginal epithelial cells, mixed samples were incubated at 4°C for one hour. Magnetic capture was performed and portions of both the bound and unbound fractions were counted using a hemocytometer.

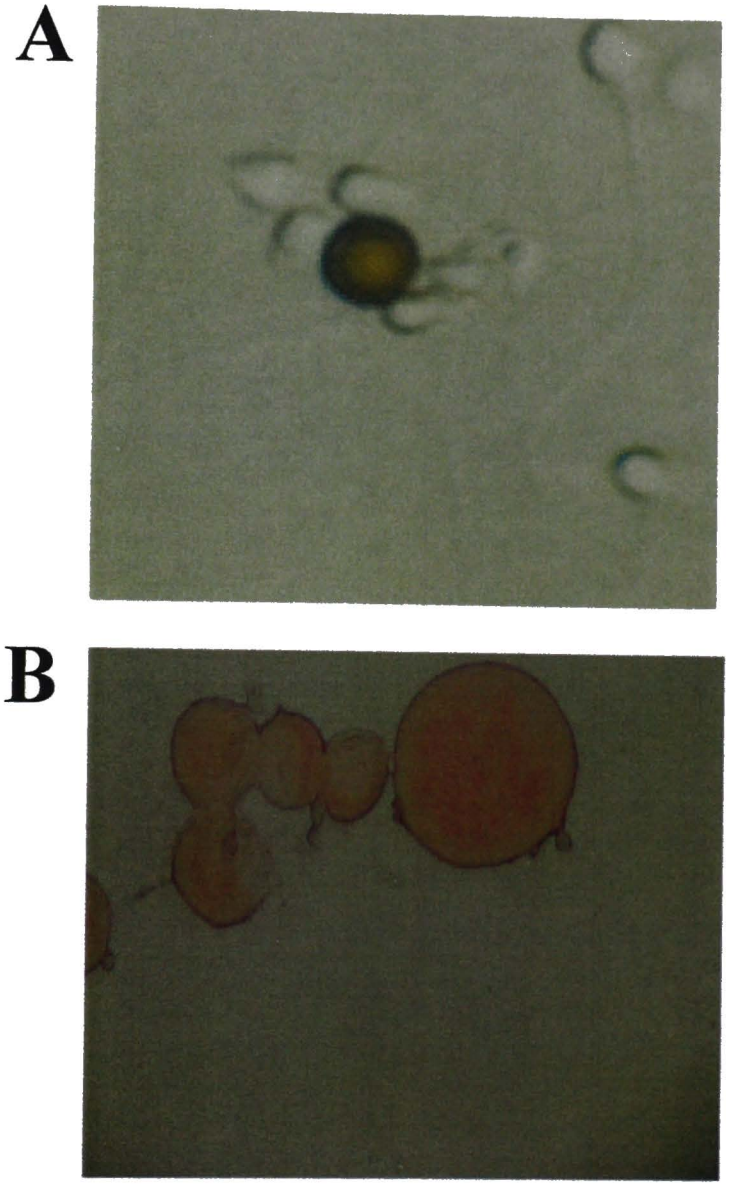


Figure 10. Photomicroscopy of Bound Spermatozoa and Unbound Vaginal Epithelial Cells. To simulate sexual assault evidence, 40,000 cultured vaginal epithelial cells were mixed with 10,000 spermatozoa and incubated with antibody/magnetic beads. After exposure to the magnet, bound and unbound fractions were examined by microscopy and cell counts were prepared. A) MHS-10 antibody/magnetic bead with multiple spermatozoa attached. B) Unbound vaginal epithelial cells (note absence of magnetic beads).

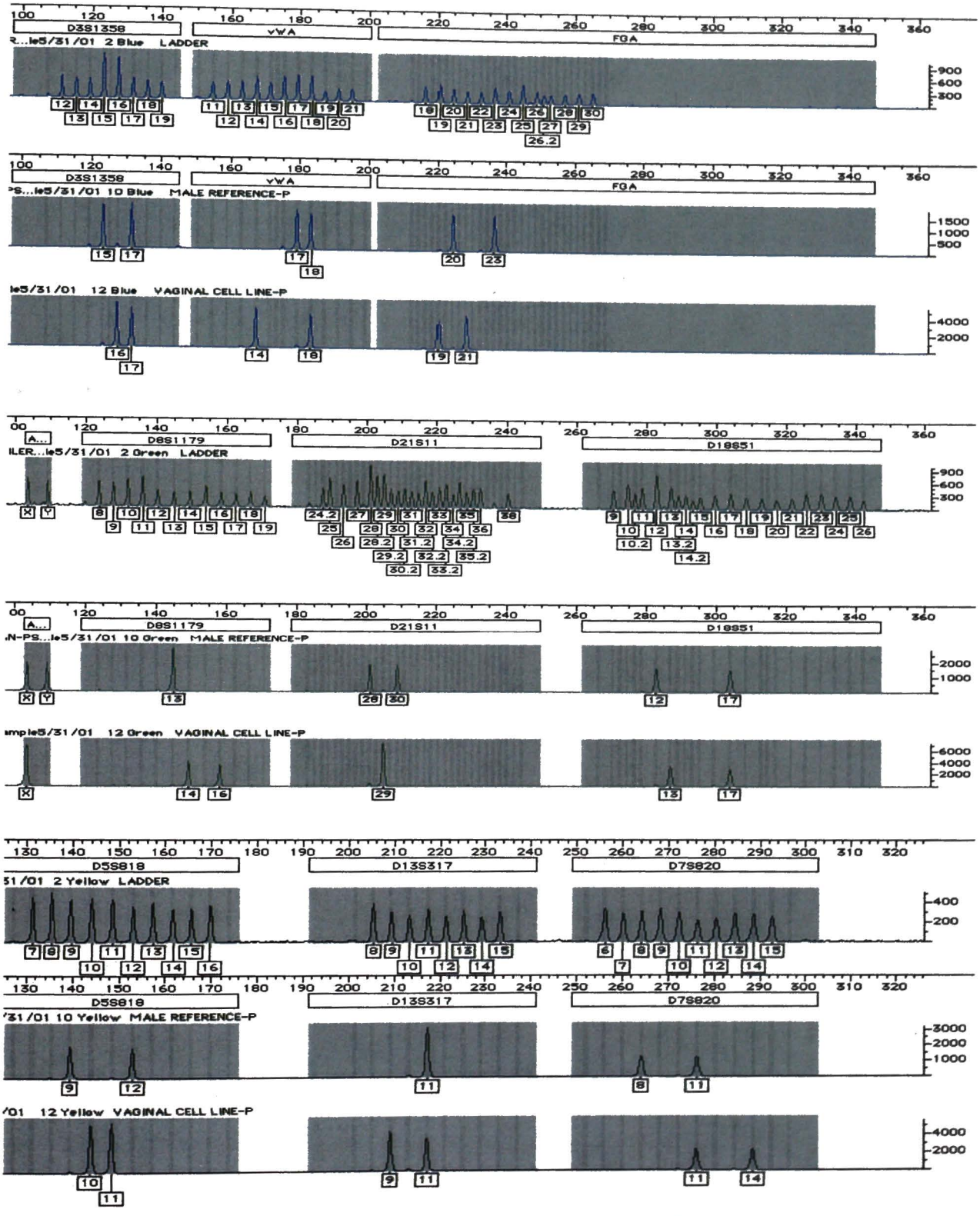


Figure 11. Profiler Plus DNA Profiles for sperm and vaginal cell DNA. DNA profiles were generated for the following loci: FGA, vWA, D3S1358, D8S1179, D18S51, D21S11, D5S818, D7S820, D13S317 and amelogenin.

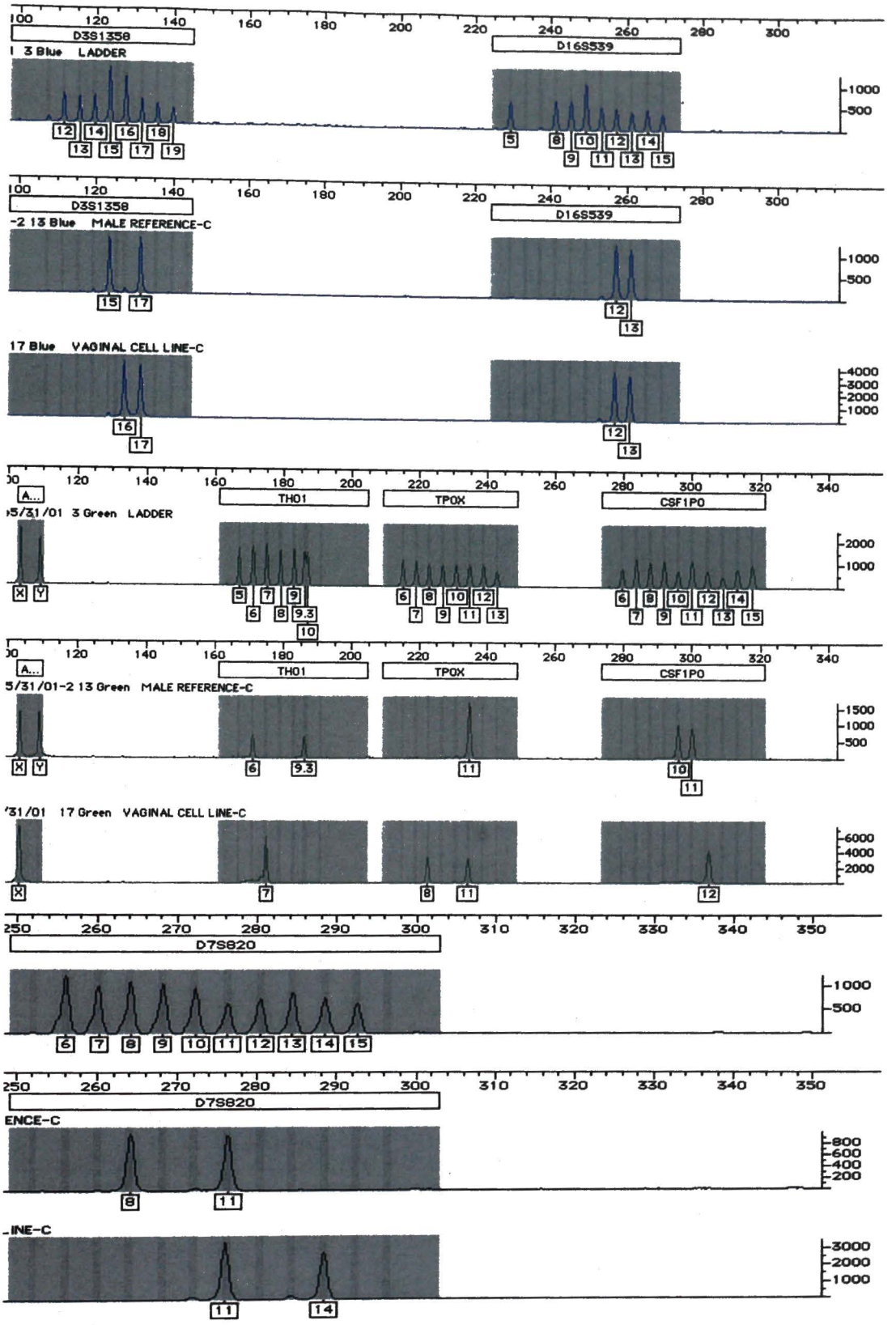
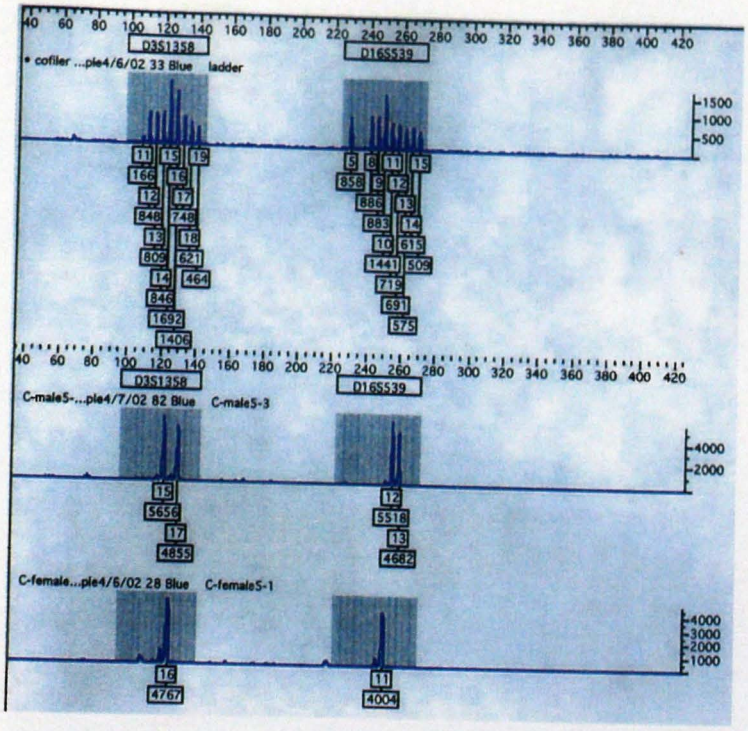


Figure 12. COfiler DNA Profiles for sperm and vaginal cell DNA. DNA profiles were generated for the following loci: D3S1350, D16S539, THO1, TPOX, CSF1PO, D7S820 and amelogenin.

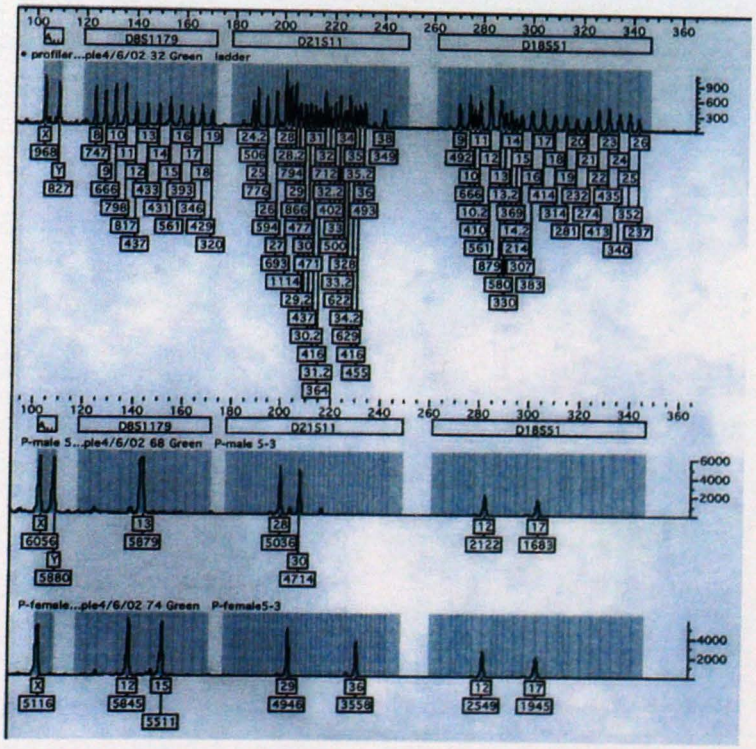
# Spermatozoa	# Vaginal Cells
10,000	0
5000	20,000
1000	4000
500	2000
300	1200
0	10,000

Table 5. Minimum Number of Spermatozoa for DNA Profiling. In a simulation of sexual assault evidence, vaginal cells were mixed with spermatozoa. Varying numbers of spermatozoa were used (from 10,000 to 300). A 4:1:4 antibody/magnetic bead: sperm: VEC ratio was used in each mixture. Following a one-hour incubation at 4°C, magnetic capture was performed and the two different cell types were fractioned. DNA was extracted from both for DNA profiling.

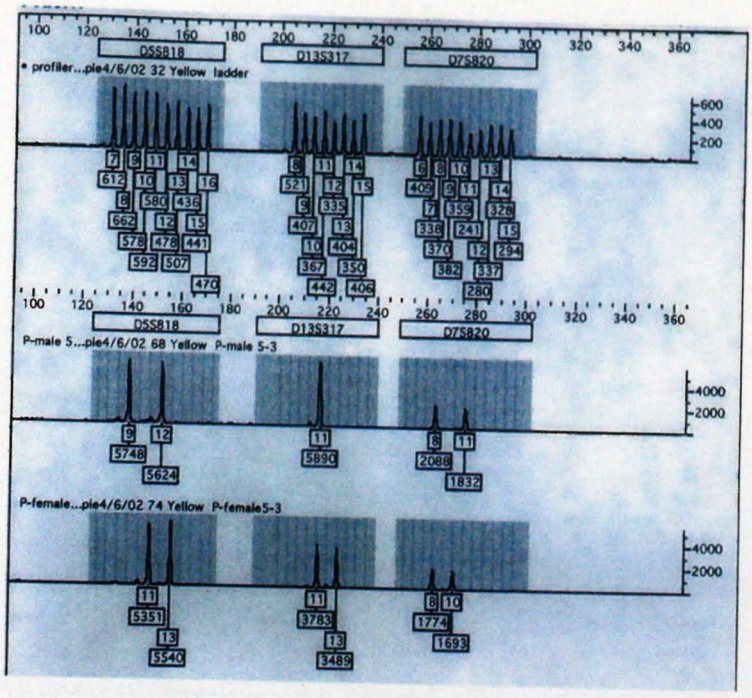
A


D3S1358
D16S539
• cofiler ...ple4/6/02 33 Blue ladder
C-male5-...ple4/7/02 82 Blue C-male5-3
C-female...ple4/6/02 28 Blue C-female5-1

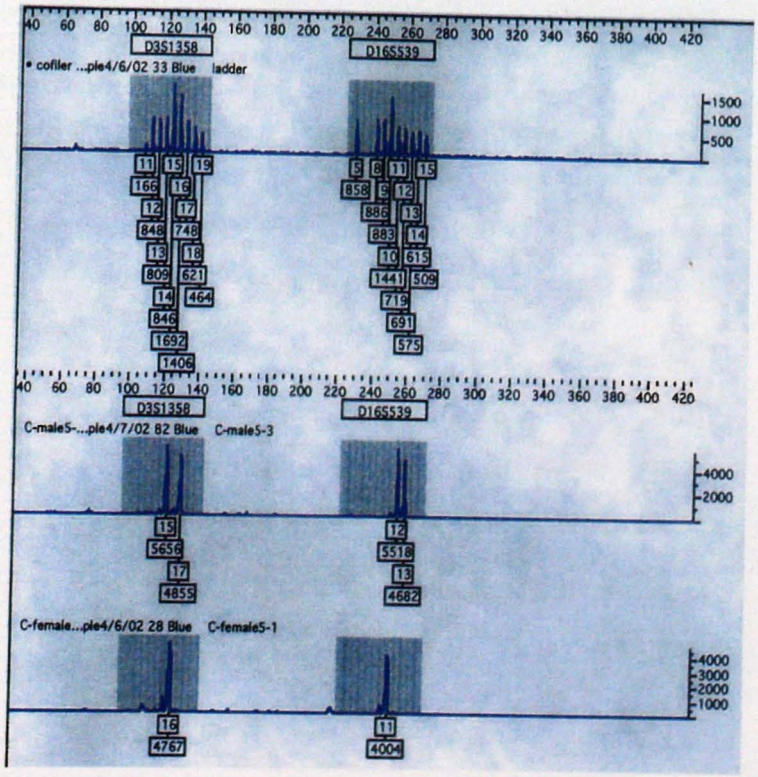
B


D8S1179
D21S11
D18S51
• profiler...ple4/6/02 32 Green ladder
P-male 5...ple4/6/02 68 Green P-male 5-3
P-female...ple4/6/02 74 Green P-female5-3

C


D5S818
D13S317
D7S820
profiler...pie4/6/02 32 Yellow ladder
P-male 5...pie4/6/02 68 Yellow P-male 5-3
P-female...pie4/6/02 74 Yellow P-female5-3

D


D3S1358
D16S539
cofiler ...pie4/6/02 33 Blue ladder
C-male5-...pie4/7/02 82 Blue C-male5-3
C-female...pie4/6/02 28 Blue C-female5-1

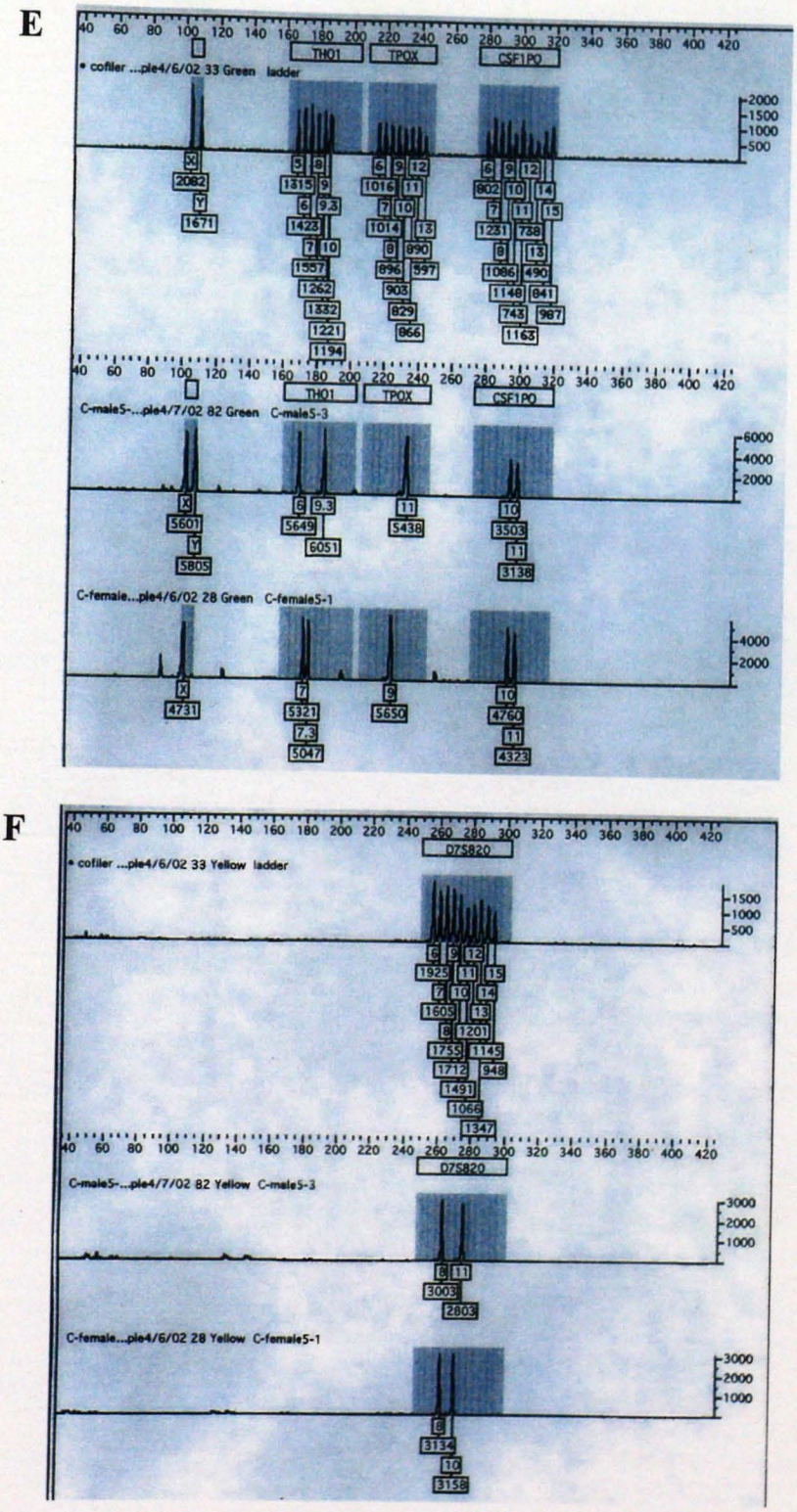


Figure 13. Panels A, B, and C represent the Profiler Loci for the sperm and vaginal epithelial DNA from a mixed sample. Panels D, E, and F represent the COfiler Loci.

DISCUSSION

The overall goal of this project was to develop a cellular sorting method (antibody/magnetic bead reagent capture system) for the recovery of sperm from sexual assault evidence. The design of the antibody/magnetic bead capture system is such that:

- It utilizes differentiating features (extracellular antigenic sites unique to sperm) so that the separation of cell types will be complete;
- It should provide a more efficient means of sperm recovery, increasing the yield of DNA from the sperm fraction;
- The DNA isolation process is compatible with the amplification of the CODIS core STR loci;
- It may be possible to automate the entire process, resulting in a more efficient, reliable and cost effective system;

The overall goal was divided into three phases: 1) Evaluate a panel of antibodies directed against sperm for their ability to bind sperm heads; 2) Attach the anti-sperm antibodies to magnetic beads in such a way as to maximize their binding efficiency; and 3) Develop conditions for lysing the sperm heads and releasing the DNA, which will allow the direct amplification of the CODIS core STR loci.

Phase 1 involved the evaluation of three different anti-sperm antibodies obtained through ATCC. Cell lines producing each of the three antibodies were grown, and milligram quantities of each antibody were purified. The first was the MHS-10 antibody developed by John C. Herr and R.M. Wright, University of Virginia Health System, and covered by U.S. Pat. 5,602,005 [8]. It is an IgG monoclonal antibody which reacts with

an intra-acrosomal antigen (designated SP-10). It does show some cross-reactivity with analogous sperm antigen from other primates [8]. It reacts with adluminal spermatids and mature sperm. The MHS-10 antibody does not react with other primate tissue other than sperm. This research demonstrates that after attachment to magnetic beads, MHS-10 has the ability to capture 90-95% of spermatozoa.

The second anti-sperm antibody that was investigated is NUH-2. It was developed by E. Nudelman et al., The Biomembrane Institute, Hyogo Medical College, and is covered by U.S. Pat. 5,227,160 [13]. NUH-2 is an IgM monoclonal antibody which reacts with a ganglioside containing disialyl I structures, and inactivates human sperm. The research shows that after attachment to magnetic beads, NUH-2 has the ability to capture approximately 80% of spermatozoa, primarily binding to the tail region.

The third anti-sperm antibody that was evaluated is HS-21. It was developed by D.P. Wolf et al. [19]. It is an IgG monoclonal antibody directed against mammalian sperm, and has been shown to react with an antigen on the acrosomal cap of mammalian sperm. The research shows that after attachment to magnetic beads, HS-21 captures approximately 60% of spermatozoa, primarily binding to the acrosomal cap.

Phase 2 involved the evaluation of commercially available magnetic beads and the development of an attachment chemistry that would maximize the binding of the antibodies to sperm. Due to its high antibody loading potential, the Dynal M450-epoxy magnetic bead was selected. Each of the three antibodies was successfully attached to the Dynal beads. This work was done by Dr. William Chapman at MiraiBio, Inc.

(formerly Hitachi Genetic Systems). The binding efficiencies of each of the anti-sperm/magnetic bead reagents were then evaluated.

To demonstrate the specificity and sensitivity of the initial antibody coated magnetic beads, the ability to capture spermatozoa was tested. Mixtures consisting of varying numbers of vaginal epithelial cells and sperm were prepared and placed into 1.5 ml centrifuge tubes. The ratio of the antibody-coated magnetic beads to cells was also varied. Both the bound and unbound portions were stained with a Christmas Tree Stain and visualized by light microscopy. The number of recovered cells, both sperm and vaginal, were counted on a hemocytometer. The MHS-10 antibody coated magnetic beads proved to be the most efficient, binding between 90-95% of the input sperm. None of the three antibodies bound the vaginal epithelial cells.

Following the magnetic capture of the sperm, a rigorous washing is desirable to ensure the separation of the sperm from any other cells or cellular debris present in the evidentiary sample. To test the strength of the magnetic binding, samples were exposed to a stress of either vortexing or sonication. After only 30 seconds of vortexing or sonication, greater than 95% of the sperm were released from the magnetic beads. This experiment demonstrated the necessity to modify the antibody/magnetic bead reagent to enhance the retention of the sperm.

Attempts were made to chemically modify the reagent to allow the formation of a more permanent covalent bond. The formation of covalent binding between the spermatozoa and the antibody through photochemical activation should enhance the retention of the spermatozoa to the magnetic beads. This enhanced binding should be

advantageous during the rigorous wash cycles performed during sperm/epithelial cell separation. In addition, the covalent attachment of the sperm to the magnetic bead should provide a mechanism to retain cellular components of the spermatozoa following the final lysis of the sperm head. This could allow the direct amplification of the lysate without the need for any additional cleanup. Two different photoaffinity labels were evaluated for the covalent attachment of the sperm to the antibody/magnetic bead reagent. One of the most popular photoaffinity label types uses the arylazide group for the light activated attachment step. When light activated, arylazides lose diatomic nitrogen producing the reactive nitrene intermediate. Nitrenes are known to form covalent bonds to neighboring molecules by the addition to unsaturated linkages or insertion into single covalent bonds (C-H or C-C). Thus, attachment of the arylazide group to one molecule allows it to be covalently coupled to a second molecule.

The photoaffinity labels can be selectively attached to either the N-terminus of the protein, or to lysine residues throughout the protein. The initial experiments involved the attachment of the photoaffinity label ANB-NOS (N-5-azido-2-nitrobenzoyloxy-succinimide). While several different concentrations were examined, both in the presence or absence of photoactivation, the binding efficiency of the MHS-10 antibody/magnetic bead was dramatically reduced to only a few percent.

The attachment of a photoaffinity label with a longer spacer group, SFAD (sulfosuccinimidyl [perfluoroazido benzamido]-ethyl 1,3 dithiopropionate), was tested. After the initial sperm binding and photo-activation, approximately 30% of input sperm appear to be covalently attached to the photoaffinity labeled MHS-10 antibody/magnetic

bead reagent. Control experiments showed that using the same photoaffinity labeled MHS-10 antibody/magnetic bead reagent, in the absence of the photoactivation, caused the release of the bound sperm after vortexing or sonication.

To simulate sexual assault evidence, cultured vaginal epithelial cells were obtained. The genetic profiles for the vaginal epithelial cell line and the anonymous sperm donor were determined for the 13 core STR loci. Additionally, it was shown that a genetic profile could be obtained with as little as 300 sperm. This experiment demonstrated the efficacy of bead capture in a mixed sample of vaginal cells and sperm.

CONCLUSION

The results of this study show that the antibody/magnetic bead capture system is a rapid, effective technique for isolating spermatozoa from a sperm-epithelial cell mixture or mock sexual assault sample. The MHS-10 antibody demonstrated the highest affinity for spermatozoa, capturing 90-95% of sperm. The photoaffinity labeled antibody/magnetic beads show promise in their ability to maintain a bond with spermatozoa even after exposure to rigorous vortexing or sonication (mimicking of action produced during washing steps). Following the proposed magnetic bead protocol for spermatozoa isolation, the filtered sample was sufficiently pure so that PCR-based analytical methods were successfully employed with little apparent cross-contamination in either the male or female DNA profiles. In addition, as few as 300 sperm cells produced a distinct and clean genetic profile.

This method also sets the stage for the automation of the entire process, which could result in a more cost effective and reliable process. Overall, the proposed technique should increase the probability of success in the analysis of sexual assault case samples.

REFERENCES

1. Anderson, DJ, Johnson, PM, Alexander, NJ, Jones, WR, Griffin, PD. Monoclonal antibodies to human trophoblast and sperm antigens: Report of two WHO-sponsored workshops, June 30, 1986 – Toronto, Canada. *J Reprod Immun* 1987; **10**:231-257.

2. Calvin, HI, Bedford, JM. Formation of disulfide bonds in the nucleus and accessory structures of mammalian spermatozoa during maturation in the epididymis. *J Reprod Fertil Suppl* (England) 1971; **13**:65-75.

3. Chen, J, Kobilinsky, L, Wolosin, D, Shaler, R, Baum, H. A physical method for separating spermatozoa from epithelial cells in sexual assault evidence. *J Forensic Sci* 1998; **43(1)**:114-118.

4. Diekman, AB, Herr, JC. Sperm antigens and their use in the development of an immunocontraceptive. *Am J Reprod Immun* 1997; **37**:111-117.

5. Gill, P, Jeffreys, AJ, Werrett, DJ. Forensic application of DNA "Fingerprints.' *Nature* 1985; **318**:577-9.

6. Gill, P, Lygo, JE, Fowler, SJ, Werrett, DJ. An evaluation of DNA fingerprinting for forensic purposes. *Electrophoresis* 1987; **8**:38-44.

7. Guisti, A, Baird, M, Pasquale, S, Balazs, I, Glassberg, J. Application of Deoxyribonucleic Acid (DNA) Polymorphisms to the Analysis of DNA Recovered From Sperm. *J Forensic Sci* 1986; **31**:409-417.

8. Herr, JC, Wright, RM. Primate intra-acrosomal sperm antigen for use in a contraceptive vaccine. U.S. Patent 5,602,005 dated Feb. 11, 1997.

9. Jeffreys, AJ, Wilson, V, Thein, SL. Hypervariable 'minisatellite' regions in human DNA. *Nature* 1985; **314**:67-73.

10. Jeffreys, AJ, Wilson, V, Thein, SL. Individual-specific 'fingerprints' of human DNA. *Nature* 1985; **314**:76-9.

11. Lee, CG, Wong, E. Developmental studies of sperm surface antigens using sperm-specific monoclonal antibodies. *J Reprod Immun* 1986; **9**:275-287.

12. Nudelman, E, Kaizu, T, Mandel-Clausen, U, Levery, SB, Hakomori, S, Tsuji, Y, Isojima, S. Monoclonal antibody NUH-2 capable of inactivating motility of human sperm, antigen defined by said monoclonal antibody and methods of using said monoclonal antibody and antigen. U.S. Patent 5,227,160 dated July 13, 1993.

13. Okabe, M, Matzno, S, Nagira, M, Ying, X, Kohama, Y, Mimura, T. Collection of acrosome-reacted human sperm using monoclonal antibody-coated paramagnetic beads. *Mol Reprod Dev* 1992; **32**:389-393.

14. Patel, D, Rickwood, D. Optimization of conditions for specific binding of antibody-coated beads to cells. *J Immun Method* 1995; **184**:71-80.

15. Wolf, DP, Boldt, J, Byrd, W, Bechtol, KB. Acrosomal status evaluation in human ejaculated sperm with monoclonal antibodies. *Biol Reprod* 1985; **32**:1157-1162.

16. Ying, X, Okabe, M, Mimura, T. Selection of acrosome-reacted human spermatozoa and their fusing ability by micro-injection into the perivitelline space of hamster eggs. *Human Reprod* 1993; **8**:1074-1078.