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Immunomagnetic Beads Coupled with Anti-PH-20 Antibodies to Isolate Sperm from DNA Mixtures for Sexual Assault Kit Application

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Immunomagnetic Beads Coupled with Anti-PH-20 Antibodies to Isolate Sperm from DNA Mixtures for Sexual Assault Kit Application

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Thesis submitted
to the Eberly College of Arts and Sciences
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in partial fulfillment of the requirements for the degree of

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ABSTRACT

Immunomagnetic Beads Coupled with Anti-PH-20 Antibodies to Isolate Sperm from DNA Mixtures for Sexual Assault Kit Application

Kayla M. Becks

Sexual Assault Kits (SAKs) account for a large portion of DNA mixture samples that are processed within a forensic laboratory, most commonly in the form of vaginal swabs. A typical vaginal swab from a SAK contains the female victim's epithelial cells and varying concentrations of sperm cells left by the perpetrator. The key to analyzing these types of samples is to separate the mixture into male and female fractions. As such, there is a demand to develop novel techniques that have the capability of recovering as much of the male fraction as possible, specifically in cases where there is a limited sperm concentration to begin with. Magnetic beads are a versatile product in that they can be coupled with a variety of substances including a silica coating or various antibodies. Several studies have explored techniques to target testis specific molecules to isolate sperm from DNA mixtures. This study targeted a protein (PH-20) that is not testis specific but is intrinsic to the head of a sperm cell.

The goal was to evaluate the efficiency of magnetic beads coupled with anti-PH-20 antibodies in isolating sperm cells from DNA mixtures. Results suggested that streptavidin (SA) coated immunomagnetic beads (IMBs) recover more male DNA than carboxylic acid coated IMBs in both male-only samples and male:female mixture samples. Overall, the SA-IMBs recovered approximately 21.7% of the total male DNA available in samples. While more research is needed to optimize the recovery of male DNA utilizing the IMB process, magnetic beads coupled with PH-20 antibodies have shown success in recovering male DNA and have considerable potential for forensic applications.

Contents

1	Introduction	1
1.1	DNA	1
1.2	Polymorphisms	2
1.3	DNA Extraction	3
1.3.1	Differential Extraction	4
1.3.2	Magnetic Bead Extraction	4
1.4	Quantitation	6
1.5	Flow Cytometry	8
1.6	Immunomagnetic Beads	10
1.6.1	Carboxylic acid coated beads	10
1.6.2	Streptavidin coated beads	11
1.7	PH-20 Protein	12
1.8	Previous Research	14
1.9	Purpose, Goals, and Objectives	15
2	Methods	17
2.1	Cell Preparation	17
2.1.1	Cell Agitation	17
2.2	Immunomagnetic Bead Preparation, Isolation, & Analysis	18
2.2.1	Carboxylic Acid Coated Beads	18
2.2.2	Streptavidin Coated Beads	19
2.2.3	Immunomagnetic Bead (IMB) Isolation	19
2.2.4	DNA Extraction	20
2.2.5	DNA Quantitation	21
2.3	Concentration Estimation	21
2.4	Immunomagnetic Bead Testing	21
2.4.1	Cell Elution Methods	22
2.4.2	Immunomagnetic Beads in Cell Mixtures	23

2.4.3	Female Cell Lysis	23
2.4.4	DNase as a Lysing Step	24
2.4.5	Immunomagnetic Beads with Female Cells	24
2.4.6	Homogenizing Semen Samples	25
2.4.7	Magnetic Beads' Ability to Bind Cells	25
2.4.8	Antibody Ability to Bind Cells	26
2.4.9	Bead Type	28
2.4.10	Statistical Analysis	29
3	Results and Discussion	32
3.1	Immunomagnetic Bead Testing	32
3.1.1	Cell Elution Methods	32
3.1.2	Immunomagnetic Beads in Cell Mixtures	33
3.1.3	Female Cell Lysis	34
3.1.4	DNase as a Lysing Step	34
3.1.5	Immunomagnetic Beads with Female Cells	35
3.1.6	Homogenizing Semen Samples	36
3.1.7	Magnetic Beads Ability to Bind Cells	37
3.1.8	Antibody Ability to Bind Cells	37
3.1.9	Bead Type	39
4	Conclusions	46
5	Appendix A	49

1. Introduction

1.1 DNA

Deoxyribonucleic acid or DNA is the genetic material that makes up all living things. The genetic information within DNA is passed from one generation to the next [1]. Nuclear DNA is contained in all nucleated cells in the body and its purpose is to make copies that will be passed down to offspring. Those copies hold the instructions for making proteins [1]. When passed down, half of an individual's DNA is inherited from their mother and the other half from their father. DNA is composed of a specific sequence of nucleotides, each nucleotide containing three components: a nitrogenous base, a deoxyribose sugar, and a phosphate group [2]. Nucleotides are linked to one another by a phosphodiester linkage to form an alternating sugar-phosphate backbone that creates the double helical structure of DNA [1]. There are four nitrogenous bases found in DNA: adenine (A), thymine (T), guanine (G), and cytosine (C). A single strand of DNA contains a series of A, T, C, and G bases. The order of these bases vary among individuals and account for the biological differences between all human beings [1]. The double helix structure is made up by the complementary pairing of the bases via hydrogen bonds, as seen in Figure 1.1[1]. Adenine-thymine share a double bond and cytosine-guanine share a triple bond. A nucleotide's complementary fashion allows each strand to serve as a template to synthesize the opposite strand. The complementary strands run anti-parallel to one another, one in the 5' to 3' direction and the other in the 3' to 5' direction, determined by the orientation of the sugar [1].

A chromosome is a structure containing nuclear DNA that is tightly wound around proteins called histones [1]. Each individual has 23 pairs of chromosomes that make up their entire genome. Every cell in a human body will contain the same 23 pairs of chromosomes, with the exception of haploid cells. One pair of chromosomes are the sex chromosomes, which can exhibit either an X chromosome or a Y chromosome [1]. A pair of chromosomes are the same size and have the same structure but are not necessarily identical; this is referred to as a homologous pair. Chromosomes consist of coding and noncoding regions. The coding regions are referred to as genes, which contain the information that codes for proteins. Genes make

Figure 1.1: DNA double helical structure [3]. The two strands run anti-parallel to each other with a sugar phosphate backbone and the nitrogenous bases, adenine, thymine, guanine, and cytosine, in the center of the structure connected by hydrogen bonds.

up approximately five percent of the human genome while noncoding regions account for the rest [1]. Throughout the noncoding regions are areas that are variable, or polymorphic, between individuals [4]. The locations on a chromosome where those polymorphisms occur are called loci and the variation in loci provides the basis by which individuals can be differentiated by their genetic profile.

1.2 Polymorphisms

The majority of human DNA, approximately 99.7%, is the same from person to person. Individuals only differ from one another in 0.3% of their DNA; these variable regions are known as polymorphisms [1]. A polymorphism is a variation in the DNA sequence that occurs on homologous chromosomes. The variations that occur at the same locus on a homologous pair of chromosomes are called alleles [4]. The alleles in a homologous pair of chromosomes can either be the same as each other (homozygous) or different from each other (heterozygous). There are different forms of variations that can be found and analyzed in the human genome [4]. The primary forms, found in the noncoding region, include sequence polymorphism and length polymorphism as shown in Figure 1.2. A sequence polymorphism is a single nucleotide polymorphism (SNP), wherein a variation at a single nucleotide within a locus occurs [4]. Length polymorphism refers to the variation in repeated units of base

pairs within a locus. The repeating units contain a set number of base pairs and those units are consecutively repeated. The variation occurs in the number of consecutive repeating units at a locus. Highly variable repeats make length polymorphism a useful tool in forensic science for inclusionary and exclusionary purposes because these can be used to differentiate individuals [4].

(A) Sequence polymorphism

```

-----AGACTAGACATT-----
-----AGATTAGGCATT-----

```

(B) Length polymorphism

```

-----(AATG)(AATG)(AATG)-----
              3 repeats
-----(AATG)(AATG)-----
              2 repeats

```

Figure 1.2: Polymorphisms [1]. Sequence polymorphism and length polymorphism are the two primary polymorphisms found in the human genome.

1.3 DNA Extraction

To analyze a sample for forensic purposes, the DNA must first be isolated from the remainder of the material in the sample (e.g. bodily fluids, extraneous proteins, etc.). DNA extraction is the process of breaking open cells to allow the DNA contained inside the nucleus of the cell to be free in solution. The first step in any DNA extraction is to add a digest buffer to the DNA sample [4]. Digest buffers typically contain a detergent used to lyse the cell, proteinase K to aid in the hydrolysis of proteins, and Ethylenediaminetetraacetic acid (EDTA) to prevent DNA degradation. The detergent used in the digest buffer will differ based on which method is being used and what type of DNA is being extracted [4]. The more common, sodium dodecylsulfate (SDS), is used to lyse the cellular membrane and denature the histone proteins attached to the DNA strands. Dithiothreitol (DTT) is used when sperm cells are suspected to be present in a sample because it reduces protein disulfide bonds and is commonly used in differential extraction. After cells have been lysed, various steps are used for the purification and isolation of the DNA depending on which extraction method is being utilized [4].

1.3.1 Differential Extraction

Differential extraction is a common method used for the isolation of DNA from a male:female mixture sample suspected of containing sperm cells. Typically, these include samples from sexual assault kits. The purpose is to separate the sperm cells from other cell types, such as vaginal epithelial cells, contained within a single sample, as illustrated in Figure 1.3 [1]. Separation is possible because sperm cells are more difficult to lyse due to disulfide bonds that make up the membrane. This makes it possible to lyse epithelial cells without disturbing the integrity of the sperm cells. With centrifugation, the sperm cells can be spun into a pellet and the supernatant containing the lysed DNA from all other cells can be removed [1]. The pellet which contains sperm cells can then be resuspended and treated with a digest buffer, containing DTT and a high concentration of proteinase K, which will break down the disulfide bonds and further degrade proteins allowing for lysis of the cells. The two fractions, the supernatant removed from the sperm cell pellet and the lysed sperm cells (female and male fractions, respectively), can then both be treated with a wash buffer to remove other debris so that they are ready for quantitation [1].

Figure 1.3: Differential Extraction [1]. A mixture containing female epithelial cells and male sperm cells is separated into male and female fractions through multiple lysis and incubation steps.

1.3.2 Magnetic Bead Extraction

The use of magnetic beads for extraction has become a popular method due to its high recovery rate and ease of automation. Magnetic beads can be used for the purification of several biomolecules due to their ability to be coupled with a ligand [5]. The target molecule

being isolated will determine what type of ligand is used. While the ligand or functional group on the surface of the magnetic bead affects the affinity, the quantity and size of the beads determines the binding capacity [6]. Magnetic separation protocols typically include three basic steps: bind with the target molecule, wash unwanted material away, and elute the target molecule in solution for further downstream application as shown in Figure 1.4. Silica coated magnetic beads are commonly used in the forensic science field due to their affinity for DNA [5]. This is an attractive quality because the affinity is dependent upon the salt concentration that allows this affinity to be reversible. In high ionic conditions, DNA binds with the silica coated magnetic bead and in low ionic conditions the DNA is released [7]. This process involves overcoming an electrostatic potential to allow Van der Waals forces to dominate. The beads used for this process are paramagnetic and as such they exhibit magnetic properties when exposed to a magnetic field with no residual magnetism once removed from the magnetic field [5].

During a magnetic bead extraction, cells of interest are lysed with a digest buffer leaving free floating DNA in solution. Magnetic beads are then introduced to a high ionic environment and DNA binds to the surface of the beads [7]. In the manual method, magnetic stands are typically used to attract beads to one side of the tube, allowing for easy removal of unwanted supernatant [7]. Once bound, the bead-DNA complex is washed several times and then the DNA is then eluted from the magnetic beads leaving pure isolated DNA that can be quantitated and analyzed.

Figure 1.4: Magnetic bead isolation [8]. The three steps in the magnetic bead extraction process include: capture, purification, and elution.

1.4 Quantitation

Quantitation is performed to determine the quantity or concentration of DNA present in a given sample. It is important to quantitate DNA because a specific amount is needed to undergo amplification and capillary electrophoresis for forensic science applications. Laboratories are required by the QAS (Quality Assurance Standards for Forensic DNA Analysis) standard 9.4 to use quantitation kits with assays that are human specific. This standard requires the quantity of human DNA to be determined [9]. Real-time polymerase chain reaction or quantitative PCR (qPCR) monitors the target product as the process occurs. The qPCR process uses DNA quantitation standards that are important for the accuracy of the data. These standards are a dilution series that create a standard curve used for optimization [4].

There are multiple nuclease assays that can be used in qPCR to target total human DNA, total human male DNA, and to target an internal PCR control (IPC). The IPC is a synthetic DNA template that is present in each sample and indicates that the components in the assay are performing correctly, giving validation to negative results [10]. Each target is identified by labeling with different dyes, such as FAM or VIC dye. Quantifiler® Trio targets total human genomic DNA with both a small and large autosomal target. The small autosomal is

80 bases long while the large autosomal is 214 bases [10]. Due to the large autosomal being more susceptible to degradation, it can be compared to the small autosomal to provide information about the DNA degradation in a sample. Each assay contains two primers per target and one TaqMan[®] MGB probe. Each probe is labeled with a fluorescent reporter dye at the 5' end as well as a minor groove binder (MGB) and a non-fluorescent quencher (NFQ) at the 3' end of the probe as shown in Figure 1.5 [11]. The function of the MGB is to increase the melting temperature of the probe so that it is not destroyed during the heating cycles of PCR. The NFQ suppresses the emission of fluorescence of the reporter dye through a Förster type energy transfer [10]. The suppression is due to the close proximity of the reporter dye and the quencher on the probe. The process of qPCR starts with the denaturation of the template DNA in order for the target specific primers, both the forward and reverse, to anneal to the target template if present in the sample [10]. The probe anneals downstream from one of the primer sites and is cleaved by the 5' nuclease activity of the Taq DNA polymerase as the primer extends the length of the target template [11]. When the probe is cleaved at its 5' end the reporter dye is separated from the quencher dye, allowing the fluorescence from the reporter dye to be expressed; once excited by a laser, the fluorescence can be detected by the qPCR instrument. Eventually, the probe is completely cleaved from the target strand to allow the primer to extend the length of the template [11]. As additional reporter dyes are cleaved from their respective probes with each cycle, the intensity of the fluorescence signal increases and is proportional to the amount of target template in the sample [11]. The real-time PCR instrument measures the accumulation of fluorescent signal during the exponential phase of the reaction [10]. This process is how the quantity of total human and total male DNA is measured through qPCR. The reaction is arranged to have an excess amount of PCR primers and DNA polymerase, allowing the target template to be the limiting factor in the reaction [10].

There are four phases that occur during the qPCR process: lag phase, exponential phase, linear phase, and plateau phase. During the lag phase the PCR product is beginning to increase but the fluorescence is too low to be detected and a baseline is set during this phase [10]. In the exponential phase the PCR efficiency has reached 100%. As the PCR products continue to increase, the ratio of AmpliTaq Gold[®] DNA polymerase to target product decreases [10]. During the linear phase one or more PCR components have dropped below a critical concentration and amplification begins to decrease. The amplification efficiency is continually decreasing during this phase [10]. In plateau phase amplification has stopped and there is no more product being produced and no more fluorescence [10].

Figure 1.5: Quantitative PCR process using TaqMan probe [11]. The DNA template denatures allowing the forward and reverse primers to bind. The Taq MGB probe anneals downstream to the forward primer and as the primers extends the Taq DNA polymerase breaks apart the probe facilitating the detection of fluorescence.

1.5 Flow Cytometry

Flow cytometry allows for the rapid measurement and analysis of large quantities of cells to be analyzed at the single cell level, thus making it a popular technique for scientists. [12]. Flow cytometry is utilized for applications including cell sorting, cell detection, protein expression, structural and morphological characterization, along with several others. A specialized flow cytometer, a fluorescence activated cell sorter (FACS), must be used for cell sorting capabilities [13]. A flow cytometer works by using a fluidics system to carry the sample into the instrument and through the flow chamber. Inside the flow chamber the sample is surrounded by sheath fluid. The fluidics system then arranges the sample into a stream of single cells through hydrodynamic focusing as the flow chamber narrows, as seen in Figure 1.6 [12]. Once the cells form a single line inside the flow chamber, they reach an interrogation point where cells intersect with a laser. The light from the laser excites the cell

upon contact and causes photons to scatter [12]. The scattered photons are then measured as forward angle scatter (FSC) and side angle scatter (SSC) and can provide information on relative size and structure of the cell, shown in Figure 1.6. A detector in the instrument also collects the fluorescence emission produced by excited fluorophores linked to the cell [13]. The emitted photons from FSC, SSC, and fluorescence emission are converted into an electrical signal that can be processed through the electronics system in the flow cytometer. The fluidics system is a series of filters and mirrors that help to guide the photons emitted from a cell to the laser's respective detector, each laser will have a specified detector [12]. The filters include bandpass, longpass, and short pass and they are used along with the dichroic mirrors to get specific wavelengths of light to a designated detector [13]. At the detector, the photons are converted to an electric current or photocurrent that can pass through the electronics system. The electronics system involves the electric current being amplified and then converted to a voltage pulse that gets converted to a digital number [14].

Figure 1.6: Flow cytometry fluidics system and optics. Left: illustrates hydrodynamic focusing of the cells through the flow chamber [14]. Right: illustrates how forward and side scatter disperse from the cell after the cell passes the laser source [15].

Once analyzed at the interrogation point cells are pushed to a waste container. Flow cytometers can contain multiple lasers used as excitation sources. The available lasers determine the type of fluorophores that can be utilized for an experiment [14]. For cell analysis, cells must have some sort of fluorescent label. The process of attaching a fluorescent label is called staining or surface staining [13]. In some cases, a fluorescently labeled secondary

antibody must be used if looking at the interaction between a cell and a non-fluorescently labeled primary antibody. Data analysis for flow cytometry uses a principle called gating. Gating allows the analyst to put a perimeter around the target population of cells to be evaluated. Cell populations are identified utilizing forward and side scatter that provides an estimation of size [16]. As in any scientific experiment, a series of controls must be used. The unstained control is used to identify the target cell population and to determine a baseline for background fluorescence. Background fluorescence is needed because cells naturally have some level of fluorescence, called autofluorescence. An isotype control is an antibody for an antigen not found on the target cell population that can determine non-specific binding [16]. An example of an isotype control could be a fluorescently labeled secondary antibody that is not specific to the target population.

1.6 Immunomagnetic Beads

Magnetic beads used for DNA isolation can be coupled with various ligands to capture a target source. It is common to pair magnetic beads with antibodies to take advantage of antigen-antibody affinity [17]. Antibodies are proteins that are produced to fight against foreign molecules in the body but antibodies can also be used for molecule specific binding. Immunomagnetic beads have been used in applications including protein purification [18] and immunoassays [19]. There have also been several studies conducted using IMB isolation in forensic science applications. In preparing the IMBs, studies have used different methods of coupling the antibody to the magnetic beads. Li et al. used the avidin-biotin interaction to bind antibodies to magnetic beads while Zhao et al. utilized carbodiimide crosslinking for binding.

1.6.1 Carboxylic acid coated beads

The common method for coupling such antibodies with magnetic beads is the use of carbodiimide crosslinkers. Carbodiimide compounds such as 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and Dicyclohexylcarbodiimide (DCC) are used to crosslink carboxylic acids to primary amines [17]. This method is advantageous because of the readily available magnetic beads containing a carboxylic acid coating. EDC is a water-soluble carbodiimide, while DCC is a water-insoluble crosslinker [17]. The carbodiimide crosslinking mechanism works through forming an amide bond by activating carboxyl groups so that they can directly react with primary amines [17]. When EDC reacts with a carboxylic acid group an intermediate is formed that is readily attacked by a primary amine group where the amide bond is formed as shown in Figure 1.7 [17]. For improved efficiency, N-hydroxysuccinimide

(NHS) is added to the EDC coupling mechanism to produce a second intermediate that is more stable to allow ample time for the primary amine to effectively bind, shown in Figure 1.7 [17]. The amide or peptide bond is then formed between the carboxylic acid coated bead and the amine group on the antibody.

Figure 1.7: Carbodiimide Chemistry. An EDC crosslinker reacts with a carboxyl group to form an unstable intermediate. The intermediate can then be attacked by an amine to form an amide bond or form a second intermediate with NHS before forming the amide bond.

1.6.2 Streptavidin coated beads

Biotin is a vitamin present in living cells, that aids in biological processes such as cell growth and the citric acid cycle. Biotin is ideal for labeling because it is small and has a valeric acid side chain that allows it to be conjugated to other molecules without affecting the biotin-avidin function [20]. When a molecule is biotin labeled it can then be immobilized through the interaction with a biotin-binding protein and then further used to bind target molecules. Due to its size and stability, biotin labels do not commonly interfere with the labeled molecule's function. The process of attaching biotin to proteins or other molecules is called biotinylation. Avidin and streptavidin are biotin-binding proteins that have the greatest known affinity for biotin of any ligand-protein complex [21]. These proteins that bind biotin can bind up to four molecules at once, shown in Figure 1.8, making biotin an ideal label for target molecules such as proteins or antibodies [22]. However, the biotin-binding protein is often conjugated or immobilized to a surface resulting in less biotin binding sites available. The bond between avidin/streptavidin and biotin forms quickly and is known to be a strong non-covalent interaction. Once the avidin-biotin complex has formed it is not affected by extreme environments, such as pH or temperatures [23]. Streptavidin is a biotin-binding protein isolated from the bacterium *Streptomyces avidinii* [21]. Streptavidin has a lower chance of nonspecific binding compared to avidin due to the absence of glycosylation

and lower isoelectric point, making it a more attractive protein to use for some applications [20]. The high specificity of the avidin-biotin interaction makes it a commonly used tool for detection and purification of proteins [22] [23]. One application of the avidin-biotin complex is to aid in binding antibodies to magnetic beads for target protein isolation. The biotin-binding protein can be immobilized on the surface of magnetic beads that are then introduced to biotin labeled antibodies to form the avidin-biotin complex that attaches the bead to the antibody.

Figure 1.8: Avidin-biotin Interaction [24]. Avidin and streptavidin have the ability to bind up to four biotin molecules. The biotin molecules will typically be conjugated to a protein or antibody and once binding occurs the avidin-biotin complex is formed.

1.7 PH-20 Protein

The PH-20 protein is a sperm adhesion molecule (SPAM1) that is found primarily in the male reproductive organs [25]. In humans the protein acts as a hyaluronidase, an enzyme that hydrolyzes hyaluronic acid and is abundant on the surface of acrosome-intact human sperm heads [26] [27]. The sperm cell structure and acrosomal membrane is shown in Figure 1.9. Hyaluronic acid is a polysaccharide made up of repeated N-acetyl-D-glucosamine and D-glucuronic acid units and is found in the extracellular matrix of connective tissues [27] [28]. Two extracellular matrix layers surround the female egg, the first consists of cumulus cells and is abundant in hyaluronic acid, the inner layer is the zona pellucida, a thick glycoprotein-rich membrane that encloses the egg and binds spermatozoa. Hyaluronidase breaks the polysaccharides into oligosaccharides [27]. The hyaluronidase activity of the sperm cell allows it to penetrate the first extracellular layer rich in hyaluronic acid to reach the zona pellucida that is vital for fertilization [27]. Once the sperm binds to the zona pellucida the PH-20 protein migrates to the acrosomal membrane, shown in Figure 1.10 [28] [29].

The PH-20 protein is bound to the membrane on the head of the sperm via a glycosyl phosphatidylinositol (GPI)-anchor [26] [28] [31]. The primary role of the PH-20 protein is to aid in the penetration of the egg for fertilization. However, presence of the protein is not

Figure 1.9: Sperm Structure [30]. The sperm contains three sections: head, midsection, and tail. On the head is an acrosomal membrane that contains surface proteins.

necessary for fertilization to occur [25]. As sperm mature, the majority of the PH-20 protein is cleaved into an amino-terminal fragment (41-48 kDa) and a carboxyl-terminal fragment (27 kDa) where a disulfide bond links the two domains [28]. It is reported that there are six genes encoded in the human genome for hyaluronidase [27] [32]. The PH-20 protein is encoded by a gene on human 7q31 [25] [32]. Although the PH-20 protein is primarily found on the surface of mature sperm head, it has also been found in other areas [25].

Previously, PH-20 was thought to be testis-specific; however, with emergence of newer techniques such as RT-PCR and RNase-protection assays it is now known to be present in other tissues in the body. The PH-20 protein transcripts found in other areas of the human body are in a lower abundance than in the testis, with the exception of cancer cells which have an abundance of hyaluronidase activity [25]. The PH-20 in cancer cells is typically found within the body tissue and is not secreted on the outer surface of the human body [33] [34]. The protein has also been studied in the female reproductive tract of mice which showed the presence of PH-20 in the epithelium where it is secreted in the luminal fluid [25]. Therefore, it is suspected to be present in the human female vaginal tract. There is controversy regarding what the specific purpose of this protein is in the vaginal tract, but it is thought that the PH-20 protein on the surface of the sperm has some affinity for the PH-20 from the vaginal fluid [25]. There is not much research on the PH-20 protein in the female reproductive tract and the studies that have been conducted have not used human samples. This is important to take into consideration when using PH-20 as a cell isolation method because it may not be appropriate to use to isolate male cells from female cells if this protein is present in both.

Figure 1.10: PH-20 Protein in Fertilization [29]. The classical model on the left shows the PH-20 surface protein penetrating the cumulus layer surrounding the egg to attach to the zona pellucida.

1.8 Previous Research

Li et al. used IMB isolation to separate sperm cells from epithelial cells using the antibody MOSPD3 [35]. Thirty cell mixture samples were tested with a consistent concentration of female buccal cells and varying concentrations of sperm cells ranging 10^3 , 10^4 , or 10^5 cells/mL. It was found that full single-sourced profiles were able to be obtained from 80% of the mixture samples that contained 10^3 sperm cells/mL and all samples with higher concentrations [35].

In a study by Zhao et al. the objective was to find a sperm-specific protein that would allow for maximum DNA isolation of male DNA in a male and female cell mixture. Four anti-sperm antibodies were tested: anti-PH-20, anti-SP-10, anti-ADAM2, and anti-JLP [36]. It was found that the magnetic beads coupled with the anti-PH-20 antibodies recovered the most male DNA and produced the most single sourced male profiles after capillary

electrophoresis [36]. The study then compared the immunomagnetic bead isolation technique with the commonly used differential extraction method. Cell mixtures were made with a 10^5 female DNA concentration and a 10^3 male DNA concentration. Based on the results, the immunomagnetic bead method recovered more male DNA when compared to the differential extraction method [36]. However, only one male to female ratio was tested to make the comparison between IMB isolation and differential extraction. It was also not taken into consideration that an actual vaginal swab may have PH-20 proteins from the female that could interfere with the immunomagnetic bead binding to the PH-20 on the sperm. More research is necessary to test the PH-20 IMBs effectiveness for recovering sperm at different male to female ratios and to assess its ability to recover sperm from vaginal swabs where competitive binding could occur.

1.9 Purpose, Goals, and Objectives

Separating DNA mixtures has been an area of forensic research for several years. One of the most common sources of DNA mixtures are the samples collected in sexual assault kits. Vaginal swabs are collected in a sexual assault kit along with test tubes, microscope slides, hairs, and fibers [37]. When a vaginal swab is taken, there is a vastly greater concentration of female epithelial cells versus sperm cells from a perpetrator, when present. Therefore, it is important to separate the male fractions from the female fractions in order to recover as much of the male fraction as possible from the DNA mixture to find a perpetrator [1].

As techniques have advanced, the demand on laboratories for DNA analysis has increased. Automation of techniques has helped to increase efficiency and throughput. More specifically, the use of magnetic beads has been used to purify DNA from samples due to its low cost and ease of automation. Typically, magnetic beads are used by exploiting the ionic conditions under which DNA will bind to silica coated beads and can then be separated from other debris [5]. Magnetic beads are a versatile product in that they can be coupled with a variety of substances including a silica coating or various antibodies. Several studies have explored techniques to target testis specific molecules to isolate sperm from DNA mixtures [35] [36]. This study targeted a protein that is not testis specific but is intrinsic to the sperm head and has shown recovery capability in previous research efforts [36]. The sole purpose of DNA analysis on a sexual assault kit is to obtain the male suspect's DNA profile and as long as this is the case, efforts will continue to be made to improve the recovery capability from DNA mixtures. When a sexual assault occurs and male DNA is present, forensic DNA analysts need to be able to recover that male DNA no matter how little is left behind. The obstacle with these types of cases is the large quantity of female DNA collected that can obscure

the recovery of the male perpetrator DNA left behind when it is in such a low quantity. Until there is a way to fully and consistently recover the male DNA, in any quantity, from a male:female mixture research will continue to optimize this process through any available means.

The goal of this study sought to evaluate the efficiency of magnetic beads coupled with anti-PH-20 antibodies in isolating sperm cells from DNA mixtures. The objectives of this research study were as follows:

1. Evaluate the efficiency of immunomagnetic beads in recovering sperm from mixtures containing both male and female DNA at different ratios.
2. Evaluate the presence of the PH-20 protein in the vaginal lining to see if it competes and inhibits the binding of the PH-20 protein on the sperm surface.
3. Evaluate if the PH-20 immunomagnetic bead isolation method recovers more male DNA from DNA mixtures when compared to the differential extraction method.

The purpose of this study was to take the findings of Zhao et al. [36] and take it a step further to see if using the carboxylic acid coated magnetic beads bound to PH-20 antibodies were more successful at recovering male DNA from mixture samples compared to the widely used differential extraction. During the execution of this study several obstacles arose and the main objective did not end up being feasible. In a sample containing semen only the IMBs recovered low quantities of DNA leaving behind the majority of the DNA to be removed in the supernatant. When tested in mixture samples IMB isolation resulted in both male and female DNA. Due to the low recovery of the IMBs in both single source and mixture samples, the IMB isolation method was not compared to the differential method. A series of small tests were run to investigate different factors that could potentially be the issue for why the IMBs were not recovering the expected amount of DNA. The testing of several factors including elution methods, magnetic beads without antibodies, and epithelial cell lysis methods led to hypothesizing that bead type affected DNA recovery. The study ultimately concluded by comparing two bead types to determine if one outperformed the other.

2. Methods

The sample size of this study included five female and five male participants. Samples were collected in accordance with the WVU Internal Review Board (1708721028). Each male participant was provided a sterile 50 mL tube to collect their semen sample and a Styrofoam cooler with an ice packs for storage and preservation of the sample. Five female participants were provided three cotton tipped swabs in a Styrofoam cooler with an ice pack for the collection of vaginal epithelial cells. Vaginal swabs were collected at the WVU Student Health Center by a Women’s Health Nurse to attempt to mimic real-world samples taken in a sexual assault kit. Each female provided three vaginal swabs to make a total of 15 vaginal swabs. The five female participants also provided two buccal swabs. Each sample was marked with an identifying number to ensure anonymity of all participants.

2.1 Cell Preparation

2.1.1 Cell Agitation

All vaginal swabs and buccal swabs collected from female participants were agitated into solution using the following procedure:

1. Break off the cotton tipped swab into a microcentrifuge tube, add 500 μ L nuclease free water.
2. Incubate at 56°C for 30 minutes, vortex every 10 minutes.
3. Remove the swab from the microcentrifuge tube and place the swab in a spin basket, centrifuge at 14,000 rpm for 5 minutes.
4. Transfer the lysate from the spin basket to the original microcentrifuge tube with cell solution.

2.2 Immunomagnetic Bead Preparation, Isolation, & Analysis

2.2.1 Carboxylic Acid Coated Beads

Magnetic beads coupled with anti-PH-20 antibodies cannot be readily purchased. Immunomagnetic beads using carboxylic acid coated beads were prepared using the following methodology: [36] [38]

Activation of Beads

1. Dynabeads[®] M-270 Carboxylic Acid (Thermo Fisher Scientific) are washed twice with 25mM MES, pH 5, for 10 minutes with good mixing.
2. Immediately before use, dissolve 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) in cold 25mM MES, pH 5 to a concentration of 50 mg/mL.
3. Prepare a 50 mg/mL solution of N-hydroxysuccinimide (NHS) in 25mM MES, pH 5.
4. Add 50 μ L of EDC solution and 50 μ L of NHS solution to the Dynabeads[®], mix well and incubate with slow tilt rotation at room temperature for 30 minutes.
5. After incubation, place the tube on the magnet for 4 minutes and remove the supernatant. Wash twice with 100 μ L of 25mM MES, pH 5. The Dynabeads[®] are now activated.

Immobilization of ligand (anti-PH-20 antibody) after activation

1. Add 60 μ g of anti-PH-20 antibody to activated Dynabeads[®], vortex to mix.
2. Incubate for at least 30 minutes at room temperature with slow tilt rotation.
3. Place the tube on the magnet for 4 minutes and remove the supernatant.
4. Incubate beads in 50 mmol/L Tris (pH 7.4) for 15 minutes to block the unreacted carboxylic acid groups.
5. Wash three times with reaction buffer (PBS) and suspend in 100 μ L of reaction buffer.

2.2.2 Streptavidin Coated Beads

To prepare streptavidin coated magnetic beads coupled to biotinylated PH-20 antibodies, Dynabead® M-280 Streptavidin (Thermo Fisher Scientific) were purchased and the manufacturer recommended protocol for immobilizing antibodies was followed as described below. The binding capacity of the magnetic beads for biotinylated antibodies is 10 μg of antibody per one mg of beads [39].

Wash Magnetic Beads

1. Resuspend beads in vial by vortexing for 45 seconds.
2. Transfer 500 μL of beads into a new microcentrifuge tube.
3. Add 1000 μL of PBS buffer, mix by vortexing for 5 seconds.
4. Place microcentrifuge tube on a magnet for 1 minute and discard the supernatant.
5. Remove microcentrifuge tube from the magnet and resuspend the washed beads in 500 μL of PBS buffer.

Immobilize Antibodies

1. Incubate beads and biotinylated antibodies (200 μL) in PBS buffer for 30 minutes at room temperature using gentle rotation.
2. Separate the antibody-coated beads with a magnet for 3 minutes.
3. Wash coated beads 5 times with PBS buffer containing 0.1% BSA.
4. Resuspend in 500 μL of PBS buffer containing 0.1% BSA.
Note: Resuspension volume depends on desired concentration.

2.2.3 Immunomagnetic Bead (IMB) Isolation

To isolate sperm cells from single source or mixture samples the following protocol was followed: [36] [38]

1. Add 10 μL of CA-IMBs or desired amount of SA-IMBs to 100 μL of cell mixture.
2. Incubate for 60 minutes at room temperature with slow tilt rotation.
3. Place the tube on a magnet for 2 minutes and remove the supernatant.

4. Wash IMB-cell complexes with 100 μ L of PBS buffer containing 0.1% BSA (x3).
5. Store in 100 μ L of PBS buffer containing 0.1% BSA.

2.2.4 DNA Extraction

This DNA extraction protocol has been modified from the manufacturer recommended protocol from Qiagen, QIAamp[®] DNA Investigator Handbook [40].

1. To the IMB-cell complexes, add 280 μ L Buffer ATL, 10 μ L proteinase K, and 10 μ L 1 M DTT (if sperm cells are present). Close the lid and vortex for 10 seconds.
2. Place the 2 mL tube in a water bath at 56°C for 1 hour. Vortex the tube for 10 seconds every 10 minutes to improve lysis. Briefly centrifuge the tube.
3. Add 300 μ L Buffer AL and vortex for 10 seconds.
4. Place the tube in a water bath and incubate at 70°C for 10 minutes. Vortex for 10 seconds every 3 minutes. Briefly centrifuge the tube.
Note: If extracting straight from IMBs, follow steps 5 and 6. If not extracting from IMBs, skip to step 7.
5. Place the tube on a magnet for 2 minutes.
6. Remove the supernatant (containing the lysed cells) and transfer into a new 2 mL tube.
7. Add 150 μ L ethanol (96-100%); vortex for 15 seconds. Briefly centrifuge the tube.
8. Transfer the entire lysate to the QIAamp MinElute column without wetting the rim.
9. Centrifuge at 10,000 rpm for 1 minute. Place the QIAamp MinElute column in a clean 2 mL collection tube, and discard the collection tube containing the flow-through.
10. To the QIAamp MinElute column, add 500 μ L Buffer AW1 without wetting the rim. Centrifuge at 10,000 rpm for 1 minute. Place the QIAamp MinElute column in a clean 2 mL collection tube, and discard the collection tube containing the flow-through.
11. Add 700 μ L Buffer AW2 without wetting the rim. Centrifuge at 10,000 rpm for 1 minute. Place the QIAamp MinElute column in a clean 2 mL collection tube, and discard the collection tube containing the flow-through.

12. Add 700 μL of ethanol (96-100%) without wetting the rim. Centrifuge at 10,000 rpm for 1 minute. Place the QIAamp MinElute column in a clean 2 mL collection tube, and discard the collection tube containing the flow-through.
13. Centrifuge at 14,000 rpm for 3 minutes to dry the membrane completely.
14. Place the QIAamp MinElute column in a clean 2 mL microcentrifuge tube and discard the collection tube containing the flow-through. Open the lid and incubate at room temperature (15-25°C) for 10 minutes.
15. Add 50 μL Buffer ATE to the center of the column.
16. Close lid and incubate at room temperature for 5 minute. Centrifuge at 14,000 rpm for 1 minute.

2.2.5 DNA Quantitation

The manufacturer recommended protocol for the Quantifiler[®] Trio DNA Quantification Kit, by Applied Biosystems was followed for all quantitation.

2.3 Concentration Estimation

To get an estimated concentration of DNA in an aliquot of biological fluid, five replicates of 10 μL were taken from the respective sample and extracted following the extraction protocol listed in Section 2.2.4. Once extracted, replicates were quantitated following the manufacturer recommended protocol for the Quantifiler[®] Trio DNA Quantification Kit, by Applied Biosystems. DNA quantities for the five replicates were averaged and a confidence interval was calculated using the median absolute deviation (MAD), with $\alpha = 0.05$, shown in Equation 2.1. When determining variance, MAD was used because it is robust and resistant to outliers.

$$\bar{X} \pm t_{\frac{\alpha}{2}} \sqrt{s^2 + \frac{s}{n}} \quad (2.1)$$

2.4 Immunomagnetic Bead Testing

Described below are the series of tests run to assess different factors in efforts to optimize the recovery of DNA by the IMBs. For all tests described, extraction and quantitation were

carried out after IMB isolation. At the beginning of the testing phase samples were vortexed during the IMB isolation procedure. From literature [41] vortexing was found to potentially break the bond between the sperm-IMB complexes and was therefore no longer used as a means of mixing the sample. For samples where vortexing was used, it will be denoted in the section.

2.4.1 Cell Elution Methods

This test was run to compare different elution incubation times from the CA IMBs. Four samples were prepared with 20 μ L of semen from one male participant and 80 μ L of nuclease-free water. The IMB isolation protocol 2.2.3 was followed with the addition of an elution step after step 4. After washing the the IMB-cell complexes, 100 μ L of Buffer ATE (Qiagen) was added and incubated at 65°C for either 10, 15, or 20 minutes. After incubation, the tubes were placed on a magnet and the supernatant was transferred into a new tube (labeled elute). Once the elute was transferred into another tube, reaction buffer was added back to the IMBs and both the elute and IMB samples for each time interval were extracted following the protocol in Section 2.2.4. For the sample incubated for 10 minutes, the supernatant was removed after IMB isolation was saved (labeled SW) and that sample was also extracted. The fourth sample prepared for this test did not go through an elution step, Section 2.2.3 was followed and the DNA was extracted straight from the beads. All samples generated were quantitated following the protocol in Section 2.2.5 and are listed in Table 2.1. Samples in this test were vortexed during IMB isolation.

Table 2.1: List of samples run to investigate the affect of elution incubation time on IMB DNA recovery.

Sample Name	Elution Incubation Time (min.)	Sample Type
T-10 Elute	10	Elute
T-10 IMB	10	IMBs after elution
T-10 SW	N/A	Sample waste removed after IMB isolation
T-15 Elute	15	Elute
T-15 IMB	15	IMBs after elution
T-20 Elute	20	Elute
T-20 IMB	20	IMBs after elution
Bead Ex IMB	N/A	No elution step

2.4.2 Immunomagnetic Beads in Cell Mixtures

To test DNA recovery in mixture samples, two 1:1 mixtures (by volume) were used for IMB isolation. The IMB isolation protocol was followed as described in Section 2.2.3, these samples were vortexed during IMB isolation. A separate 1:1 mixture sample was extracted using differential extraction following the QIAamp DNA Investigator Kit protocol for Isolation of Total DNA from Sexual Assault Specimens. All three samples run during this test contained 20 μL of semen, 20 μL of vaginal cells, and 60 μL of nuclease-free water. Due to the presence of epithelial cells, an epithelial lysis step was used after IMB isolation to lyse any epithelial cells that may have been picked up by the IMBs. These steps were added after the IMBs were washed (step 4) in Section 2.2.3. The following were the additional steps for epithelial cells lysis:

1. Add 100 μL SDS to the sample, incubate at 37°C for 30 minutes.
2. Place tube on magnet for 2 minutes and remove the supernatant.
3. Store in 100 μL of PBS buffer containing 0.1% BSA.

2.4.3 Female Cell Lysis

To evaluate the best procedure for lysing epithelial cells from the IMBs, different variations of the lysing protocol were tested. The variations included: lysing before or after IMB isolation, incubation time (30 minutes or 60 minutes), incubation temperature (37°C or 56°C), and addition of proteinase K with the SDS. For the samples in which epithelial cells were lysed before the IMB isolation, the epithelial lysis protocol was performed before the IMB's were added to the sample for the isolation protocol. These alterations of procedure were implemented in the protocol for epithelial cell lysis in Section 2.4.2. The samples used for this test are described in detail in Table 2.2.

Table 2.2: List of samples run to investigate the effects of various factors (order of lysis, presence of proteinase K, and incubation temperature and time) on the lysing of female cells. All samples contained 30 μL of semen, 30 μL of vaginal epithelial cells, and 40 μL of nuclease-free water.

Sample Name	Composition M:F volume (μL)	Lysis Step	Proteinase K	Incubation Temperature ($^{\circ}\text{C}$)	Incubation Time (min.)
LB-30	30:30	Before	Yes	56	30
LB-60	30:30	Before	Yes	56	60
LA-K56	30:30	After	Yes	56	30
LA-S37	30:30	AFter	No	37	30

2.4.4 DNase as a Lysing Step

As an alternative to lysing the female epithelial cells from the cell mixtures, a DNase step was tested to evaluate if using a DNase procedure instead of an SDS lysis procedure yields more DNA. The DNase degrades any free floating DNA that may be attached to cells isolated by the IMBs after IMB isolation is complete. Ten samples were prepared for this test all containing 20 μL of semen, 30 μL of vaginal epithelial cells, and 80 μL of nuclease-free water. Five of the samples went through regular IMB isolation described in Section 2.2.3 and straight to extraction (labeled NoDN), the other five samples were processed with the addition of the DNase procedure (labeled DN) before going to extraction. The DNase steps were added after the IMBs were washed (step 4) in Section 2.2.3, the following were the additional steps for the DNase procedure:

1. Add 7 μL of nuclease-free water, 1 μL of DNase1 digest buffer, and 1 μL of DNase.
2. Incubate at 37°C for 30 minutes.
3. Add 1 μL of 50 mM EDTA to terminate digest.
4. Incubate at 65°C for 10 minutes.

2.4.5 Immunomagnetic Beads with Female Cells

To evaluate the recovery of female epithelial cells by the IMBs two tests were run. First, samples containing female cells in the absence of sperm cells were tested and then mixture samples were tested to evaluate the affect of changing the epithelial cell type. Vaginal epithelial versus buccal epithelial was tested because there is research that shows the PH-20 protein may be present in female vaginal fluid [25]. Therefore, if the IMBs were binding vaginal cells due to the presence of the PH-20 protein then a mixture of semen and buccal cells may result in less competitive binding with the IMBs leading to higher male DNA recovery. Four samples were prepared for the vaginal versus buccal epithelial test. For the two female only samples the IMB isolation protocol in Section 2.2.3 was performed. The two samples analyzed with female only were run during separate tests. The mixture samples went through both the IMB isolation protocol along with the DNase alteration in Section 2.4.4. Samples are detailed in Table 2.3.

Table 2.3: List of samples run to investigate female epithelial cells affect on IMB recovery. All samples had nuclease-free water added to a final volume of 100 μ L. Sample V-ISO IMB was vortexed during IMB isolation.

Sample Name	Volume Female (μ L)	Volume Male (μ L)	Female Epithelial Cell Type	Lysis Step
Fe-Ab	30	0	Vaginal	N/A
V-ISO IMB	20	0	Vaginal	N/A
S:V1	30	20	Vaginal	DNase
S:V 2	30	20	Vaginal	DNase
S:B 1	30	20	Buccal	DNase
S:B 2	30	20	Buccal	DNase

2.4.6 Homogenizing Semen Samples

Due to inconsistent data obtained from the test evaluating DNase as a lysis method, an attempt was made to see if non-homogeneous semen was responsible. In an effort to test this hypothesis a 1:1 mixture was prepared with a single semen sample and PBS buffer. Six 20 μ L samples were prepared with the semen-PBS mixture and were brought to a final volume of 100 μ L with nuclease-free water. The samples went through IMB isolation following the protocol in Section 2.2.3. One sample was left out and went through extraction and quantitation without IMB isolation as a control.

2.4.7 Magnetic Beads' Ability to Bind Cells

The Dynabeads® (Thermo Fisher Scientific), without antibodies, were tested with various samples both male:female mixtures and male only. They were put through the same IMB isolation protocol as described in Section 2.2.3. Four samples were tested with this protocol: two samples were 1:1 mixture samples (by volume) and two were semen only as listed in Table 2.4. Samples were run at different times during separate tests, accounting for variation of their composition. This test was run to determine if the magnetic beads themselves recover cells from samples. No effort was made to separate the male and female portions of the mixture samples that were run. Sample "Bead" in this group was vortexed.

Table 2.4: List of samples prepared to test the Dynabeads[®] M-270 Carboxylic Acid without antibodies. Samples varied in cell composition and volumes, concentration of DNA was unknown. All sample final volumes were 100 μ L, nuclease-free water was used to get to the final volume.

Sample Name	Sample Composition
Bead	20 μ L male + 20 μ L female
T-NoAb	30 μ L male + 30 μ L female
NoAb	20 μ L male (1:1) with PBS
Sem-NoAb	30 μ L male

2.4.8 Antibody Ability to Bind Cells

Microscopy

Microscopy was used to confirm binding between the sperm and IMBs. To accomplish this, sample “3139_S1” was prepared with 60 μ L semen and 40 μ L of nuclease-free water. Immunomagnetic bead isolation was performed following Section 2.2.3. After the wash step, 10 μ L of PBS was added to the beads and mixed, then 5 μ L of the beads was removed and placed into a new tube “Stain S1”. Christmas tree staining was completed on “Stain S1” and was then observed under microscopy. Approximately 4 sperm cells were visualized attached to beads under the microscope, but the majority of the beads had no sperm bound. This led to the need to show that the antibodies being used do bind to sperm cells. To accomplish this, flow cytometry was used to show the binding ability of the antibodies.

Flow Cytometry

Sperm cells and epithelial cells were both tested using flow cytometry on the LSR Fortessa to determine their ability to bind with primary antibodies. All cells were tested with the anti-SPAM1 antibodies (ab193009) previously used and with SPAM1/PH20 antibody-Biotin (LSC370366) used later in this study. The primary antibodies being used were not fluorescently labeled, therefore, fluorescently labeled secondary antibodies were used to achieve flow cytometry results. The antibodies used for flow cytometry are described in Table 2.5. Three types of samples were run: unstained control, secondary only control, and the test sample. An unstained control was run for each cell type and a secondary only control was run for each antibody being tested.

Table 2.5: List of antibodies tested during flow cytometry. The secondary antibodies listed for each primary antibody were used for fluorescent purposes to be detected by the LSR Fortessa. Alexa647 dye was detected with the 628nm laser and the Anti-Rabbit IgG used a Phycoerythrin (PE) dye detected with the 561nm laser.

	Anti-SPAM1 Antibody	SPAM1\PH20 Antibody - Biotin
Catalog Number	ab193009	LS-C370366
Sequence	aa173-222	aa36-490
Concentration	0.5 mg/mL	0.25 mg/mL
	R-Phycoerythrin AffiniPure	
Secondary Antibody	F(ab') Fragment Donkey Anti-Rabbit IgG (H+L)	Alexa Fluor® 647 Streptavidin

Preparing Cell Samples for Flow Cytometric Analysis

A 1:1 aliquot was prepared with 500 μ L of semen sample 1128 and 500 μ L of PBS buffer, pH 7.4. Three separate unknown concentrations of semen were tested, attempting to target 1×10^6 , 500,000, and 100,000 sperm cells. A normal range for sperm count among males is 15 million to 200 million sperm per milliliter of seminal fluid [42]. The concentrations were made based on the assumption that there are approximately 100 million sperm cells per milliliter. For epithelial cell preparation, again three unknown concentrations were used at volumes of 100 μ L, 80 μ L, and 30 μ L. The unstained controls and the secondary only samples for both cell types were prepared with the highest concentrations. All samples had a final volume of 100 μ L.

Surface Staining of Cells in Tubes for Flow Cytometric Analysis [43]

1. Pellet cells by centrifuging at max speed for 3 minutes.
2. Remove supernatant, leaving 30 μ L behind to resuspend.
3. Wash cells with 1 mL PBS with 0.5% BSA (Reaction Buffer).
4. Remove supernatant, leaving 30 μ L behind to resuspend.
5. Add 100 μ L of primary antibodies (1 μ g) to appropriate tube, incubate on ice for 30 minutes in the dark.
6. Add 1 mL Reaction Buffer and pellet cells.
7. Remove supernatant, leaving 30 μ L behind to resuspend.

8. Add 100 μL of secondary antibodies to appropriate tube, incubate on ice for 30 minutes in the dark.

Note: The R-PE antibody was diluted to a dilution range of 1:25 and the Alexa647 was diluted to a concentration of 1 ng/ μL based on product specifications.

9. Add 1 mL Reaction Buffer and pellet cells.
10. Remove supernatant, leaving 30 μL behind to resuspend.
11. Wash cells two more times with Reaction Buffer.
12. Add 100 μL 0.4% paraformaldehyde in PBS, incubate overnight at 4°C in the dark.
13. Day of analysis: pellet cells and resuspend in 300 μL of Reaction Buffer.
14. Store in the dark at 4°C until ready to use.

2.4.9 Bead Type

Throughout this research the IMBs being used were carboxylic acid (CA) coated beads crosslinked to PH-20 antibodies. To evaluate if a different bead mechanism would be superior at recovering DNA from samples, streptavidin (SA) coated beads were used to bind to biotin labeled PH-20 antibodies. This test was performed to evaluate which bead-antibody mechanism recovers the most male DNA. The CA IMBs were prepared following the protocol in Section 2.2.1 and the SA IMBs were prepared following the protocol in Section 2.2.2. Once both bead types were prepared they underwent the same IMB isolation protocol outlined in Section 2.2.3. There were two tests comparing the different bead types. The first test was run with semen only samples containing 60 μL of semen (1128) and 40 μL of nuclease-free water. The prepared SA IMBs had a different bead-antibody concentration than the CA beads. To optimize the procedure two SA IMB conditions were tested, one condition kept the quantity of beads (0.3 mg) comparable to the CA IMBs and the second condition kept the antibody quantity (6 μg) comparable to the CA IMBs. Five replicates were run per bead type condition (CA IMB, SA-bead IMB, SA-antibody IMB). A control sample of 60 μL of semen was extracted with no IMB isolation.

To further compare CA IMBs to SA IMBs, another test was run with a smaller volume of cells and a known concentration. For this test three cell types were tested: semen, vaginal epithelial cells, and male:female (M:F) mixtures. To determine the concentration of an aliquot of cells for both the male and female samples the procedure in Section 2.3 was followed. The intervals calculated using Equation 2.1 under Section 2.3 were used to take into account the variation within a single aliquot of sample. The assumed concentration

of the aliquot was the average of the five replicates found in Table 2.6. The male semen (3139) aliquot was diluted, based on the average small autosomal quantity, to the same concentration in ng/ μ L as the female vaginal epithelial cell (3630) aliquot. After dilution the male and female small autosomal concentrations were 13.65 μ L and the Y was 10.06 μ L. Once both male and female aliquots were the same concentration, mixture samples were prepared with 30 μ L of male, 30 μ L of female and 40 μ L of nuclease-free water. Due to the discrepancy in the volumes used to prepare the single cell samples versus the mixture samples an adjustment was made to scale the results. The adjustment was made based on the assumption that the sample aliquots had homogenous DNA concentrations. This was done by dividing all DNA quantities by the volume of sample added and then dividing by 20 μ L.

Table 2.6: Results of the concentration estimation test. The average starting concentration is the average concentration before the male sample was diluted.

Participant ID	Average Starting Concentration (μ L)	Confidence Interval (μ L)
3139 (M)	18.132 (Sm) 13.362 (Y)	6.951 – 29.313
3630 (F)	13.65 (Sm)	8.688 – 18.612

Each cell type was tested with both the CA IMBs and the SA IMBs in replicates of five. The controls for both the semen and epithelial cells was 20 μ L of each going through extraction and quantitation without any IMB isolation. The control for the mixture samples was a differential extraction. For an extra quality control measure 20 μ L of each bead type was extracted and quantitated to assure that the IMBs themselves were not contaminated. Once both bead types were prepared as previously described, the samples underwent IMB isolation as described in Section 2.2.3. For the volume of SA IMBs used during isolation, the bead quantity was kept the same. There was no female lysis step used for the mixture samples.

2.4.10 Statistical Analysis

Mann-Whitney

The Mann-Whitney-Wilcoxon (MWW) test is a nonparametric test that compares two independent groups when data is not normally distributed and there is a small sample size (n). Often, it is seen as an alternative to a two independent-samples t-test, and can be used in the presence of non-normality. With some assumptions, specifically that the two

samples differ only in location (i.e., they otherwise have the same shape and variance), then the MWW test can be considered as a test on the difference of medians. This procedure pools the observations from the two populations and then ranks each observation from low to high. Once ranked the values for each population are summed (R = sum of the ranks) and then a test statistic (U) is calculated as shown in Equation 2.2 [44]. To compare the use of DNase versus no female lysis procedure a Mann-Whitney test was used due to the small sample size and observation of an extreme outlier.

$$U_1 = n_1 n_2 + \frac{n_1(n_1 + 1)}{2} - R_1 \quad (2.2)$$

Analysis of Variance

Analysis of variance (ANOVA) evaluates differences between means by taking into account the variance between groups and also the variance within a given population. The purpose of ANOVA is to compare sample means from multiple (>2) populations [45]. The null hypothesis is that the population means are all equal, while the alternative hypothesis is that the population means are not equal as shown in Equation 2.3. When the null hypothesis is rejected there are at least two means that differ [45]. ANOVA does not specify which population means are different, therefore, a post hoc analysis must be run to assess all possible pairings of the populations in question. A post hoc analysis will determine which population means differ from one another [45].

$$\begin{aligned} H_0 : \mu_1 &= \mu_2 = \mu_i \\ H_1 : \mu_1 &\neq \mu_2 \neq \mu_i \end{aligned} \quad (2.3)$$

Assumptions of ANOVA include residuals of each population must be normally distributed and each population must have equal variance. The populations also must be independent of one another because there needs to be two independent estimates of the variance to be used as the ratio in the test statistic [45]. When the populations are normally distributed the MSB/MSW ratio (mean square between/mean square within) is the required F distribution which represents the distribution of the ratio of variances. The assumption of equal variance is necessary for the MSB/MSW ratio to be valid as the test statistic for the given hypothesis [45]. However, the ANOVA test is known to be robust and can handle some deviation from normality and equal variances without a significant effect. If there are largely skewed distributions or extreme outliers, the test results could be biased [45].

ANOVA was used to statistically compare bead types. The first portion of the bead type test was compared using one-way ANOVA with bead type (CA IMB, SA-bead IMB, SA-antibody IMB) as the independent variable. Two-way ANOVA was used to compare the second portion of the bead type test to evaluate bead type and cell type as the independent variables. This will determine if there is an interaction between bead type and cell type on quantity of DNA recovered by the IMBs. If the interactions between factors was significant the simple effects were tested, which is testing the levels of one factor within the levels of the other factor. To adjust for multiple comparisons, the Benjamini-Yekutieli (BY) method was used. The BY method controls the False Discovery Rate, so that the expected number of false positive comparisons is maintained at the selected significance level.

3. Results and Discussion

3.1 Immunomagnetic Bead Testing

3.1.1 Cell Elution Methods

This test was run to compare different elution incubation times. Samples had 20 μL of semen and the incubation times were, 10, 15, and 20 minutes. All these samples were vortexed at some point in the process either during or after IMB isolation which was later found to break the sperm-antibody complex which may account for the resulting low DNA quantities. Table 3.1 exhibits that eluting the cells from the beads does not recover all the cells bound to the beads which can be seen by comparing the eluted sample to its respective IMB sample. Comparing the eluted samples from incubation times 10, 15, and 20 minutes did not appear to show much of a difference in DNA quantity. It was determined from this test that the best way to recover all the DNA picked up by the IMBs was to extract straight from the beads without an elution step. As shown by sample “T-10 SW” (the first wash removed after IMB isolation) there are more cells in the sample that are not being recovered by the beads. The beads were not recovering the majority of sperm cells in the sample.

Table 3.1: Using low pH solution to elute cells from IMBs. Different incubation times were used to determine the optimal elution procedure. An elution incubation time of 15 minutes resulted in the highest DNA quantity of the three times tested. The wash sample T-10 SW was the lysate removed from the IMBs after the isolation procedure, which has the highest quantity of DNA.

Sample Name	Large Autosomal ng/ μ L	Small Autosomal ng/ μ L	Y Chromosome ng/ μ L
T-10 IMB	0.14	0.09	0.10
T-10 Elute	0.44	0.30	0.31
T-10 SW	8.01	7.46	6.38
T-15 IMB	0.95	0.69	0.63
T-15 Elute	1.13	0.80	0.75
T-20 IMB	0.24	0.14	0.18
T-20 Elute	0.49	0.29	0.29
Bead Ex IMB	0.53	0.31	0.35

3.1.2 Immunomagnetic Beads in Cell Mixtures

Immunomagnetic beads were tested with 1:1 (male:female by volume) samples. The results in Table 3.2 show that the IMBs in a 1:1 mixture only recover low quantities of DNA, while the differential extraction method recovers a larger quantity of male DNA. These samples were vortexed during the process of isolation which may contribute to the low DNA recovery for the IMB samples. Assuming the quantity of starting DNA was approximately the same, the IMBs only recovered 0.84% of what the differential method recovered via the procedure used. Along with being vortexed, the lysis procedure used may have contributed to the low DNA quantities.

Table 3.2: Immunomagnetic beads were tested in male:female mixtures and compared to the male sample recovered from a differential extraction. The male differential sample produced a large quantity of male DNA and the IMB samples produced very low quantities of male DNA.

Sample Name	Large Autosomal ng/ μ L	Small Autosomal ng/ μ L	Y Chromosome ng/ μ L
1:1 IMB	1.47	1.03	0.42
1:1 IMB2	0.58	0.34	0.31
1:1 Diff-male	35.17	36.53	43.13

3.1.3 Female Cell Lysis

Mixture samples were tested with variations of the lysis procedure, both before IMB isolation (LB) and after IMB isolation (LA). The samples that were lysed before isolation produced DNA quantities that were slightly higher than the samples lysed after IMB isolation. However, overall none of the lysis variations produced over 1 ng/ μ L of DNA in Y or small autosomal targets, shown in Table 3.3. This may be a result of the long incubation times and high temperature. It is possible that sperm cells bound to the IMBs were becoming unbound during these steps.

Table 3.3: Mixture samples were used to test different variations of the female lysis procedure. Lysing before IMB isolation produced slightly higher DNA recovery. Overall, all lysis procedure variations resulted in low DNA quantities.

Sample Name	Large Autosomal ng/ μ L	Small Autosomal ng/ μ L	Y Chromosome ng/ μ L
LB-30	0.78	0.54	0.46
LB-60	1.07	0.92	0.64
LA-K56	0.16	0.10	0.07
LA-S37	0.77	0.56	0.21

3.1.4 DNase as a Lysing Step

The DNase step was used as an alternative to lysing the female cells via SDS. Comparing the use of DNase to the use of no lysis procedure the samples with no lysis step produced the highest DNA quantities shown in Table 3.4. Results from a Mann-Whitney test concluded that for participants 7238 (M) and 0001 (F) using DNase versus using no lysis procedure do not behave similarly ($p=0.0079$). This can be further visualized in Figure 3.1. It was also seen that the sample “NoDN2” may be an outlier as it produced a high quantity of DNA compared to the rest of the “NoDN” samples. Removing the possible outlier from the statistical calculation still results in a significant difference between groups ($p=0.0159$).

Table 3.4: Mixture samples were used to test DNase as a lysis alternative. Overall the samples that did not go through the DNase procedure produced the highest DNA quantities ($p=0.0079$).

Sample Name	Large Autosomal ng/ μ L	Small Autosomal ng/ μ L	Y Chromosome ng/ μ L
NoDN1	3.29	2.76	2.52
NoDN2	11.70	11.23	11.33
NoDN3	3.44	3.49	1.85
NoDN4	3.21	3.58	2.44
NoDN5	2.75	2.65	1.72
DN1	0.52	0.46	0.27
DN2	1.16	1.20	0.88
DN3	1.05	0.97	0.79
DN4	0.92	0.93	0.69
DN5	1.01	0.95	0.67

Figure 3.1: Boxplot of samples treated with DNase versus no lysis treatment. The DNase group resulted in lower DNA quantities overall.

3.1.5 Immunomagnetic Beads with Female Cells

Vaginal cells were also tested with the IMBs. These samples simply showed that female cells were being recovered by the IMBs even in the presence of no sperm cells shown in

samples “Fe-Ab” and “V-ISO”. Vaginal epithelial versus buccal epithelial did not show much difference in the amount of male DNA recovered. Detailed results shown in Table 3.5.

Table 3.5: Samples were prepared to asses recovery of female epithelial cells with IMBs. Female only samples, Fe-Ab and V-ISO, show that IMBs recover epithelial cells. Comparing the buccal mixtures to the vaginal cell mixtures, there was little difference in the quantity of male DNA that is recovered from the mixtures.

Sample Name	Large Autosomal ng/ μ L	Small Autosomal ng/ μ L	Y Chromosome ng/ μ L
Fe-Ab	12.16	8.48	0.00
V-ISO	5.88	4.20	0.00
S:B1	1.98	1.80	1.41
S:B2	2.24	2.08	1.53
S:V1	3.03	2.99	1.76
S:V2	1.34	1.05	0.83

3.1.6 Homogenizing Semen Samples

Assuming the homogeneity of the 1742 semen aliquot, a 20 μ L sample of 1742 with PBS had approximately 11.27 ng/ μ L of male DNA. The five replicates had an average recovery of 2.34 μ L. Given the assumption for sample 1742, the IMBs recovered 20.76% of the DNA available in the 20 μ L samples.

Table 3.6: Five 20 μ L semen replicates run with IMB isolation recovered between 1.66-2.94 ng/ μ L of male DNA. The 20 μ L sample that did not do through IMB isolation contained 11.27 ng/ μ L of male DNA.

Sample Name	Large Autosomal ng/ μ L	Small Autosomal ng/ μ L	Y Chromosome ng/ μ L
POS 1	3.41	2.71	2.85
POS 2	2.21	1.72	1.66
POS 3	2.72	2.31	2.35
POS 4	3.37	2.95	2.94
POS 5	2.20	1.87	1.90
1742_Sem_20 μ L	12.61	9.88	11.27

3.1.7 Magnetic Beads Ability to Bind Cells

Samples “Bead” and “T-NoAb” used magnetic beads without antibodies in place of IMBs with 1:1 mixture samples. The magnetic beads picked up both male and female DNA. Samples “Sem-NoAb” and “NoAb” were semen-only samples, in different volumes, put through IMB isolation with magnetic beads. Shown in Table 3.7 it was observed that the magnetic beads are recovering sperm cells. The DNA quantity difference between the two semen-only samples could have been due to the difference in volume of semen in the samples. The results from this test clearly showed that magnetic beads recover cells without antibodies present. With these results it is unclear if the results from the previous test were a result of the antibodies binding cells or just the magnetic beads binding to the cells.

Table 3.7: The magnetic beads themselves, without antibodies, bind cells.

Sample Name	Large Autosomal ng/uL	Small Autosomal ng/uL	Y Chromosome ng/uL
Bead (vortexed)	33.6	38.02	7.88
T-NoAb	12.29	9.67	5.28
Sem-NoAb	5.14	3.3	3.87
NoAb	0.03	0.02	0.03

3.1.8 Antibody Ability to Bind Cells

Flow Cytometry

Sperm cells and vaginal epithelial cells were tested with two antibodies: the PH-20 antibody used for the CA-IMBs and the PH-20 antibody with a biotin label used for the SA-IMBs. This test was completed using one male and one female participant and the results here cannot be generalized for all individuals’ samples. All samples tested using flow cytometry collected measurements from 10,000 events or cells. The cells sampled were gated based on relative size so that the target cells were what was being measured instead of debris. In Figure 3.2 the left graph shows the gating of the cells and the right graph shows the histogram with relative fluorescence on the x-axis and cell count on the y-axis. The percentages of fluorescence and median fluorescence intensities (MFI) reported are relative to the number of cells measured. With the CA antibodies, sperm cells showed some non-specific binding between the fluorescently labeled secondary antibody and the sperm cells. The fluorescence in the secondary only control was 10.7% and in the target sample it was 82.6% showing that for this individual the sperm cells were binding to the PH-20 antibodies. Epithelial

cells showed fluorescence percentages of 0.4% (non-specific) and 33.1% (target) indicating that there was some binding between vaginal epithelial cells and the PH-20 antibody. For the biotynalated PH-20 antibody, sperm cells showed fluorescence percentages of 5.2% (non-specific) and 45.2% (target), again showing that theses sperm cells do bind to the antibodies. Epithelial cells produced 0.2% (non-specific) and 1.3% (target) for biotynalated PH-20 antibodies. A summary of these results can be found in Table 3.8. These results are specific to the participant samples used and the two specific antibodies tested. Different PH-20 antibodies from different companies may produce different results. All graphs and tables produced by the samples using flow cytometry can be found in Appendix A.

Figure 3.2: Semen sample (1×10^{10} cells) with secondary and primary PH-20 antibody. Of the 10,000 cells samples 82.6% showed fluorescence with a median fluorescence intensity of 439.

Table 3.8: Summary table of all secondary only and target samples run for sperm and epithelial cells with the two antibodies. The increase between the fluorescence percentage in the non-specific (secondary control) to the target sample shows that the primary antibodies used are binding to the target cell.

	PH-20 Antibody				PH-20 Antibody (Biotin)			
	Non-Specific Binding		Target		Non-Specific Binding		Target	
	Fluorescence Percentage	Median Fluorescent Intensity	Fluorescence Percentage	Median Fluorescent Intensity	Fluorescence Percentage	Median Fluorescent Intensity	Fluorescence Percentage	Median Fluorescent Intensity
Sperm Cells	10.7%	99	82.6%	439	5.1%	89	45.2%	196
Epithelial Cells	0.4%	143	33.1%	464	0.2%	493	1.3%	717

3.1.9 Bead Type

The streptavidin-biotin IMBs recovered 20% of the DNA assumed to be present in samples from the “1128 (+)” aliquot. Carboxylic acid-amine IMBs pulled out 13% of the DNA present in the samples shown in Table 3.9. An ANOVA test was run and a significant difference was found between bead types CA and SA ($p=0.0031$). The Benjamini-Yekutieli method was utilized as the post hoc test to evaluate the specific interactions. Results are shown in Table 3.10. The CA beads recovered a significantly different quantity of DNA compared to both SA bead types ($p=0.0090$) which can also be seen in Figure 3.3. Changing the antibody quantity versus the bead quantity for the SA beads did not show a significant difference ($p=1.0000$). It should be noted that this experiment considered a single male participant. For more generalized conclusions, this experiment should be repeated with more biological replicates.

Table 3.9: The SA beads recovered approximately 20% of male DNA concentration from samples while CA beads recovered approximately 13% of male DNA concentration from samples. Using the bead quantity versus the antibody quantity batches of SA beads for IMB isolation did not make a statistical difference ($p=1.0000$) in the male DNA recovered for sample 1128. Percentages were calculated based on the assumption that all 60 μL samples had approximately the same quantity of DNA as the 1128(+) sample.

Sample Name	Large Autosomal ng/ μL	Small Autosomal ng/ μL	Y Chromosome ng/ μL	Average Y Chromosome ng/ μL	Percentage of Total
CA 1	3.18	2.12	2.53		
CA 2	4.32	3.57	3.92		
CA 3	5.61	4.27	4.10		
CA 4	1.83	7.97	3.99		
CA 5	4.66	4.14	3.97	3.70	13.48
SA 1-B	7.06	5.61	5.64		
SA 2-B	5.89	4.30	4.55		
SA 3-B	6.71	4.66	5.52		
SA 4-B	8.35	6.32	7.05		
SA 5-B	6.74	5.05	5.55	5.66	20.61
SA 1-A	6.54	5.57	5.81		
SA 2-A	5.34	3.97	4.28		
SA 3-A	7.47	5.73	5.99		
SA 4-A	6.46	4.89	5.10		
SA 5-A	7.54	6.15	6.04	5.44	19.82
1128 (+)	32.91	25.43	27.47		

Table 3.10: Interactions from the post hoc test (Benjamini-Yekutieli method). For participant 1128 the recovery of DNA between the CA beads and SA beads was statistically different. The variation in antibody versus bead quantity for the SA beads showed no statistical difference.

Interactions	P-value
CA – SA-A	0.0090
CA – SA-B	0.0090
SA-A – SA-B	1.0000

Figure 3.3: Boxplot of CA IMB versus SA IMB results. The CA IMBs resulted in lower DNA quantities.

Three different cell types were evaluated during this test: sperm cells, vaginal epithelial cells, and mixtures (M:F). Carboxylic acid IMBs recovered 8.28% of the male DNA concentration in the semen only samples and 22.94% of the female DNA concentration in the epithelial only samples. Streptavidin-biotin IMBs recovered 24.81% of the male DNA concentration in the semen only samples and 16.06% of the female DNA concentration in the epithelial only samples. In 1:1 mixture samples CA IMBs recovered an average small autosomal concentration of 7.87 ng/ μ L and an average Y concentration of 1.09 ng/ μ L; SA IMBs recovered an average small autosomal concentration of 5.79 ng/ μ L and an average Y concentration of 2.47 ng/ μ L. Detailed results can be found in Table 3.11. Note that these numbers are specific for samples 3139 (male) and 3630 (female).

For semen only samples SA-IMBs recovered higher quantities of DNA compared to CA-IMBs and adversely CA-IMBs recovered higher quantities of female DNA when compared to SA-IMBs in epithelial only samples. Trends for this data are seen in Figures 3.4 and 3.5. Based on the assumption that all aliquots were homogenous in concentration the adjusted DNA concentrations were plotted at the left side of Figure 3.4. A two-way ANOVA test was performed and both cell type and bead type showed significant differences for the Y chromosome ($p=1.04 \times 10^{-8}$, $p=1.77 \times 10^{-4}$ respectively). The Benjamini-Yekutieli method used for post hoc analysis indicated that for the Y chromosome there was a significant difference between sperm cells and vaginal epithelial cells ($p=5.05 \times 10^{-8}$) and there was not

a significant difference between mixture samples and sperm cell only samples ($p=0.5276$). Finally, an interaction analysis was performed for more detailed results which showed a significant difference between CA-IMBs and SA-IMBs in semen only samples ($p=2.07 \times 10^{-5}$) and between SA-IMBs in mixtures versus semen only samples ($p=0.0190$). A sensitivity study was run after initial analysis to evaluate how removing the outlier would affect the data. After removal of the outlier in the mixture samples all interactions were significant, as shown in Table 3.12. For the small autosomal two-way ANOVA showed a significant difference in the cell type but no difference in the bead type. The interaction analysis of the small autosomal adjusted data showed a significant interaction between sperm cell and epithelial cell recovery with CA-IMBs ($p=0.0365$). Once the outlier was removed the interaction between bead type in semen only samples became significant ($p=0.0245$). All individual comparison p-values can be found in Table 3.12.

Table 3.11: The streptavidin-biotin IMBs recover more male DNA and less female DNA overall when compared to carboxylic acid IMBs.

Sample Name	Large Autosomal ng/ μ L	Small Autosomal ng/ μ L	Y Chromosome ng/ μ L	Average Y Chromosome ng/ μ L	Percentage of Total
S20CA1	1.11	0.66	0.85	0.83	8.28
S20CA2	1.06	0.60	0.81		
S20CA3	1.10	0.71	0.87		
S20CA4	1.62	1.10	1.25		
S20CA5	1.08	0.94	0.39		
S20SA1	3.22	2.67	2.68	2.50	24.81
S20SA2	3.32	2.46	2.66		
S20SA3	3.84	2.90	2.62		
S20SA4	3.92	3.11	2.83		
S20SA5	2.19	1.62	1.70		
S20C	23.36	12.44	20.40		
				Average Small Autosomal ng/ μ L	Percentage of Total
E20CA1	6.68	3.57	0.01	3.13	22.94
E20CA2	6.45	3.63	0.00		
E20CA3	4.55	3.30	0.00		
E20CA4	5.56	3.77	0.00		
E20CA5	1.67	1.38	0.00		
E20SA1	3.06	2.55	0.00	2.19	16.06
E20SA2	4.30	3.31	0.19		
E20SA3	1.63	1.17	0.01		
E20SA4	1.80	1.18	0.00		
E20SA5	3.20	2.76	0.00		
E20C	9.56	4.55	0.00		
				Average Y Chromosome ng/ μ L	Percentage of Total
MCA1	17.53	9.81	2.20	1.90	18.86
MCA2	15.48	10.44	2.28		
MCA3	10.20	6.24	1.86		
MCA4	11.72	7.88	1.66		
MCA5	8.91	4.99	1.50		
MSA1	15.80	7.79	2.85	2.47	24.56
MSA2	12.93	6.32	2.77		
MSA3	13.68	6.94	2.91		
MSA4	13.80	7.09	3.59		
MSA5	1.14	0.81	0.23		
MC-DM	6.69	3.73	5.31		
MC-DF	11.07	6.04	1.53		
IMB_CA	0.00	0.00	0.00		
IMB_SA	0.00	0.00	0.00

Figure 3.4: These plots show the interactions that bead type and cell type have on DNA concentration both with and without the outlier. The top graphs show the small autosomal data and the bottom graphs show the Y chromosome data with the adjusted data on the left and data excluding the outlier on the right. The change can be seen in the error bars for mixture SA IMB samples.

Figure 3.5: This plot is the mixture data with the Y chromosome DNA quantity subtracted from the small autosomal DNA quantity to compare the recover of male versus female DNA. The CA-IMBs are recovering more female DNA than male DNA from this set of mixture samples.

Table 3.12: Results from the interaction analysis of bead types (CA, SA) and cell types (m-mixture, sp-sperm cells, ep-vaginal epithelial cells). When the outlier is removed all interactions become significantly different, specifically CA-IMBs with mixtures versus semen only samples and mixture samples with CA versus SA IMBs for the Y chromosome. For the small autosomal removing the outlier changes the significance of the semen only samples with CA versus SA IMBs.

Interaction	Y Chromosome Adjusted	Y Chromosome (outlier removed)	Interaction	Small Autosomal Adjusted	Small Autosomal (outlier removed)
	p-value			p-value	
m,CA – sp,CA	0.3457	0.0355	sp,CA – ep,CA	0.0365	4.28×10^{-3}
m,CA – m,SA	0.3587	1.29×10^{-3}	sp,CA – sp,SA	0.1101	0.0245
sp,CA – sp,SA	2.07×10^{-5}	7.69×10^{-9}	ep,CA – ep,SA	0.6053	0.3281
m,SA – sp,SA	0.0190	0.0355	sp,SA – ep,SA	1.0000	1.0000

4. Conclusions

During the extent of this project several small-scale experiments were run testing different aspects of the IMB isolation process including: elution methods, mixture samples, female cell lysis, antibody-cell binding, and bead type. While evaluating elution methods it was determined that more DNA is recovered when skipping the elution step and extracting straight from the IMBs. When IMBs were tested in cell mixture samples containing male and female cells there was an attempt to lyse only the female cells to obtain a single source male profile. However, lysing female cells using SDS and high temperature incubation resulted in low DNA recovery overall in both the small autosomal and Y chromosome DNA. Factors that may have contributed to the low DNA recovery may have been the incubation temperatures or incubation times. This was not explored any further. Instead, a different method was attempted to try and mitigate the amount of female DNA being recovered. A DNase procedure was used to degrade any DNA that may have been attached to the IMB-sperm complexes. To evaluate if this procedure was useful it was compared to samples that did not go through any female cell removal step. In cell mixture samples, using DNase compared to extracting DNA straight from the IMBs (without a lysis step) did not behave similarly ($p=0.0079$). This experiment was only tested on one mixture pairing containing two individuals' DNA. This should not be used to generalize for all samples. More biological replicates comparing between individuals would need to be run to make any generalized statements.

Based on Table 3.5 the reason for female cells binding to the IMBs may not be solely because vaginal cells express the PH-20 protein (not tested here). There may be an interaction occurring between the female epithelial cells and the magnetic beads themselves. The flow cytometry results did show some binding between the PH-20 protein obtained from Abcam and vaginal epithelial cells from one participant, but since the magnetic beads themselves also pick up cells more research needs to be performed to evaluate why IMBs when attached to PH-20 antibodies are binding epithelial cells. Contradictory to these findings, one study using PH-20 antibody-bound beads [46] reported that in male:female mixture samples, male DNA was recovered at quantities 10-fold higher than that of the female.

Results showed that DNA recovery is affected by bead type and cell type, based on a small sample size and sample replicates, not biological replicates. Carboxylic acid coated

beads were compared to streptavidin coated beads to determine if one outperformed the other. For semen only samples one test showed a significant difference between the two bead types. However, a second test only showed a significant difference when a single outlier was removed. To verify that SA-IMBs recover more DNA in semen only samples when compared to CA-IMBs, further testing will need to be done with a larger sample size to mitigate the effect of any outliers and more biological replicates would need to be tested to generalize this further. When considering the trends presented here, the SA-IMBs do not show a significant difference in the recovery of sperm cells versus epithelial cells but CA-IMBs recovered more female DNA than male DNA in mixture samples and when comparing the respective single source samples. While these findings are only based on one biological replicate and cannot be generalized to all samples, recovering more female DNA in a cell mixture defeats the proposed purpose of the IMB isolation to isolate sperm cells from cell mixtures. A significant difference was also found between SA IMBs recovery of male DNA in mixture samples versus semen only samples. This could be due to competitive binding or just an overload of cells in the samples, but either way further investigation is needed. Of the total DNA available in semen-only samples, SA-IMBs recovered approximately 21.7% of the DNA. This low percentage of recovery by antibody-bead complexes is similar to another research group's findings where 23.5% of total DNA was recovered [46].

The streptavidin magnetic beads with the biotin labeled antibodies seemed to be more successful at recovering higher quantities of male DNA when compared to the carboxylic acid-amine beads. Larger studies need to be conducted to confirm this. One potential reason for the differences in binding could be the sequence expression difference between the two antibodies used in this study. The PH-20 antibody expresses sequence aa173-222 while the biotinylated PH-20 antibody expresses aa36-490 (NCBI Accession No. NP_694859). The biotin labeled antibody used with the SA-IMBs has a longer chain of the PH-20 protein sequence therefore, it could be more specific. This could also potentially be why the CA-IMBs recover more female cells due to having a shorter PH-20 expression sequence leading to more non-specific binding. These are not claims of this study, but should be further investigated.

In summary based on this research utilizing the specific methods presented, DNA recovery is more optimal when extracted straight from the immunomagnetic beads after IMB isolation and preliminary data suggests that using streptavidin coated beads bound to biotinylated antibodies may be efficient at recovering sperm cells for utilization in male:female mixture samples. While IMBs recover some male DNA from cell mixtures, there are still several questions that need to be investigated in an attempt to optimize the IMB isolation process. Studies have shown successful separation of sperm cells from mixture samples [20] [36]. The

utilization of IMBs to recover sperm cells from cell mixtures is very promising but until more improved methods are evaluated that recover larger percentages of the total DNA available, IMBs will not be a realistic alternative to differential extraction. For IMB isolation to be implemented in forensic applications they would need to be able to outperform differential extraction in separating male from female cells and thus far that has not been accomplished. The goal in forensic DNA analysis is to capture the smallest amount of male DNA that may be present in a sexual assault specimen where the female DNA far outweighs the male portion and research will continue to optimize this process.

5. Appendix A

Figure 5.1: Semen sample (1×10^{10} cells) control with no stain. There is no fluorescence in the in the Alexa 647 and PE ranges with the respective fluorescence percentages of 0.5 and 0.6 and median fluorescence intensities of 50 and 58.

Figure 5.2: Semen sample (1×10^{10} cells) secondary antibody control with PE. Out of the 10,000 sperm cells sampled 10.7% showed fluorescence and there was a median fluorescence intensity of 99.

Figure 5.3: Semen sample (1×10^{10} cells) with secondary and primary PH-20 antibody. Of the 10,000 cells samples 82.6% showed fluorescence with a median fluorescence intensity of 439.

Figure 5.4: Semen sample (1×10^{10} cells) secondary antibody control with Alexa 647. Out of 10,000 sperm cells sampled 5.1% showed fluorescence and there was a median fluorescence intensity of 89.

Figure 5.5: Semen sample (1×10^{10} cells) with secondary and primary PH-20 antibody with biotin label. Of the 10,000 cells samples 45.2% showed fluorescence with a median fluorescence intensity of 196.

Figure 5.6: Vaginal epithelial cell (100 μ L) control with no stain. There is no fluorescence in the in the Alexa 647 and PE ranges with the respective fluorescent percentages of 0.2 and 0.4 and median fluorescence intensities of 481 and 119.

Figure 5.7: Vaginal epithelial cell (100 μ L) secondary control with PE. Out of 10,000 sperm cells sampled 0.4% showed fluorescence and there was a median fluorescence intensity of 143.

Figure 5.8: Vaginal epithelial cell (100 μ L) secondary and primary PH-20 antibody. Out of 10,000 sperm cells sampled 33.1% showed fluorescence and there was a median fluorescence intensity of 464.

Figure 5.9: Vaginal epithelial cell (100 μ L) secondary control with Alexa 647. Out of 10,000 sperm cells sampled 0.2% showed fluorescence and there was a median fluorescence intensity of 493.

Figure 5.10: Vaginal epithelial cell (100 μ L) secondary and primary PH-20 antibody with biotin label. Out of 10,000 sperm cells sampled 1.3% showed fluorescence and there was a median fluorescence intensity of 717.

References

- [1] John M. Butler. *Fundamentals of Forensic DNA Typing*. Academic Press, 2009.
- [2] J. D. Watson and F. H. C. Crick. Molecular structure of nucleic acids: A structure for deoxyribose nucleic acid. *Nature International Journal of Science*, 171:737–738, 1953.
- [3] Leslie A. Pray. Discovery of dna structure and function: Watson and crick, 2008.
- [4] John M. Butler. *Forensic DNA Typing: Biology, Technology, and Genetics of STR Markers*. Academic Press, 2 edition, 2005.
- [5] Siun C. Tan and Beow C. Yiap. Dna, rna, and protein extraction: The past and the present. *Journal of Biomedicine and Biotechnology*, 2009.
- [6] Thermo Fisher Scientific. Dynabeads types and uses.
- [7] Yasuhisa Seo, Daisuke Ichida, Shingo Sato, Kohji Kuroki, and Tetsuko Kishida. An improved method for the diatom test utilizing dna binding ability of silica. *Journal of Forensic Sciences*, 59(3):779–784, 2014.
- [8] Thermo Fisher Scientific. Genecatcher magnetic beads.
- [9] Quality assurance standards for forensic dna testing laboratories, 2011.
- [10] Applied Biosystems. *Quantifiler HP and Trio DNA Quantification Kits User Guide*, 2017.
- [11] Thermo Fisher Scientific. How taqman assays work.
- [12] Michael Brown and Carl Wittwer. Flow cytometry: Principles and clinical applications in hematology. *Clinical Chemistry*, 46:1221–1229, 2000.
- [13] Andrea Cossarizza et al. Guidelines for the use of flow cytometry and cell sorting in immunological studies. *European Journal of Immunology*, 47:1584–1797, 2017.
- [14] Thermo Fisher Scientific. How a flow cytometer works.

- [15] BD Biosciences. *BD LSRFortessa Cell Analyzer Users Guide*, 2010.
- [16] Bio-Rad Laboratories. Flow cytometry basics guide.
- [17] Thermo Fisher Scientific. Carbodiimide crosslinker chemistry.
- [18] Hailing Zheng, Wei Zhanga, Hailiang Yang, Chao Maa, Yang Zhou, and Xianjun Dai. An immunomagnetic bead enrichment technique to improve the detection efficiency for trace silk protein, its application. *Journal of Cultural Heritage*, 38:46–52, 2019.
- [19] Jing-Yuan Hou, Tian-Cai Liu, Guan-Feng Lin, Zhi-Xiong Li, Li-Ping Zou, Ming Li, and Ying-SongWu. Development of an immunomagnetic bead-based time-resolved fluorescence immunoassay for rapid determination of levels of carcinoembryonic antigen in human serum. *Analytica Chimica Acta*, 734:93–98, 2012.
- [20] XinRan Cheng and Kagan Kerman. *Biochemistry Research Updates*. Nova Science Publishers Inc, 2012.
- [21] Oded Livnah, Edward A. Bayer, Meir Wilchek, and Joel L. Sussman. Three-dimensional structures of avidin and the avidin-biotin complex. *Proceedings of the National Academy of Sciences*, 90(11):5076–5080, jun 1993.
- [22] Carlos E. Argarana, Irwin D. Kuntz, Steven Birken, Richard Axel, and Charles R. Cantor. Molecular cloning and nucleotide sequence of the streptavidin gene. *Nucleic Acids Research*, 14(4):1871–1882, 1986.
- [23] Zsuzsanna Szekeres, Melinda Herbth, and J. Prechl. *Biochemistry Research Updates*. Nova Science Publishers Inc, 2012.
- [24] Thermo Fisher Scientific. Avidin-biotin interaction.
- [25] Patricia A. Martin-DeLeon. Epididymal spam1 and its impact on sperm function. *Molecular and Cellular Endocrinology*, 250:114–121, 2006.
- [26] Khalida Sabeur, Gary N. Cherr, Ashley I. Yudin, Paul Primakoff, Ming-Wen Li, and James W. Overstreet. The ph-20 protein in human spermatozoa. *Journal of Andrology*, 18(2):151–158, 1997.
- [27] Daichi Baba, Shin ichi Kashiwabara, Arata Honda, Kazuo Yamagata, Qing Wu, Masahito Ikawa, Masaru Okabe, and Tadashi Baba. Mouse sperm lacking cell surface hyaluronidase ph-20 can pass through the layer of cumulus cells and fertilize the egg. *The Journal of Biological Chemistry*, 277(3):30310–4, 2002.

- [28] Michael Gmachl, Sandrine Sagan, Sigrid Ketter, and Gnther Kreil. The human sperm protein ph-20 has hyaluronidase activity. *Federation of European Biochemical Societies*, 336:545–548, 1993.
- [29] Masaru Okabe. The cell biology of mammalian fertilization. *The Company of Biologists*, 140:4471–4479, 2013.
- [30] Lamelle Research Laboratories. Effects of oxidative stress.
- [31] Ying Lin, Kimberly Mahan, William E. Lathrop, Diana G. Myles, and Paul Primakoff. A hyaluronidase activity of the sperm plasma membrane protein ph-20 enables sperm to penetrate the cumulus cell layer surrounding the egg. *The Journal of Cell Biology*, 125(5):1157–1163, 1994.
- [32] Antonei B. Csoka, Gregory I. Frost, and Robert Stern. The six haluronidase-like genes in the human and mouse genomes. *Matrix Biology*, 20:499–508, 2001.
- [33] Caitlin O. McAtee, Joseph J. Barycki, and Melanie A. Simpson. Emerging roles for hyaluronidase in cancer metastasis and therapy. *Advances in Cancer Research*, 123:1–34, 2014.
- [34] Vinata B. Lokeshwar, Marvin J. Young, Gulten Goudarzi, Naoko Iida, Ashley I. Yudin, Gary N. Cherr, and Marie G. Selzer. Identification of bladder tumor-derived hyaluronidase: Its similarity to hyal1. *Cancer Research*, 59:4464–4470, 1999.
- [35] Xue-Bo Li, Qing-Shan Wang, Yu Feng, Shu-Hua Ning, Yuan-Ying Miao, Ye-Quan Wang, and Hong-Wei Li. Magnetic bead-based separation of sperm from buccal epithelial cells using a monoclonal antibody against mospd3. *International Journal of Legal Medicine*, 128:905–911, 2014.
- [36] Xing-Chun Zhao, Le Wang, Jing Sun, Bo-Wei Jiang, Er-Li Zhang, and Jian Ye. Isolating sperm from cell mixtures using magnetic beads coupled with an anti-ph-20 antibody for forensic dna analysis. *PLoS ONE*, 11(7):1–9, 2016.
- [37] Sexual assault kits using science to find solutions.
- [38] Life Technologies Corporation. *Dynabeads M-270 Carboxylic Acid*, 2012.
- [39] Thermo Fisher Scientific Inc. *Dynabeads M-280 Streptavidin*, 2018.
- [40] Qiagen. *QIAamp DNA Investigator Handbook*, 2012.

- [41] Pamela Marshall. Optimization of spermatozoa capture during the differential extraction process for str typing with the potential for automation, 2002.
- [42] Trevor G. Cooper, Elizabeth Noonan, Sigrid von Eckardstein, Jacques Auger, H.W. Gordon Baker, Hermann M. Behre, Trine B. Haugen, Thinus Kruger, Christina Wang, Michael T. Mbizvo, and Kirsten M. Vogelsong. World health organization reference values for human semen characteristics. 16(3):231–245, 2010.
- [43] Kathleen Brundage. *WVU Flow Cytometry Core & Single Cell COre Facility Training Manual*.
- [44] Wayne W. LaMorte. Mann whitney u test (wilcoxon rank sum test).
- [45] Rudolf J. Freund, Donna Mohr, and William J. Wilson. *Statistical Methods*. Academic Press, 2010.
- [46] Chelsie N. Testerman, Jordan Cox, Kemper Gibson, James P. Landers, and Tracey Dawson Cruz. Antibody-mediated separation of seminal male/female mixtures from sexual assault samples (abstract). Abstract presented at AAFS (2019).