FORENSIC TECHNIQUES FOR THE ISOLATION OF SPERM CELLS FROM MIXED CELL FRACTIONS: A REVIEW

By

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A thesis submitted in fulfilment of the requirements for the degree of

Master of Forensic Science (Professional Practice)

in

The School of Veterinary and Life Sciences
Murdoch University

Supervisor: Mr Brendan Chapman

Semester 2, 2017
Declaration

I declare that this thesis does not contain any material submitted previously for the award of any other degree or diploma at any university or other tertiary institution. Furthermore, to the best of my knowledge, it does not contain any material previously published or written by another individual, except where due reference has been made in the text. Finally, I declare that all reported experimentations performed in this research were carried out by myself, except that any contribution by others, with whom I have worked is explicitly acknowledged.

Signed: Shannen Jane Blackwell
Acknowledgements

I would like to extend my acknowledgements to a number of people;

Firstly to Mr. Brendan Chapman, thank you for your continued support and mentorship throughout the writing of this dissertation, as well as for the blind faith you showed in my writing abilities.

To Sean Hutchinson, thank you for starting this journey off. Without your work, I wouldn’t have even known where to begin.

To my family and friends, thank you for the constant encouragement and support that you provided throughout this process and for listening to me whinge endlessly about how much work I have to do. Also, a special thank you to my partner Julian, who reminds me every day that it will all be worth it.
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Part One

Literature Review

A Review of Forensic Methods for the Isolation of Sperm Cells from Mixed Cellular Fractions
Abstract

The process of separating one specific cell type from a mixed cellular sample has been utilised extensively by the scientific community. In particular, separating sperm cells from mixed cell samples, containing both male and female DNA, is typically employed within the forensic sciences. This review will examine the different sperm cell DNA isolation techniques which are used by forensic analysts, with a particular focus on differential extraction and Y-chromosome targeted DNA profiling, as these are currently the most widely used techniques. Throughout the literature, many modifications have been made to the original methods which were proposed, in an effort to increase the scientific value of both of these methods. Any method modifications which have been introduced will be reviewed, and their benefit for the forensic community will also be discussed. Although these methods have their uses, it is discovered by this review that they are both limited in their applicability, and by their overall success rates. For this reason, a more recently published technique of using immunomagnetic beads (IMBs) to separate the DNA fractions is proposed as an alternative method to the separation techniques currently employed by forensic DNA laboratories.

**Keywords:** DNA Analysis, forensic, differential extraction, Y-chromosome STR, immunomagnetic beads
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<td>ABS</td>
<td>Australian Bureau of Statistics</td>
</tr>
<tr>
<td>ADAM2</td>
<td>Disintegrin and Metalloprotease</td>
</tr>
<tr>
<td>AKAP3</td>
<td>A Kinase Anchor Protein 3</td>
</tr>
<tr>
<td>AP</td>
<td>Acid Phosphatase</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyltrimethylammonium Bromide</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>IMBs</td>
<td>Immunomagnetic beads</td>
</tr>
<tr>
<td>JLP</td>
<td>JNK-associated Leucine Zipper Protein</td>
</tr>
<tr>
<td>LCM</td>
<td>Laser Capture Microdissection</td>
</tr>
<tr>
<td>MOSPD3</td>
<td>Motile Sperm Domain-containing Protein 3</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NIPSVS</td>
<td>National Intimate Partner and Sexual Violence Survey</td>
</tr>
<tr>
<td>Pro K</td>
<td>Proteinase K</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
</tr>
<tr>
<td>PTC</td>
<td>Paternity Testing Corporation</td>
</tr>
<tr>
<td>PTSD</td>
<td>Post-traumatic Stress Disorder</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>RSID™-Semen</td>
<td>Rapid Stain Identification of Human Semen</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SP-10</td>
<td>Sperm protein-10</td>
</tr>
<tr>
<td>SPAM1</td>
<td>Sperm Adhesion Molecule 1</td>
</tr>
<tr>
<td>SPRED</td>
<td>Separation Potential Ratio for the Extraction Differential</td>
</tr>
<tr>
<td>SVSA</td>
<td>Seminal Vesicle-specific Antigen</td>
</tr>
<tr>
<td>tACE</td>
<td>Testicular Isoform of the Angiotensin-converting Enzyme</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Tris hydrochloride</td>
</tr>
<tr>
<td>TRITON X</td>
<td>Polyethylene Glycol Tert-octylphenyl Ether</td>
</tr>
<tr>
<td>TSI</td>
<td>Time Since Intercourse</td>
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<tr>
<td>Y-STR</td>
<td>Y-chromosome Short Tandem Repeat</td>
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1. INTRODUCTION

The term “sexual assault” refers to any unwanted, coerced, or forced sexual contact that occurs between two or more people, without the victim’s consent.\(^1\) Although most people associate sexual assault with rape, the definition also covers; attempted rape, grabbing and fondling and verbal threats. The most recent crime data published by the Australian Bureau of Statistics (ABS) indicates that the number of sexual assaults occurring nationwide is on the rise; a 22% increase in sexual assaults was recorded from 2010 to 2016, with a 5% increase documented from 2015 to 2016 alone.\(^2\) The data also reveals that females account for at least 82% of the total number of sexual assault victims.

Given that sexual assaults are typically underreported, with only approximately 30% of cases conveyed to the authorities, most statistics are established by obtaining data through anonymous surveys.\(^1\) According to the National Intimate Partner and Sexual Violence Survey (NIPSVS) (2010 Summary Report) conducted in the United States, 35.6% of women have experienced some form of sexual assault by an intimate partner.\(^3\) In comparison, a review conducted in 2014 found that the prevalence estimate for non-partner sexual violence against females was 7.2% worldwide.\(^4\)

These statistics are staggeringly high, and are also especially disturbing given the substantial physical and mental consequences suffered by victims. Survivors of sexual assault are at a greater risk for post-traumatic stress disorder (PTSD), suicidal ideation and attempts, bipolar conditions and obsessive-compulsive disorders.\(^1,5\) They are also more likely to; suffer from
depression, anxiety, and sexual dysfunction, have relationship and social adjustment problems, and have substance abuse issues.\textsuperscript{1,6-8} Given the severe trauma that sexual assault victims suffer, and will continue to experience throughout their lives, it is of vital importance that the perpetrator of the crime is caught and sentenced. The main aspects of building a sexual assault case involve collecting evidence from the crime scene, the victim and also reference samples from the suspect(s).

Following a sexual assault, the victim will often present themselves to a hospital for forensic evidence recovery. Typically, this involves the collection of: biological evidence, victim’s statements, observations by medical staff and photographs of injuries.\textsuperscript{9} The collection of biological samples, from areas which may contain cellular material that has been transferred from the perpetrator, are prioritised and often include the collection of vaginal, anal and oral swabs.\textsuperscript{10} Vaginal swabs are often deemed as the most important evidence to be collected as finding sperm deoxyribonucleic acid (DNA) on vaginal swabs provides substantial evidence that sexual contact has occurred.\textsuperscript{9,11-13} Biological samples which are collected, that contain both male and female cells, are termed mixed cell samples. These samples often contain a variety of components including: female buccal and/or epithelial cells, saliva, vaginal secretions, spermatozoa, seminal plasma and also red and white blood cells.\textsuperscript{14} Each mixed cell sample that is collected will also contain male DNA and female DNA in varying concentrations. Typically, there will be an excess of female cells and only a very small proportion of male cells; this is due to the nature of the sampling procedure.
After the samples are collected, microscopy is used to visualise the cells and determine whether spermatozoa are present in the sample. Once the presence of semen has been confirmed, DNA profiling can be performed and potentially lead to identification of the perpetrator. Prior to DNA profiling being performed, the male and female portions of the mixed samples must be separated so that profiling can be performed on the two separate DNA fractions. If the fractions are not separated, the excess number of epithelial cells in the sample will lead to complications during DNA profiling, making interpretation difficult.

This dissertation will focus on techniques which are available for the isolation of sperm cell DNA from mixed cell samples. Differential extraction and Y-chromosome short tandem repeat (Y-STR) profiling are the two key techniques that are currently implemented in forensic DNA laboratories, and consequently, these will be considered in detail. The limitations of these methods will then be discussed to convey to the reader why new techniques are required.

An extensive number of techniques have been published within the literature on the topic of sperm cell DNA isolation, including: laser capture microdissection (LCM), filtration-based methods, microdevices, and flow cytometry. However, the practice of using immunomagnetic beads (IMBs) will be suggested as the future of sperm cell DNA isolation. Finally, a study outline will be proposed, based on a recent study by Hutchinson, that aims to determine whether a primary antibody-secondary antibody IMB technique, which is aimed at the sperm adhesion molecule 1 (SPAM1) antigen, could be successful at isolating sperm cell DNA from mixed cell samples. Before moving onto the topic of current sperm isolation
techniques, the preliminary and confirmatory tests available to detect semen in forensic samples will be discussed.

2. TESTS FOR SEMEN

2.1. Preliminary Tests for Semen

Most of the currently available preliminary tests screen for the presence of the protein components of semen, specifically: prostate specific antigen (PSA),\(^{24-29}\) semenogelin I and II,\(^{30}\) prostatic acid phosphatase (AP),\(^{29,31}\) zinc,\(^{32}\) and seminal vesicle-specific antigen (SVSA).\(^{33}\) More recently, a number of test kits have become available which can rapidly detect the presence of these proteins. These test kits include ABAcard\(^\text{®}\) p30 (Abacus Diagnostics, Inc., West Hills, CA), which detects PSA, and Rapid Stain Identification of Human Semen (RSID™-Semen) (Independent Forensics, Hillside, IL), which detects human semenogelin. These kits been compared by Boward and Wilson\(^{34}\) and Hobbs et al.,\(^{35}\) who confirmed that they are both sensitive, relatively specific and cost-effective. However, the ABAcard\(^\text{®}\) 30 was shown to perform better than the RSID™-Semen test kit. None of the targeted proteins are entirely sperm-specific, which can potentially result in false negatives, and therefore, they cannot be used as confirmatory tests.\(^{36,37}\) Additionally, Hooft et al.\(^{32}\) describe that the applicability of these proteins as biomarkers for semen is significantly time-dependant, as they degrade quite rapidly after ejaculation. The tests are only deemed reliable within a 24-hour window post-ejaculation, and therefore, preliminary screening for semen using these tests is generally performed in the field to find areas of interest at crime scenes.
2.2. Confirming the Presence of Semen

The current method for confirming the presence of semen involves transferring a portion of the sample onto a microscope slide, performing histological staining of the cells and then searching for spermatozoa using microscopic examination.\(^{36}\) This is the method of choice because spermatozoa are the longest-surviving constituent of seminal fluid, and therefore, can be visualised days after the sexual assault.\(^{38}\) However, it should be kept in mind that the presence of spermatozoa is dependent on time since intercourse (TSI) and the absence of spermatozoa does not necessarily mean that a sexual assault did not occur.\(^{39}\) If the perpetrator is azoospermic, or is vasectomised, there will also be no observable spermatozoa.\(^{32}\)

Two of the most frequently used stains are hematoxylin/eosin and nuclear fast red/picroindigocarmine (Christmas Tree Stain).\(^{40,41}\) These stains are not specific for spermatozoa, and therefore, there can be background staining of non-specific cells which can make visualisation of the sperm cells difficult.\(^{36}\) Differentiation between cell types can also be difficult due to the low contrast levels generated between sperm cells and non-sperm cells. The results of a study by Allery et al.\(^{40}\) found that the Christmas Tree Stain and the hematoxylin/eosin stain were both effective methods for detecting spermatozoa. However, the Christmas Tree Stain was able to detect more spermatozoa per slide than the hematoxylin/eosin stain and showed a higher contrast between the sperm cells and non-sperm cells. This makes it the preferred choice for forensic DNA laboratories.
2.3. Determining the Number of Cells per Slide

Most of the studies which are covered in this review include a step where the microscope slides are viewed using light microscopy and the number of sperm cells are counted. This is typically performed prior to the isolation technique being employed, and then afterwards, to determine whether cell lysis has occurred and can be used to demonstrate the success of the method by providing a percentage recovery. The cells are occasionally counted using a hemocytometer, however, other studies employ a method which classifies the number of spermatozoa present on the slide into a number of discrete categories, based on visualisation of the cells using microscopy. The number of sperm cells on the slide can be classified as follows: 1) ++++ many in every field, 2) +++ many, or some, in most fields, 3) ++ some in most fields, easy to find, 4) + hard to find, or 5) 0 = none. In laboratories around the world, this is currently the most widely used method for evaluating the number of sperm cells on a slide. However, a recent study found this method to be highly subjective, particularly when the slides that were being visualised had low sperm counts. Therefore, if a study discussed in this dissertation uses this method for cell counting, it should be noted that there is some subjectivity involved and the results may not be reproducible. After the presence of semen has been confirmed, and the amount which is present has been classified, DNA analysis of the collected swabs can be performed.

3. DNA ANALYSIS

When methods for isolating sperm cells were first introduced, restriction fragment length polymorphism (RFLP) analysis was being used for DNA profiling. Samples which were
assessed using this method required approximately 5 µg of DNA for a profile to be produced.\textsuperscript{46} The DNA analysis technique that is currently employed is multiplex short tandem repeat typing, which is able to amplify DNA present in a quantity of approximately 100 pg.\textsuperscript{46} Although the sensitivity of the DNA analysis has increased significantly, the success of the analysis is still dependent on a number of factors including the: TSI, sampling conditions and storage conditions.\textsuperscript{47} According to Vuichard et al.,\textsuperscript{47} the male autosomal DNA cannot be detected if it is below a ratio of 1:20 male/female DNA, which is irrespective of the amount of DNA that is recovered. When a mixed cell sample is subjected to STR analysis, if the female DNA is present in excess of the male DNA, the amplification of female cells will be favoured by the PCR reaction and the male profile will not be visible.\textsuperscript{48} Even if the male DNA is at a high enough concentration to be visible, interpretation of mixture profiles can still be very difficult.\textsuperscript{49} To fix this issue, prior to samples being analysed, the female and male DNA are separated from each other into two fractions. The main goals of sperm cell isolation techniques are to: 1) extract the sperm successfully from the swab, 2) retain the sperm throughout the isolation process (high yield), and 3) to minimise carryover of other cell types into the sperm fraction (high purity).\textsuperscript{50} When the fractions are submitted for STR analysis, the result will be two single-source DNA profiles corresponding to the victim and the perpetrator.\textsuperscript{10}

4. CURRENT METHODS FOR THE ISOLATION OF SPERM CELLS

Presently, most forensic laboratories utilise the differential extraction method, in a modified form to the original method, to obtain male DNA from vaginal swabs.\textsuperscript{13} Given that this technique is the most routinely used, this section of the review will primarily focus on differential
extraction and any variations which have been applied to the technique since its inception. Y-STR profiling is also employed within forensic DNA laboratories, and will be reviewed extensively.

4.1. The Differential Extraction Method

The differential extraction method was first proposed by Gill et al., and since its introduction, there have been numerous alterations made to the original method to increase cell yield and decrease carryover between the two fractions. Ensuring both these conditions are met will result in a clear male DNA STR profile being produced. Alterations to the differential extraction method include: introducing extra washing steps after centrifugation, altering the initial lysis buffer, treating epithelial cells with DNase to reduce carryover, and a one-step buffer has also been proposed. The most recently published article has developed a semi-quantitative method to compare methods and determine which has a higher separation potential.

4.1.1. Origination of the Differential Extraction Method

The first article that published data from a forensic perspective, in relation to the isolation of sperm cell DNA from mixed cell fractions, was written by Gill et al. in 1985. In this study, a two-step procedure termed differential extraction was able to successfully isolate the male DNA fraction from semen-contaminated vaginal swabs that were collected 6.5 hours post-coitus. The first step of the method involved an initial 30 minute incubation period in a TNE Buffer: 0.01 M Tris hydrochloride (Tris-HCl), 0.01 M Ethylenediaminetetraacetic acid (EDTA), 0.1 M Sodium chloride (NaCl) (pH 8), containing 20 µg mL\(^{-1}\) proteinase K (Pro K) and 2% sodium dodecyl sulfate
(SDS). This step allows for preferential lysis of the fragile female epithelial cells, while the robust sperm cells remain intact due to the presence of disulphide cross-links in their nucleus which makes them resistant to treatment with proteinase K.\textsuperscript{50,51} Subsequent centrifugation of the sample allows sperm cells to be pelleted and then the female DNA-containing supernatant can be removed and collected.

The second step of the procedure was an overnight incubation at 37°C, in the same buffer described above, with the addition of 0.039 M Dithiothreitol (DTT). The addition of DTT breaks down the disulphide cross-links in the sperm nucleus and allows the DNA to be released.\textsuperscript{50,51} The DNA was then purified and the sperm DNA was analysed via RFLP analysis. One of the main benefits to this separation technique is that both the female and male DNA fractions are collected, which means that a DNA profile can be generated for both the victim and the perpetrator(s).\textsuperscript{53} Being the pilot study in this field, only 11 samples were analysed and hence, further studies with a larger sample size were required to validate this technique.

4.1.2. Microscopic Evaluation of the Method

In 1989, Iwasaki et al.\textsuperscript{54} evaluated the proteinase K pre-treatment described by Gill et al.\textsuperscript{45} by microscopically observing the cells after incubation. Vaginal swabs were prepared from autopsied cases, the cells were eluted and then mixed with semen. The samples were then treated as per the initial lysis stage and centrifugation step proposed by Gill et al.\textsuperscript{45} However, rather than releasing the male DNA, a sample was taken directly from the pellet and fixed onto a microscope, stained using the Christmas Tree Stain and observed using light microscopy.
Treatment with the proteinase K buffer resulted in the intact semen being easily visualised amongst digested epithelial cells. This provides visual evidence that treatment with proteinase K is able to completely digest epithelial cells, while leaving sperm cells intact.

4.1.3. Replication of the Differential Extraction Method

Two years after Gill et al.\textsuperscript{45} originally published their results, they expanded the study\textsuperscript{55} for over 100 semen-contaminated vaginal swabs collected from five donor couples. The authors decided midway through the experiment that a wash step should be introduced after the centrifugation step, as they observed that many of the samples remained contaminated with female DNA after treatment with DTT. The wash step included incubation of the pelleted material with the initial lysis buffer, re-pelleting of the sperm cells via centrifugation, and then the secondary lysis buffer was added to lyse the sperm cells.\textsuperscript{55} The success rate of the procedure averaged 62%, however, it was found to vary greatly amongst donors.

The authors hypothesised that the variable success rates were due to a number of factors:

1. The samples were collected using swabs, meaning that only a small area of the vagina is sampled, which is not necessarily representative of the entire vagina. In a study conducted by Rutter et al.\textsuperscript{56}, the results showed that a number of swabs that were collected immediately after intercourse had very low levels of AP, even though high levels should have been observed due to the fresh deposition of semen. It was concluded that this is due to the uneven distribution of semen in the vagina following intercourse and swabs
were subsequently being collected from areas with low concentrations of semen, and therefore, low concentrations of AP.

2. The variation in sperm production for each sample. The literature reports that the amount of sperm produced in each ejaculate varies both within individuals and also within a population. This variation in sperm concentration across individuals will have an obvious downstream effect on the success rate of the technique, as a higher starting amount of male DNA is more likely to result in a male DNA profile following separation. This phenomenon was observed by Gill et al., as the success rate of the procedure varied amongst different donors and also across samples provided by the same donor.

3. Physical activity of the female participant after intercourse. Previous to this study, Willott and Allard reported that retention of semen in the vagina after intercourse is dependent on the post-coital activity of the female. This conclusion was also reported by Davies and Wilson, who found that donors who had intercourse at night, slept for eight hours, and then provided vaginal swabs had higher levels of AP in the vagina that those who were active for a few hours prior to providing the sample. This observed phenomenon is termed vaginal drainage and it results in the seminal constituents, i.e. sperm, AP, and PSA, becoming diluted by vaginal secretions. This ultimately results in a decreased chance of observing a male DNA profile.

Although all of these factors play a role in the overall success rate of the differential extraction method, the observed success rate of 62% is still not very efficient. The observed success rate is likely due to ineffective separation of the male and female fractions. Contamination of the
fractions can occur in one of two ways, either there is carryover of male DNA into the female fraction, or there is carryover of female DNA into the male fraction.

4.1.4. Ineffective Separation of the Male and Female Fractions

There are a number of ways in which the male DNA can carry over into the female fraction. The simplest mechanism through which this can occur is if the sperm pellet is agitated during any of the washing steps, which results in sperm DNA moving into the supernatant.\textsuperscript{53} Repeated washing of the sperm cells and extra centrifugation steps, to decrease the chance of epithelial cell carryover, may damage the sperm cells and cause carryover of the male DNA into the supernatant.\textsuperscript{60} The male DNA can also carry over if the initial lysis buffer is able to lyse the sperm cells during incubation, resulting in free sperm DNA in the supernatant.\textsuperscript{47} The carryover of male DNA into the female fraction is important to avoid because it results in a decrease in the amount of male DNA that can be collected. This can have devastating consequences if the male evidential sample is already present in low quantities, as a decrease in the DNA yield may result in no male DNA profile being observed, as it drops below detection point.\textsuperscript{60}

The main reason that female epithelial cells may be observed in the sperm fraction is due to an inefficient initial lysis step which allows epithelial cells to remain non-lysed.\textsuperscript{61} When centrifugation is performed, the non-lysed female epithelial cells will be pelleted with the sperm cells and subsequent treatment of the cells with DTT will result in the male fraction containing female DNA.\textsuperscript{47} This can have two outcomes for DNA analysis: a mixed profile will be observed - if the male and female cells are present in equal proportions; or a single-source profile will be
observed that will match the victim - if the epithelial DNA is present in excess of the sperm DNA.\textsuperscript{60,62}

4.1.5. Variations to the Differential Extraction Method

Many differential extraction techniques have been generated that alter the initial method designed by Gill et al.\textsuperscript{45} The aim of these methods is to entirely separate the two DNA fractions, with no cross-contamination in either fraction, which can then provide a single-source STR profile for both the victim and the perpetrator.\textsuperscript{53} Therefore, most of the variations aim to resolve one, or more, of the issues which can cause crossover of DNA, i.e. the number of washing steps, unsuccessful lysis of epithelial cells by the initial lysis buffer or premature lysis of the sperm cells during the initial lysis stage.

4.1.5.1. Alterations to the Differential Extraction Initial Lysis Buffer

The methods which have already been discussed have been unable to produce a male DNA fraction that does not contain female DNA. This means that the initial lysis step has been ineffective at lysing all the epithelial cells. A number of researchers have attempted to improve the recovery of sperm cells by making alterations to the initial buffer components including: proteinase K concentration, SDS concentration and detergent type. They also attempt to optimise the incubation temperature and time and investigate whether the addition of a cellulase can increase elution of cells from the swabs.
4.1.5.1.1. Mild Preferential Lysis

The first method proposed to optimise the differential extraction method aimed to reduce the amount of male DNA carryover into the female fraction by minimising the number of washing and centrifugation steps and altering the initial lysis buffer. The authors proposed a procedure which they termed “mild differential lysis” because the initial lysis stage is less stringent than the original method by Gill et al. Although the other components of the initial lysis buffer remain the same, the concentration of proteinase K was altered depending on the number of spermatozoa in the sample, which was classified using the method by Davies and Wilson. To reduce the number of washes, the male and female fractions are not physically separated. Instead, the lysis buffer allows for destruction of a large portion of the female epithelial cells, and the ratio of sperm DNA to female epithelial DNA becomes almost equal. Subsequent DNA analysis will therefore result in a mixed profile, with the two fractions being amplified almost equally. The results of the experiment indicated that “mild differential lysis” was able to decrease the amount of female DNA in the sample, while the male DNA concentration remained the same. Wiegand et al. classified this as a success as the male DNA yield was increased, however, this technique still results in the production of a mixed profile, which can be difficult to interpret. Given that the number of spermatozoa were classified based on the subjective cell counting technique, it is unlikely that this method would be accurate at determining the exact amount of proteinase K which should be added to make the fractions equal in concentration, and also, there would be difficulties with replicating results across laboratories.
4.1.5.1.2. Collective Increase in Pro K Concentration, Incubation Temp and Time

The method proposed by Yoshida et al.\textsuperscript{63} aimed to improve the initial lysis buffer by simultaneously increasing the proteinase K concentration, incubation time, and incubation temperature. The samples were initially incubated at 70°C for three hours, rather than the thirty minute incubation step used by Gill et al.\textsuperscript{55} The initial lysis solution was also altered to contain: TNE Buffer, 100 µg mL\textsuperscript{-1} proteinase K and 1% SDS, meaning that the proteinase K concentration is five times higher than the concentration used by Gill et al.\textsuperscript{55} The remainder of the wash steps, incubation times and temperatures were kept the same. The DNA typing results showed that the male fraction contained only male DNA and therefore, the buffer had been successful for its intended purpose; to completely eliminate the epithelial cells. However, the female fractions showed cross-contamination with male DNA, meaning that the sperm cells were prematurely lysed during the initial stage. If a sample contains a low-level of starting male DNA, as is the case for sexual assault swabs, sperm lysis during the first stage can be detrimental to the results and no male profile may be observed. Therefore, this altered method would not be recommended for use on sexual assault swabs.

4.1.5.1.3. The Effects of Adding a Cellulase to the Initial Lysis Buffer

A novel approach authored by Voorhees et al.\textsuperscript{64} attempted to increase the elution of both epithelial and sperm cells from swabs, by the addition of a cellulase-based enzyme mixture from \textit{Aspergillus niger} (\textit{A. niger}). \textit{A. niger} cellulase releases the cells by breaking down the cellulose polymers which the cells are adsorbed to on the swab.\textsuperscript{65,66} A number of samples were dried for 12 days and then incubated with either citrate buffer, \textit{A. niger} cellulase (250 µg mL\textsuperscript{-1}) in a citrate
buffer, or a differential extraction buffer. The results showed that cellulase-containing citrate buffer was similar in effectivity as the differential extraction buffer and the recoveries were 18.3% and 23%, respectively. Also, they were both more effective than samples which were incubated in citrate buffer only.

Voorhees et al. also chose to determine whether different cellulases, from three different micro-organisms: A. niger, Trichoderma Reseei (T. reseei), and Trichoderma viride (T. viride), would be better suited for cell elution. Each cellulase has a different mode of action to break down the cellulose polymers in the swab and they also have different enzyme activities, meaning they should have different effectivities of eluting cells. Given that T. viride has the highest enzymatic activity, it was not surprising that it was the most effective at eluting cells from swabs. In comparison to swabs treated with A. niger cellulase, it was twice as effective at recovering cells. Voorhees et al. therefore proposed that future studies should focus on T. viride cellulase-treated buffers.

Just one year later, Norris et al. furthered this work by eluting cells from samples using either citrate buffer, T. viride cellulase solution, a differential extraction buffer containing 2% SDS, or a solution containing 1% Sarkosyl. Of the samples which had been treated with cellulase, half were treated with 1% Sarkosyl to determine whether detergent and cellulase would have an additive effect when combined in solution. Surprisingly, incubation with T. viride cellulase alone could only recover 19% of the sperm cells. In comparison, Voorhees et al. recovered 21% of the male cells using the A. niger cellulase, which was only half as effective as the T. viride
cellulase. Although an unexpected result, this can be explained by observing the study design of each experiment. Norris et al.,\textsuperscript{67} used swabs which were dried for four weeks while Voorhees et al.,\textsuperscript{64} used swabs which had been dried for two days. As part of their study design, Voorhees et al.\textsuperscript{64} observed the effect that drying time had on the success of the cellulase-treated buffer by leaving swabs to dry for varying time intervals and then performing the incubations. They found that, regardless of which buffer was used, the number of sperm cells recovered decreased as the drying time increased. Therefore, it is likely that the decreased enzyme activity observed by Norris et al.,\textsuperscript{67} is due to the increased drying time they employed.

Norris et al.\textsuperscript{67} found that samples incubated in citrate buffer alone were able to recover 7.6\% of the sperm cells, which is similar to the 9.4\% recovered using cellulase in Voorhees et al.’s\textsuperscript{64} study. Treatment with 1\% Sarkosyl was able to recover 44\% of the male cells, which was very similar recovery to the differential extraction buffer containing 2\% SDS (43\%). This means that the differential extraction buffer was over twice as effective as the cellulase-treated buffer at recovering sperm cells. In contrast, Voorhees et al.’s\textsuperscript{64} results showed that the cellulase-treated buffer had a similar recovery percentage as the differential extraction method. It is possible that this is due to the detergent which is used in the differential extraction buffer as the study by Voorhees et al.\textsuperscript{64} used 1\% Sarkosyl and Norris et al. incorporated 2\% SDS.

Finally, the buffer containing cellulase and 1\% Sarkosyl was able to recover 53\% of the male cells, which is a 9\% improvement on the next best buffer, indicating that the enzyme and the
detergent are additive. Given that exposure to 1% Sarkosyl was able to recover 44% of the cells, it can be concluded that detergent plays a significant role in sperm cell recovery.

4.1.5.1.4. Optimising Detergent Concentration

Norris et al.\textsuperscript{67} also chose to compare both 1% and 2% solutions of SDS and Sarkosyl to determine which is more efficient at cell elution. The results were compared with samples incubated in a conventional differential extraction buffer\textsuperscript{45} with 2% SDS. The samples which had been incubated in the differential extraction buffer performed worse than the samples treated with detergent, indicating that the addition of detergents increases cell yield. Importantly, the results showed that SDS performed better than Sarkosyl at both concentrations, meaning that the type of detergent which is selected plays an important role in male cell recovery. Optimal recovery of cells occurred when a 2% SDS concentration was used (79%). Also, it can confirm that the results observed by Norris et al.,\textsuperscript{67} when comparing the cellulase buffer and differential extraction buffer, differ from the results of Voorhees et al.\textsuperscript{64} due to the different detergents they employ.

Hennekens et al.\textsuperscript{10} also investigated the optimal concentration of SDS by using the traditional differential extraction method\textsuperscript{45} but altering the SDS concentration to 1%, 0.5%, 0.25% and 0%. The method was also modified to include four washing and re-pelleting steps using the initial buffer, and an extra washing step with TE buffer. The number of wash steps was increased so high based on the findings of Comey et al.,\textsuperscript{68} who reported that an increase in the number of wash steps could decrease the amount of epithelial cell DNA found in the sperm cell fraction.
The authors found that when SDS was at 0% there was minimal impact on the proteinase K to lyse epithelial cells which indicates that, in contrast to the findings of Norris et al., the addition of a detergent does not have a significant impact on sperm cell recovery. It is possible that when the cells are subjected to a SDS or Sarkosyl solution only, they are increasing sperm cell recovery, however, when used in combination with differential extraction they could have a less noticeable effect. Hennekens et al. also found that both sperm and epithelial cells were able to carry over into the opposite fractions, however, there was not an extensive amount observed.

4.1.5.1.5. Optimising Detergent Types

Given the differing recovery abilities of SDS and Sarkosyl, Norris et al. hypothesised that other detergents could be more effective than SDS. The following detergents, at a 1% concentration, were investigated: SDS, Sarkosyl, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), cetyltrimethylammonium bromide (CTAB), and polyethylene glycol tert-octylphenyl ether (Triton-X 100). Again, SDS was shown to be more effective than the other detergents at eluting the sperm cells and was able to recover 75.6% of the sperm cells. In comparison, samples which were eluted using the differential extraction buffer were only able to recover 40% of the cells and the other detergents performed dismally compared to SDS.

4.1.5.1.6. Optimising Proteinase K Concentration

Based on their previous discoveries, Norris et al. proposed that since detergent increases cell elution, it must be one of the other components of the differential extraction buffer which results in the decreased effectivity of samples incubated in the buffer by Gill et al. As such,
they decided to observe what happened when proteinase K was added to samples which had already been incubated for two hours. Samples were incubated in a 1% SDS solution, which showed a 76% sperm cell recovery, and then incubated for a further 0 – 20 minutes after the addition of proteinase K. Surprisingly, the results showed that addition of proteinase K immediately reduced the amount of sperm cells to 52%, a 24% decrease in recovery, in less than 1 minute. As the samples were left to incubate for a longer period of time, the sperm cell recoveries decreased. The epithelial cell recoveries also decreased over time and within 10 minutes, they were completely removed.

Hennekens et al. employed the differential extraction method from Gill et al.,45 except that the proteinase K concentration was altered to: 0, 10, 20, 150 and 300 µg mL\textsuperscript{-1}, to determine what effect proteinase K has on sperm cell recovery. When the proteinase K concentration was altered to 0 µg mL\textsuperscript{-1}, a large proportion of epithelial cells remained after incubation, meaning that proteinase K is required to lyse epithelial cells. However, the results also showed that the when a proteinase K concentration of 300 µg mL\textsuperscript{-1} was applied for 2 hours, it had minimal effects on the lysis of sperm cells. The amount of male DNA observed in the non-sperm fraction did not alter significantly between any of the concentrations used.\textsuperscript{10} This is in complete contrast to the findings of Norris et al.\textsuperscript{67} and the only difference between these two sections of the experiment is that Norris et al.\textsuperscript{67} used 42°C as the incubation temperature while Hennekens et al.\textsuperscript{10} used 56°C, which should not have such a significant effect on the outcome. Interestingly, Yoshida et al.\textsuperscript{63} observed a carryover of male DNA into the female fraction using their altered method with a proteinase K concentration of 100 µg mL\textsuperscript{-1}. However, according to the results of Hennekens
et al., it would not have been the increased proteinase K concentration which is responsible for this outcome. This means the excess sperm cell lysis may be due to the increased incubation time or temperature.

4.1.5.1.7. Optimising Incubation Temperature and Time

To optimise the recovery of sperm cells, Norris et al. wished to determine the effect of altering incubation temperature. Samples were therefore eluted using 1% SDS with the following incubation temperatures: 25°C, 42°C or 56°C. As expected, SDS was able to recover more sperm cells than the other two buffers, at each different temperature which was used. The optimal temperature was recorded to be 42°C (88%), however, incubation at 25°C gave almost equal recovery (86%) and as the temperature was raised above this, to 56°C, there was an observed decrease in the amount of DNA recovered (68%). Using the optimal conditions discovered throughout Norris et al.’s experiment, time alterations from 15 to 120 minutes were observed for samples incubated with an altered differential extraction buffer. Increasing the incubation time only increased the sperm cell yield by 7%, from 80% – 87%, which was not significant.

Hennekens et al. also used the traditional buffer described by Gill et al. to observe the effects when the incubation time was altered. Samples were tested for 30 minutes, 2 hours, 4 hours and overnight. Hennekens et al. found that effective lysis of the epithelial cells was observed after 30 minutes. Importantly, the amount of male DNA in the non-sperm fraction was not altered by incubation times. This indicates than an increased incubation time does not lead to the lysis of sperm cells during the initial incubation process. To determine the optimum
temperature, the conditions were kept the same as above, except that the incubation time was set for 2 hours and the incubation temperatures were varied to 22°C, 37°C, 42°C, and 56°C. Their results showed that altering the incubation temperature did not have a significant effect on the amount of male DNA which was able to carry over into the female fraction. This did not replicate the results of Norris et al., but there is no obvious conclusion as to why these two results were so different.

4.1.5.1.8. The Effect of Drying Time/Storage

To determine the effects of extending the drying time, Norris et al. dried mock casework samples for 12, 24 and 32 weeks. The results showed that treatment with a differential extraction buffer provided similar recoveries at each drying time, however, samples treated with SDS observed an overall decline in sperm cell recovery as the age of the sample increased. At 12 weeks and 24 weeks drying time, the detergent-spiked solutions were still twice as effective at eluting sperm than differential extraction buffer, however, once the samples had been dried for 32 weeks, the methods had relatively similar extraction efficiencies. Voorhees et al. also observed similar results when their swabs were left to dry for various lengths. Voorhees et al. and Norris et al. both found that the yield recovered by the traditional differential extraction buffer, or the citrate buffer, did not vary significantly as the drying time was increased, however, the samples treated with a cellulase-treated buffer or a detergent solution both showed a decrease in sperm cell recovery over time. The authors of both papers propose that either the cellulase, or detergent concentration needs to be optimised for older samples.
A more recent study by Hennekens et al.\textsuperscript{10} also investigated whether leaving the samples to dry for an extended period of time would have an impact on the amount of carryover observed between the fractions. They also assessed whether the amount of carryover could be altered by changing the four conditions described already (proteinase K, SDS, incubation temperature and incubation time). The results showed that drying the samples resulted in a much higher carryover between the two fractions. Importantly, the amount of male DNA which was lost to the female fraction was approximately 45%. However, the results of this study also showed that altering the differential extraction conditions did not alter the carry over a significant amount. Hennekens et al.\textsuperscript{10} therefore propose that this loss of sperm cells is likely due to other factors, that are not related to the method itself, such as DNA damage or enzyme activity within the sample. One of the most vital things that can be taken from any of these proposed methods, and the traditional differential extraction method, is that they are unable to successfully recover 100% of the spermatozoa from any of the swabs.

\textit{4.1.5.2. Differex™}

Differex™ was first introduced by Tereba et al.\textsuperscript{50} in 2004 and is produced by the Promega Corporation (Madison, WI). It is currently used by many forensic DNA laboratories as an alternative to the traditional differential extraction procedure.\textsuperscript{13} Tereba et al.\textsuperscript{50} believed that one of the best methods for obtaining high sperm cell purity, and high sperm cell yield, is to treat the mixed cell samples with proteinase K, in the absence of DTT and then follow this with subsequent centrifugation of the sample. Therefore, they hypothesised that it was not the
extraction process of the traditional differential extraction procedure that is the problem, but instead, it is the separation strategy which traditional methods employ that is causing the carryover of epithelial and sperm cells. This therefore led to generation of the Differex™ System.

The Differex™ System incorporates a proteinase K-selective digestion and centrifugation step in its protocol and then aims to increase the yield and purity of sperm cell DNA by eliminating the wash steps which are required for traditional methods. To separate the two fractions, a combination of phase separation and differential centrifugation is employed. A non-aqueous separation solution is used during centrifugation of the combined digested sample and buffer, which pulls the sperm cells from the solid matrix and forms a sperm cell pellet at the bottom of the tube. The soluble DNA remains in the aqueous buffer which, due to differences in density, sits on top of the non-aqueous separation solution and can be removed for purification and downstream DNA analysis. A yellow dye which partitions into the epithelial cell fraction is also included in the system which allows for easier separation of the aqueous phase. The sperm and epithelial fractions are then purified using the DNA IQ™ System (Promega, Madison, WI) or the traditional organic method of phenol/chloroform.

Tereba et al. reported that the total time required for analysis, from addition of the proteinase K to obtaining purified DNA, can be as little as two hours. The results showed that when DNA was amplified from samples which had been stored at room temperature for four years, there was sufficient DNA extracted to produce an STR profile for both the male and female fractions. However, there was carryover observed for both the female and male fractions. The carryover
of epithelial cells into the male fraction was only minor and a clear genotype could still be observed, while the carryover was more noticeable into the female fraction. The authors hypothesised that this could have occurred due to lysis of the sperm cells during storage, or during premature lysis of the cells during proteinase K incubation.\textsuperscript{50} The Differex\textsuperscript{™} System was also applied to swabs which had been obtained 11 hours post-coitus, and had been stored at room temperature for four years.\textsuperscript{50} Again, enough DNA was collected to produce a male and female profile and there was no observable carryover of sperm cells into the female fraction. However, there was carryover of epithelial cells into the male fraction (approximately 10 – 15\%) which was hypothesised to be due to inefficient digestion of the epithelial cells.\textsuperscript{50}

The efficiency of the Differex\textsuperscript{™} System was evaluated by Tsukada et al.,\textsuperscript{70} who assessed the system in combination with four different purification methods and compared those results with the two-step differential extraction method.\textsuperscript{63} Samples containing known amounts of male and female DNA were generated and subjected to analysis with the Differex\textsuperscript{™} System. The results showed that all of the methods were able to extract enough DNA to be allele typed when the sperm counts were 50,000 and 500,000. Importantly, none of the different purification methods resulted in any signal peaks being observed when sperm counts were lower than 5,000. In terms of turn-around time, the two step method required 1 – 2 days for full analysis to be performed, while only 2 – 3 hours was required for the Differex\textsuperscript{™} System. The extraction efficiency was generally equivalent for the Differex\textsuperscript{™} System when combined with any of the four purification methods\textsuperscript{70} and it is also easy to employ.\textsuperscript{71}
In 2008, the Differex™ System was assessed by comparing it with the Chelex-100® method, which is used routinely at the Swedish National Laboratory of Forensic Science, SKL. Swabs containing equal numbers of sperm and epithelial cells were transferred to cotton swabs and then extracted using either the Chelex-100® based method or four different Differex™ methods. The first Differex™ method assessed was performed as described in the Promega instructions (Proteinase K: 270 μg mL⁻¹). The results of this method were assessed and the method was altered to try to enhance the efficiency. This was performed another two times, resulting in four Differex™ methods being employed, with each method slightly improving over the previous method. The final method was able to recover approximately 0.6 ng μL⁻¹. The authors concluded that, after the modifications had been applied, the Differex™ System gave comparable results to the Chelex-100® method. However, Valgren et al. noted that, when trying to observe the cells using microscopy, the separation solution formed droplets which made it difficult to obtain an even microscope smear. Due to the inefficient smear producing value of the system, the SKL chose not to employ the Differex™ System for future casework analysis.

In 2011, Vuichard et al. investigated a number of different differential extraction procedures by sending mock casework samples to nine different laboratories, who were to conduct the DNA isolation as they normally would, and then send back all the DNA extracts for qPCR and profiling. Vuichard et al.’s laboratory then determined the amount of male and female DNA which was recovered by each of the nine laboratories. Each of the laboratories employed different extraction protocols, differing in the: components of the initial and secondary cell lysis buffers, number of washes employed, methods used for purification and concentration devices which
were used. All of the methods employed were able to enrich the sperm cell fraction but not remove the epithelial cells completely. Laboratories five, six, and seven recovered the least amount of male and female DNA out of all of the laboratories, however, they do not employ the same DNA isolation protocols. Vuichard et al. propose that this is due to inefficient elution of cellular material from the swabs, which would explain why both fractions were not very well represented by these methods. Observation of the STR profiles demonstrated that a full DNA profile, without contaminating epithelial cells, was only achieved twice out of the possible 18 samples. This was achieved by laboratories four and nine, both of which employ the Differex™ System. However, six of the nine laboratories were able to observe male DNA profiles which were mixed with female profiles. The main result observed by this study was that the success of the analysis varied markedly amongst laboratories. This could prove to be an issue if laboratories need to compare their results, and therefore, a single method needs to be developed which can be utilised in exactly the manner in each laboratory.

4.1.5.2.1. Advantages and Disadvantages of Differex™

Given that the traditional differential extraction procedure generally requires an overnight incubation step, this method is preferential due to the speed at which samples can be processed. Also, the traditional differential extraction methods require a number of centrifugation steps, which can lead to the premature lysis of sperm cells, while this method requires only one centrifugation step. Although the preliminary study by Tereba et al., and those which follow, are performed in single tubes, the samples could easily be transferred to a 96-well plate where automated DNA purification can occur across multiple samples at one time,
increasing processing time. Tereba et al.\textsuperscript{50} also propose that a 96-well unit would become available which would allow digestion and centrifugation to occur on the same unit, which can then be submitted for DNA purification in the same 96-well plate. This would decrease the possibility of contamination and lysis of cells during transfer steps.

Although there are a number of advantages to this method, it is still limited. There is still observed carryover of the male and female cells into opposing fractions which ultimately means that this method is ineffective at separation. Both Tsukada et al.\textsuperscript{70} and Valgren et al.\textsuperscript{69} found that when removing the separation solution from the sperm pellet, some sperm can be lost to the aqueous fraction. Also, Vuichard et al.\textsuperscript{47} concluded that the excessive loss of male DNA, through the differential methods employed by the nine laboratories, is not acceptable and alternative methods should be proposed.

4.1.5.3 Treatment of Epithelial Cells with DNase

The methods which have already been discussed in this dissertation are generated on the premise that the female and male fractions must be physically separated from each other for successful profiling to occur.\textsuperscript{72} As already described, this requires a number of centrifugation and washing steps which can have a detrimental effect on sperm cell yield. The methods proposed in this sub-section aim to eliminate the washing steps by performing an initial gentle lysis incubation, treating the epithelial cells with DNase to degrade the female DNA, removing an aliquot of the soluble DNA for PCR analysis and finally, lysing the sperm cells with DTT.\textsuperscript{72,73}
The use of DNase to degrade the victim’s DNA was first described by Garvin et al.\textsuperscript{72} who added DNase I to the buffer which selectively degrades the epithelial cells and only causes minimal loss from the sperm cell fraction. Observation of the solution after treatment showed that the soluble DNA was decreased by over 1000-fold, while the sperm heads remained intact. Garvin et al.\textsuperscript{72} proposed that the loss of cells from the sperm fraction can be accounted for by the degradation of male non-sperm cells that are susceptible to proteinase K digestion. The authors also selected a buffer which uses Triton X-100, in replacement of SDS, so that the DNase I can be added to the detergent/proteinase K buffer that is used for elution of the sperm cells. This means that a buffer change is not required for elution and then extraction.\textsuperscript{72}

The protocol involves a ten minute incubation of the sample, followed by removal of an aliquot from the upper part of the liquid, which is the female fraction that will be submitted for PCR analysis. Another four hour incubation is performed and then DNase I is added to the solution to degrade the female DNA. A solution containing an excess of EDTA is then added to inactivate the DNase I and then the sperm cells are lysed with DTT. Garvin et al.\textsuperscript{72} hypothesised that the decrease in handling and centrifugation steps should result in a higher sperm DNA yield. This protocol was compared to the standard selective lysis protocol proposed by Gill et al.\textsuperscript{45} [1985], with the addition of three washing steps using reagents supplied by Qiagen. Also, for further comparison of the new method, five vaginal swabs which had been collected from rape victims were processed in parallel using the Differex™ System.
Both Garvin et al.’s\textsuperscript{72} method, and the differential extraction method, gave clear male STR profiles from swabs taken soon after coitus, which was also observed for swabs taken 36 hours post-coitus. Neither of the methods could give clear male STR profiles after 70 – 100 hours post-coitus. In particular, for a swab collected 61 hours post-coitus, the nuclease method proved superior to the preferential lysis method as it provided three of the four loci, while the preferential lysis method was unable to determine the male profile at any of the STR loci.\textsuperscript{72} Of the five swabs collected from the Police, two gave excellent male profiles with both methods, possibly due to a high ratio of sperm cells to epithelial cells in the starting sample. Another swab was unable to be profiled by either method. The two remaining swabs showed that the nuclease method was superior to the Differex\textsuperscript{™} method. The Differex\textsuperscript{™} method gave a mixed profile for one swab, and for the other swab only a female profile was produced. The nuclease method was able to provide a clear male profile for the STR system used for one swab, and 11 of the 13 alleles could be typed for the other swab. Garvin et al.\textsuperscript{72} concluded that all of the three methods tested are able to effectively separate the two fractions, however, this nuclease-based approach gives a superior male fraction.

The use of an alkaline lysis method for DNA extraction was first introduced into the forensics field by Rudbeck & Dissing,\textsuperscript{74} who found that semen stains could be extracted by the addition of 20 \( \mu \text{L} \) 0.2M NaOH and a five minute incubation step at 75°C. The extraction process is then stopped by the addition of 180mL 0.04 M Tris-HCl, pH 7.5. In the article by Hudlow et al.,\textsuperscript{73} swabs are placed in a mild alkaline solution (0.1 N NaOH) to generate the non-sperm lysates and the non-sperm DNA is then removed by neutralization and enzymatic digestion steps. The swabs
are then placed in a 1 N NaOH solution for 5 minutes at 75°C to generate the sperm fraction lysates and to simultaneously inactivate the DNase activity. Mock casework samples were used in this study, as well as; post-coital swabs which had been frozen after collection and, swabs which had previously been submitted for analysis with the differential lysis procedure.

The experiment consists of two main sections; performing the alkaline lysis method in a single tube to determine optimal lysis conditions for the epithelial cells and sperm cells, and then applying this optimised method to a 96-well plate containing samples.\textsuperscript{73} Although the authors were able to determine the minimum concentration of NaOH required for epithelial cell lysis without lysing sperm cells, semen has a buffering capacity, and therefore, this value could be different for different samples.\textsuperscript{75,76} Application of the method on the 96-well scale did not yield optimum sperm DNA, and therefore, the lysis and DNase digestion times were altered again and the authors also chose to include an additional DNase digestion step and corresponding post-digestion rinse steps to reduce carryover of non-sperm DNA.

The results of the 96-well lysis procedure were compared to mock sexual assault swabs which were extracted using the standard differential extraction method.\textsuperscript{45} Comparison of the total yields of male DNA extracted using each method showed that the methods gave similar results, however, the authors believe that STR typing results are a better indicator of the success of these methods. Both techniques were able to obtain full, or major, male profiles from samples 0.1 µL of semen. When the amount of semen fell below 0.01 µL, the samples only gave female profiles, or contained only a small number of male alleles. The final part of the experiment used
swabs which had already been subjected to the standard DTT extraction method, and subjected them to analysis with this method to determine whether spermatozoa remained bound to the substrate. This is based on the findings by Voorhees et al.\textsuperscript{64} and Norris et al.\textsuperscript{67} who both found that DTT is not 100% effective at removing the sperm cell DNA. The results showed that the DTT method was only able to capture 50% of the spermatozoa and, the remaining DNA could be removed successfully using this newly proposed method.

In 2012, Garvin et al.\textsuperscript{13} authored an article which used the Erase Sperm Isolation Kit (Erase), produced by Paternity Testing Corporation (PTC) Labs, to treat the residual female DNA, which remains on the sperm pellet, without having to introduce a buffer change. As with the standard differential lysis procedure,\textsuperscript{45} this method includes a proteinase K/detergent lysis step to elute the sperm cells from the swab and to digest the non-sperm cells. It is also subject to centrifugation of the sperm pellet and removal of the non-sperm containing supernatant. The nuclease is then added after this step to remove any DNA which remains on the sperm pellet which should, theoretically, reduce contamination of the fractions.\textsuperscript{13} The nuclease is then inhibited and the sperm cells are lysed into solution. Garvin et al.\textsuperscript{13} evaluated the success of the Erase Kit by comparing it against standard methods which are used by five separate crime laboratories. Three of the laboratories use modified versions of the differential lysis method proposed by Gill et al.\textsuperscript{45} and the other two used Differex\textsuperscript{™}.

The results demonstrated that each of the methods utilised were able to give female STR profiles, however, there were minute amounts of male PCR signal observed in the female STR
profiles. Garvin et al.\textsuperscript{13} hypothesise that this is due to the lysis of semen components which are non-sperm, i.e. macrophages and male epithelial cells. The standard methods gave a higher DNA yield than the Erase Kit 80\% of the time, however, each method was able to obtain enough male DNA to provide an STR profile. The STR profiles produced using the Erase kit were superior to the profiles generated using the other methods.\textsuperscript{13} The standard methods were only able to provide full male profiles 25\% of the time, while the Erase Kit was able to give full male profiles 80\% of the time. Most importantly, the profiles observed from the Erase Kit predominantly did not contain any contaminating female DNA. The samples which were only spiked with 1,500 sperm were unable to be processed successfully with the standard methods, which gave a mixed profile, but were able to be successfully processed with the Erase Kit.

Garvin et al.\textsuperscript{13} also tested the technique on post-coital vaginal swabs which collected from volunteers after 5 minutes, 6 hours, 24 hours, 34 hours, 48 hours or 58 hours. The swabs were treated with either Differex\textsuperscript{TM} or with Erase. The Erase Kit was able to provide a full male STR profile at each of the time points while those analysed with the Differex\textsuperscript{TM} System gave full male profiles from five minutes to six hours post-coitus, but gave either mixtures or female-only profiles after six hours. Evidence from four sexual assault cases was also collected for analysis with a standard differential lysis method, and a small amount of evidence that remained was treated with Erase. In all of the cases which were analysed, the Erase Kit was able to produce better separation of the fractions.\textsuperscript{13} Perhaps one of the most important indicators that this method is successful is that Garvin et al.’s\textsuperscript{13} laboratory now employs the Erase Kit, instead of the conventional differential lysis method for separating male and female fractions.
4.1.5.3.1. Advantages and Disadvantages of Treatment with DNase

The methods described under this sub-section have mostly only provided advantages over the previous methods described. Treatment of swabs with nuclease-based approaches is able to provide an effective reduction in the amount of epithelial cells which carry over into the male fraction.\textsuperscript{60} Also, they have been shown to be more effective than the standard differential lysis procedures currently employed by laboratories.\textsuperscript{13,73} In comparison to the traditional methods employed, these methods are preferential as they require only a number of pipetting steps, which reduces the occurrence of contamination and loss of sperm cell DNA.\textsuperscript{72,73} The steps which are used in this study are also amenable to existing robotic workstations, meaning that future designs could eliminate handling altogether. Also, DNase I has been shown to have a minimal effect on the sperm cells, which could be critical if the samples which are being analysed only have a very small amount of starting male DNA.\textsuperscript{72} Hudlow et al.\textsuperscript{73} also demonstrated that the method can be applied to a 96-well plate and, with the method itself only taking approximately four hours, this will greatly increase throughput and also increase productivity of the labs which are able to employ this method. The protocol of the Erase Kit is also very easy to follow and the results observed across laboratories were quite similar, which is not observed for the differential lysis procedure.\textsuperscript{72} Although they have all of these advantages, nuclease-based methods are still unable to completely separate the male and female fractions. However, the results obtained are similar, if not better, than those obtained by the standard differential lysis procedures, and therefore, given the long list of advantages which come with this method, use of these methods is encouraged.
4.1.5.4. The Introduction of a One-Step Buffer

The method proposed by Lounsbury et al.\textsuperscript{53} aimed to eliminate the need for a second incubation step by adding the proteinase K directly to the elution buffer. The study looked at mock sexual assault samples which were either; fresh (dried for one week), aged up to one year or contained a low starting evidence amount of sperm cell DNA (<500 cells). Swabs which contained known amounts of male and female DNA were incubated in the one-step buffer the swab was subsequently removed from the eluted cell sample. The sample was centrifuged, the supernatant removed, the sperm pellet rinsed using the one-step buffer and the sample centrifuged again to re-pellet the sperm cells. The rinsing step was replicated four times and then the DNA from the samples was extracted.

Lounsbury et al.\textsuperscript{53} hypothesised that this new one-step buffer method would be able to obtain similar yields as what is described by the two-step method\textsuperscript{63} and that, by optimising the pH of the buffer, they could increase the yield observed. It was discovered that the buffer worked optimally at a pH of 8.5, the optimal temperature was 42°C, and also, an incubation time above 30 minutes was not warranted as the recovery of sperm cells plateaued at this time.\textsuperscript{53} Importantly, Lounsbury et al.\textsuperscript{53} found that, only when the four components were used in tandem, could the sperm cell recovery be so greatly enhanced. The sperm cell recovery for this method was almost twice the amount recovered using the standard differential lysis procedure, and the method is also able to lyse the epithelial cells effectively. The theory that vortexing of the sample throughout incubation could increase sperm cell recovery was also disproved and
the results actually showed the opposite, there was a decrease in sperm cell recovery with an increase in vortexing. The authors hypothesise that this is due to mechanical lysis of the sperm cells due to damage.

The results of the STR analysis showed that the STR profiles, for each method, were quite similar from 50,000 cells down to 1,000 cells. However, as the initial cell count became lower the one-step buffer proved more effective at producing loci (14 of 16) than the conventional differential extraction buffer (4 of 16 loci). Once the cell count was down to 250 cells, neither of the methods were able to obtain STR profiles. Lounsbury et al. hypothesised that the yield of DNA collected using this method could be limited by the number of handling steps used in this method: four wash steps, one tube transfer step and pipetting.

The main finding of Lounsbury et al.’s study was that the one-step buffer was able to successfully recover 89% of the sperm cells, while the two-step system recovered 81% and the conventional differential extraction buffer could only recover 45%. These methods were then applied to swabs which had been stored for up to one year. After the samples had been stored for a year; the one-step buffer and two-step system were still able to recover 68% of the sperm cells, while the conventional differential extraction method was only able to recover 22%. Therefore, on aged samples, the one or two-step buffer methods can provide a 300% increase in the amount of sperm cells recovered, when compared to the differential extraction method. It is also completed in approximately half the time.
4.1.5.5. Invention of the SPRED Calculation

One of the more recent articles published was authored by Klein & Buoncristiani\textsuperscript{46} and, in the article, they introduce a semi-quantitative method for analysing the success of some of the methods which have been covered by this dissertation. The methods which have already been discussed have a range of “successes” at preventing the carryover of male DNA into the female fraction and vice versa. The methods which place a focus on maximizing sperm cell recovery (i.e. yield) include those by; Voorhees et al.,\textsuperscript{64} Norris et al.,\textsuperscript{67} Allard et al.,\textsuperscript{29} and Lounsbury et al.\textsuperscript{53} Other methods place a distinct focus on efficient removal of epithelial cells from the sperm fraction, i.e. getting a clear sperm fraction.\textsuperscript{13,42,48,63,72} Klein & Buoncristiani\textsuperscript{46} propose an approach which is able to give a semi-quantitative value of the efficiency of each method, in relation to sperm cell recovery and epithelial cell removal as the two factors involved. The calculation which is introduced has been termed the Separation Potential Ratio for the Extraction Differential (“SPRED”) and it is able to compare two methods against each other by calculating their overall method efficiencies.\textsuperscript{46} Three different differential extraction methods were assessed in this study: two versions of the DTT-based procedure and Erase, which was employed according to manufacturer’s instructions.

Using data from qPCR analyses, the yield of male and female DNA in the sperm fraction was calculated, and also the amount of female DNA in the non-sperm fraction. The amount of male DNA which was recovered by each method was calculated by, dividing the average amount of male DNA recovered in the sperm fraction, with the estimated starting mass on the swab and then multiplying it by 100 to get a percentage of estimated recovery. An estimation of the amount of female DNA which carried over into the sperm fraction was calculated by, dividing
the amount of female DNA in the sperm fraction, with the estimated amount of female DNA which started on the swab. This was then converted to a percentage, giving the estimated percentage carryover of female DNA.

The success of a differential extraction method is dependent on both the percent recovery of male DNA and percent carryover of epithelial DNA. The SPRED of the sperm fraction, which is a ratio, is calculated by dividing the percentage of sperm DNA recovery by the percentage of female DNA carryover. Therefore, the separation potential of a method can be characterised by its SPRED. The higher the sperm cell recovery, the higher the SPRED value, and therefore, a higher SPRED value is indicative of a better separation potential of the method. Although a number of methods have been shown to increase the removal of non-sperm DNA, Klein and Buoncristiani propose that researchers are unlikely to use these as they may have a “perceived risk” that the sperm DNA yield will be lowered. This calculation will enable methods to be directly compared to each other, so that assumptions do not have to be made. The SPRED is calculated using the three different methods described above, to give them a separation potential value.

4.1.5.5.1. Erase vs Standard Differential Extraction with One Initial Lysis Step

Utilisation of the Erase procedure on swabs resulted in a lower sperm cell recovery than that of the differential extraction method, which were 4.3% and 26%, respectively. Conversely, the sperm fraction showed a lower estimated carryover of female DNA when the Erase Kit was used (0.0048%), rather than the differential extraction method (0.12%). Therefore, although a
smaller recovery of sperm was recorded, the ratio of male DNA to female DNA in the Erase fraction was higher than the conventional differential extraction method. The SPRED calculation was then applied and the estimates were given as 896 for the Erase Kit and 217 for the differential extraction method. This means that, although the Erase method produced a lower yield of DNA, the separation potential of the method was much higher than the differential extraction method. This calculation also shows ways in which the method can be improved. Given that the percentage of DNA recovered is the numerator in the SPRED calculation, it is possible that more productive effort would be better aimed at reducing the epithelial cell carryover to increase the SPRED. However, if samples contain vast amounts of female DNA, but only a small amount of male DNA, then the yield of male DNA would be vital for obtaining enough DNA for an STR profile to be generated, regardless of its contamination level.

4.1.5.5.2. Erase vs Standard Differential Extraction with Two Initial Lysis Steps

Erase was then compared to a standard differential lysis method which used a two-step initial epithelial cell lysis. Addition of an initial lysis step has previously been used in methods proposed by Comey et al. and Yoshida et al., as an attempt to reduce carryover of epithelial cell DNA. Studies conducted after these suggested that, although they do remove epithelial cells, they may also harm the yield. In a more recent study by Hennekens et al., the premature lysis of sperm cells was investigated and it was found that alterations to the initial lysis conditions did not necessarily alter the amount of sperm cell lysis during the initial cell lysis stage. In this study, an additional wash step is introduced to determine whether or not this can affect the SPRED value.
The results showed that introduction of the extra initial lysis step resulted in a 5.5-fold decrease in the amount of non-sperm DNA in the sperm fraction and there was only a slight reduction in sperm DNA recovery. The addition of this extra initial lysis step was responsible for increasing the SPRED from approximately 217 to 1300. This results in a higher SPRED than Erase, which was 896, meaning that is has a better separation potential, and there is also a higher amount of sperm DNA recovered. These results indicate that the addition of extra initial lysis steps to the standard differential lysis method, which decrease the epithelial cell carryover, could be extremely beneficial.

The authors conclude that, although this method can apply to methods under a specific set of conditions, it does not necessarily directly measure the effectiveness of the methods which are assessed. Given the vast amount of differential extraction procedures which have been generated over the years, it is important that a study like this exists which is able to assess the recovery ability of each method. This calculation was used by the authors to determine the SPRED of three different methods and compare their effectivities. However, this calculation can be applicable to all methods that have been generated and it is possible that the SPRED of each method could be determined, allowing direct comparison of all current methods. Also, any new alterations which are made to the existing techniques can be directly compared with the previous method.
4.1.6. Recommendations for Differential Extraction

Although the differential extraction method was lacking in effectivity when it was first introduced,\textsuperscript{45} the research that has been conducted since its inception has led to the development of faster, more successful methods. In particular, treatment of the epithelial cells with DNase I appears to generate the purest male DNA fractions, which is the ultimate goal of these techniques.\textsuperscript{72,73} Importantly, it has already been shown that these techniques are amenable to robotic workstations, which will likely be the future pathway of forensic science. In comparison to traditional differential extraction methods, one of these methods has already been applied to a 96-well plate, which will increase throughput extensively.\textsuperscript{73} It was also able to produce results in as little as four hours, decreasing the turnaround time of samples significantly. DNase I has also been incorporated into a kit which is easy to follow, and would allow laboratories to compare their results directly.\textsuperscript{13} Future studies using differential extraction should therefore focus on: validating current methods using DNase I, generating novel methods, applying them to a wide range of mock casework samples and then modifying them for high throughput analysis.

4.2. Y-STR Analysis

STR sequences exist throughout the human genome and they comprise of short nucleotide sequences (i.e. di-, tri- and tetrancleotides) which are repeated a number of times.\textsuperscript{77} The number of times the sequence is repeated will vary amongst individuals and the fragments of DNA which are amplified using PCR will therefore vary in length. Amplification of these loci will result in the generation of fragments of specific lengths, which correspond to the number of
repeats multiplied by the length of the nucleotide repeat. STR sequences which are known to be highly polymorphic amongst individuals can be useful for discriminating forensic stains\textsuperscript{77} and, although it was known that autosomal STRs could be highly polymorphic, there was no evidence of polymorphic STRs on the Y-chromosome prior to 1992.

4.2.1. The Introduction of Y-STRs

The concept of targeting polymorphic STRs on the Y-chromosome was first proposed by Roewer et al.,\textsuperscript{78} who discovered that the 27H39LR (DYS19) Y-chromosome specific locus was reasonably polymorphic when compared with two autosomal STRs on chromosome 12. Amplification of all three loci showed (GATA)\textsubscript{n} repeats which varied in repeat number. The two chromosome 12 STRs had six and eight alleles typed, respectively, while amplification of the DYS19 locus resulted in four alleles being typed.\textsuperscript{78} Although not as informative as the STRs from chromosome 12, the authors proposed that if it were used in combination with other Y-STRs, a high degree of informativeness could be achieved. To follow on from this study, later research conducted by Muller et al.,\textsuperscript{79} Gomolka et al.,\textsuperscript{80} and Santos et al.\textsuperscript{81} demonstrated that different populations had considerable allele frequency diversity at the DYS19 locus.

Almost immediately after its inception, Y-STR analysis was applied to a forensic case. In this study by Roewer and Epplen,\textsuperscript{77} a mixed dinucleotide polymorphism ((GT)\textsubscript{n}(GA)\textsubscript{m}), that is located in the HLA-DRB genes, was used in tandem with the three loci described by Roewer et al.\textsuperscript{78} The HLA-DRB alleles could not be amplified by PCR, and therefore, the notion of this section of the Y-chromosome as a marker was discarded. However, the DYS19 locus was successfully amplified
and importantly, was only amplified in male DNA containing samples. In this article, amplification of this Y-STR was able to help exclude a suspect from a sexual assault case based on different allele designations. Also, the sperm DNA which was collected from the sexual assault swab was shown to be degraded, however, because the product of the DYS19 locus is short, it could still be amplified.\textsuperscript{77} Given that differential extraction is notoriously ineffective at isolating sperm cells from degraded samples, this method could be a possible alternative for older samples.

4.2.2. The Continued Discovery of Y-STR Markers

Given Roewer and Epplen’s\textsuperscript{77} conclusion that Y-STRs are a useful forensic tool, and Roewer et al.’s\textsuperscript{78} conclusion that more Y-STRs need to be discovered to increase the discrimination power of the method, researchers set out to discover as many highly polymorphic Y-STRs as possible. From Y-STRs first being employed, to the most recent study published, hundreds of Y-STRs have been discovered. Given the enormous amount of literature covering the introduction of new Y-STRs and the applicability of current Y-STRs, Table 1 is included to briefly summarise the introductory articles (from 1994 – 2002). Articles which are of particular importance, and require a more extensive review, will be discussed in future paragraphs.
Table 1: Brief summaries of Y-STR articles (from 1994 - 2002).

<table>
<thead>
<tr>
<th>Author(s) &amp; Year</th>
<th>Y-STRs Targeted</th>
<th>Main Finding(s)</th>
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<tr>
<td>Chen et al.(^{82}) 1994</td>
<td>DXYS15</td>
<td>This microsatellite was mapped to both the X and Y chromosomes, meaning that amplification of this STR resulted in two products being observed. These were designated as either DXYS156X or DXYS156Y, depending on which chromosome they were located on.</td>
</tr>
<tr>
<td>Mathias et al.(^{83}) 1994</td>
<td>DYZ1, DYZ2, YCAI, YCAII, and YCAIII</td>
<td>DYZ1 and DYZ2 provided different patterns for each individual observed, from a panel of 91 – except for two. Amplification of YCAI resulted in a single product, specific for the Y-chromosome. Amplification of YCAII resulted in two products on the Y-chromosome, designated as YCAIIa and YCAIIb. Amplification of YCAIII resulted in two products on the Y-chromosome, designated as YCAIIa and YCAIIb.</td>
</tr>
<tr>
<td>Roewer et al.(^{84}) 1996</td>
<td>DYS19, DYS389I and DYS389III, DYS390, DYS391, DYS392, and DYS393.</td>
<td>DYS390 was the most polymorphic and DYS389I was the least polymorphic, while the others lie somewhere in between. Therefore, each Y-STR has its own discriminatory power. It was concluded that the areas of variance on the Y-chromosome are equally as polymorphic as autosomal STRs.</td>
</tr>
<tr>
<td>Cooper et al.(^{85}) 1996</td>
<td>DYS19, DYS389-I and DYS389-II, DYS390 and DYS391</td>
<td>DYS390 and DYS391 were the least polymorphic, DYS19 and DYS389II were the next least polymorphic and DYS389I was the most polymorphic. The main conclusion was that haplotypes do frequently alter at Y-STRs.</td>
</tr>
<tr>
<td>Kayser et al.(^{86}) 1997</td>
<td>DYS19, DYS385, DYS389I/II, DYS390, DYS391, DYS393, DYS388, DYS392, DYS288, YCAI, YCAII, YCAIII and DXYS156Y.</td>
<td>DYS385, YCAII and YCAIII were the most variable loci, while the gene diversities of DYS288, DYS388, DXYS156Y and YCAI are low, and the remainder lie somewhere between these extremes. Using a 10 Y-STR analysis, the discriminatory capacity of the analysis was able to approach 100%, meaning that individualisation of a profile can almost be achieved using only Y-STRs.</td>
</tr>
<tr>
<td>De Knijff et al.(^{87}) 1997</td>
<td>DYS19, DYS389 I/II, DYS390, DYS391, DYS393, and DYS392</td>
<td>Analysis of the generated population datasets showed that haplotype diversity within populations is less than haplotype diversity between populations.</td>
</tr>
<tr>
<td>Prinz et al.(^{88}) 1997</td>
<td>DYS19, DYS390, DYS389I and DYS389II</td>
<td>Quadruplex Y-STR analysis demonstrated that a male haplotype could be generated when the male/female DNA ratio is 1:2000, meaning that Y-STR analysis is applicable for samples which have an excess of epithelial cells.</td>
</tr>
<tr>
<td>Author(s) &amp; Year</td>
<td>Y-STRs Targeted</td>
<td>Main Finding(s)</td>
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<tr>
<td>Cerri et al.\textsuperscript{85} 2003</td>
<td>DYS19, DYS390, DYS391, DYS392, DYS385, DYS389I and II</td>
<td>The main conclusion is that Y-STR analysis is able to detect a male contributor best if the sample contain a small amount of male DNA, and an excess of epithelial cells.</td>
</tr>
<tr>
<td>Gusmao et al.\textsuperscript{90} 1999</td>
<td>As per Prinz et al.\textsuperscript{88} + DYS393</td>
<td>Found that PCR amplification of a pentaplex system was susceptible to producing unspecific DNA fragments, especially for the DYS19, DYS389 and DYS393 loci. However, employing a number of optimisation steps allowed for these artefacts to be removed.</td>
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<tr>
<td>Dekairelle et al.\textsuperscript{91} 2001</td>
<td>As per Gusmao et al.\textsuperscript{90} + DYS385/II, DYS391 and DYS392</td>
<td>Samples which had already been subjected to differential extraction, and were unable to generate an autosomal DNA profile, could be successfully typed using a Y-STR pentaplex.</td>
</tr>
<tr>
<td>Sibille et al.\textsuperscript{92} 2002</td>
<td>DYS393, DYS389, Amelogenin</td>
<td>DYS393 was the most sensitive marker, followed by DYS389, and the amelogenin marker was the least sensitive. The authors propose that the decreased sensitivity of the amelogenin marker was due to allelic drop out, which can be observed when there is an unbalanced ratio of male/female DNA. If true, drop out of this allele could be observed during normal autosomal STR typing.</td>
</tr>
<tr>
<td>Redd et al.\textsuperscript{93} 2002</td>
<td>DYS449, DYS453, DYS454, DYS455, DYS456, DYS458, DYS459, DYS464, DYS446, DYS447, DYS450, DYS452, and DYS463, and DYS448.</td>
<td>14 novel Y-STR markers were identified and the gene diversities were investigated. The DYS464 STR marker was discovered to be the most polymorphic marker to be described in the literature. Again, the multi-copy markers had a higher gene diversity than single-copy markers.</td>
</tr>
<tr>
<td>Butler\textsuperscript{94} 2002</td>
<td>“Extended Haplotype” loci + DYS437, DYS438, DYS439, DYS447, DYS448, DYS388, DYS426, GATA A7.1 (DYS460), and GATA H4.</td>
<td>A novel Y-STR 20-plex analysis is generated which has the potential to increase the power of discrimination, compared to other multiplexes which have been used. “Extended Haplotype” = DYS19, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, DYS385, and YCAII</td>
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4.2.3. Allele Frequencies

The first Y-STR haplotype was generated by Roewer et al.,\textsuperscript{84} who selected only four of the microsatellites in their study for haplotype analysis: DYS19, DYS389-I and DYS389-II and DYS390.
Y-STR haplotypes were generated for 89 Dutch males and 70 German males, and it was determined that the allele frequencies differed significantly between the two different population groups. Therefore, it is possible for individuals to be differentiated as either Dutch or German, depending on their observed alleles. In comparison, Cooper et al.\textsuperscript{85} generated haplotypes, using the five Y-STRs indicated in Table 1, for 174 individuals. The allele frequencies for each of the Y-STRs was calculated and it was discovered that there were significant differences found between the East Anglian, Nigerian, and Sardinian populations. They also discovered that haplotype diversity within populations is less than haplotype diversity between populations.\textsuperscript{85} Although allele frequencies can be compared to determine the region that someone descends from, simple statistical analysis cannot be applied. This is due to the structural relationships observed between haplotypes, meaning that the product rule cannot be imposed.\textsuperscript{85} To be able to apply statistics to the haplotypes, haplotype population databases need to be generated from diverse populations.

\textbf{4.2.4. Population Databases}

Kayser et al.\textsuperscript{86} and de Knijff et al.\textsuperscript{87} both performed population studies in 1997, on the same dataset. Studies conducted by Muller et al.,\textsuperscript{79} Gomolka et al.,\textsuperscript{80} and Santos et al.\textsuperscript{81} demonstrated that neighbouring populations showed similar allele frequency patterns. It is hypothesised that this is due to non-restricted gene flow which occurs between these two populations, as opposed to populations which are separated in space.\textsuperscript{86} Both Kayser et al.\textsuperscript{86} and de Knijff et al.\textsuperscript{87} propose that the population databases should include haplotypes using as many markers as possible and should also be as extensive as possible (world-wide). Therefore, they collected DNA samples
from 3825 males, from 48 different sub-populations, which were analysed using the Y-STR loci described in Table 1.

The population and family data for each of these Y-STRs was reported by Kayser et al., with the main objective being to set up databases which are locus-specific and can be applied for forensic casework samples. Given the low gene diversities of DYS288, DYS388, DXYS156Y and YCAI it was concluded that they are not recommended for forensic applications. DYS385, YCAII, and YCAIII are all bi-local Y-STRs and, given that their amplification results in two STR products which have different repeat lengths, they have enhanced variabilities and therefore higher discriminatory powers. As already described by Mathias et al., because the YCAII and TCAII locus are dinucleotide repeat sequences, they are short in length, and therefore, are applicable for older forensic samples which may be degraded.

Kayser et al. then used the following subset of seven Y-STRs to produce a haplotype which they termed Y haplotype 1 (Yh1): DYS19, DYS389I, DYS389II, DYS390, DYS391, DYS392, and DYS393. Their results showed that application of this Y-STR analysis provided a reasonably high discriminatory power. Obviously this is dependent on the population which is selected, and within the local European populations, it is discriminative of 74% to 90% of the population. In more distant populations, it is still able to discriminate at approximately 63%. When extra Y-STRs are added to the haplotype (DYS385, YCAII and YCAIII), the discrimination capacity increases and approaches 100%, meaning that individualisation of a profile can almost be
achieved using only Y-STRs. From a forensics perspective, the more Y-STRs which are included, the higher the discriminatory power, which is the ultimate goal of profiling.

Knijff et al.\textsuperscript{87} also used the data from the Yh1 haplotype for their analysis. The haplotype data was pooled into nine different geographical regions, the allele frequencies for each of the different loci was calculated, and then a weighted allele frequency was calculated at each marker, for each of the geographical regions. Each of the markers, aside from DYS391, showed a distinct allele frequency pattern; the most frequent allele was located in the middle and then, as they moved away from the middle, they became less frequent.\textsuperscript{87} For almost all of the loci which were observed, the most common allele typed was different for each distinct region. This provides evidence for the argument, proposed by Cooper et al.,\textsuperscript{85} that haplotype diversity within populations is less than haplotype diversity between populations. This is why generating population databases, and observing population allele frequencies, is so important.

4.2.5. The Application of Y-STR Analysis to Forensics

Prinz et al.\textsuperscript{88} were the first authors to conduct a study targeted at the separation of sperm cells from mixed cell samples, by employing Y-STR haplotype analysis. They hypothesised that the sperm cells which remain in the swab after treatment with differential lysis\textsuperscript{48} could be extracted to obtain further sperm DNA, which could subsequently be analysed using Y-STRs. Prinz et al.\textsuperscript{88} generated a quadruplex Y-STR analysis, composed of the YSTRs shown in Table 1, which was applied to a pure male DNA sample and also to a mixed sample containing a 1:2000 (male/female DNA) mixture. The resultant haplotypes were then compared and it was
discovered that the haplotypes were exactly the same, regardless of the presence of epithelial cells, meaning that Y-STR analysis is applicable for sexual assault samples. This was corroborated in a study by Cerri et al.\textsuperscript{89} who found that mixed cell samples, which were unable to be profiled using autosomal STR analysis, could still be typed using Y-STR analysis. Although accurate statistical analysis cannot be performed, Y-STRs remain a useful tool for suspect exclusion.\textsuperscript{88} If the haplotypes do match, the individual can be included as a suspect, however, if they don’t match, then they can be excluded.

4.2.5.1. Generating Y-STR Haplotypes when Autosomal Profiling Fails

In 1999, Gusmao et al.\textsuperscript{90} developed a Y-chromosome STR pentaplex system which the authors hypothesised could be applicable for forensic casework samples whereby a male/female mixture is being analysed and also, in cases where azoospermic individuals are suspects. Within the pentaplex system, the allele sizes for DYS19 and DYS30 can overlap, and therefore, they were labelled with different dyes. Gusmao et al.\textsuperscript{90} generated haplotypes from two azoospermic individuals to demonstrate that Y-STR analysis is applicable to samples with very minor concentrations of spermatozoa. At the time of this publication, it was reported that azoospermic individuals only represent approximately 1-2\% of all sexual assault cases.\textsuperscript{90} However, given that vasectomies result in azoospermic individuals, an increase in the number of men being vasectomized will subsequently increase the proportion of azoospermic individuals in the population. Being able to haplotype azoospermic individuals is an important discovery within forensic science. This is because differential extraction methods are targeted at sperm cells, and
therefore, will be unable to produce a male profile if there is a very limited number of spermatozoa.

The STR pentaplex system designed by Gusmao et al.\textsuperscript{90} was then applied to samples which had already been subjected to differential extraction and were unable to produce a male DNA profile.\textsuperscript{91} The samples were subjected to a rapid PSA test prior to utilising the differential extraction method, and were only used if they tested positive, meaning that semen is present in the sample. These preliminary results showed that, in cases where the PSA test was positive, the differential extraction procedure failed to give a male DNA profile 35\% of the time.\textsuperscript{91} The Y-STR pentaplex was then applied to remaining DNA extract from the differential extraction procedure, and another two Y-STR PCR reactions were employed so that, overall, nine Y-STR loci were amplified. The other reactions were a DYS385I/II singleplex, and a DYS391 and DYS392 duplex.\textsuperscript{91} The analysis was applied to 42 semen traces, which could not be amplified using autosomal STR profiling, and an extra eight stains from other rape cases. Dekairelle et al.\textsuperscript{91} were able to produce a Y-STR haplotype from 48\% of the samples. Given this information, the authors suggest that cold-cases could be re-opened for analysis using Y-PCR analysis.

Sibille et al.\textsuperscript{92} also progressed the work of Gusmao et al.\textsuperscript{90} by demonstrating that samples which gave negative cytology, as would be observed in azoospermic cases, or in cases which have a prolonged TSI interval, could still be amplified using a Y-STR marker. This is because sperm cells degrade over time, giving a negative cytology, but male epithelial cells deposited during the assault can still persist in the sample.\textsuperscript{92} 104 cervicovaginal, anal or oral swabs which were
subjected to cytology, and tested negative for spermatozoa, were collected from alleged sexual assault victims. Cells were eluted from the swab, the DNA was extracted, then subjected to singleplex Y-STR analyses using DYS393, DYS389 and the amelogenin loci.\textsuperscript{92} The results showed that 28.8\% of the samples which gave negative cytology were able to be amplified using Y-STR markers. Typically, if the cytological examination demonstrates that there is no spermatozoa present, the case may not hold up in court, however, confirming the presence of male DNA with Y-STRs could still be used.\textsuperscript{92} Sibille et al.\textsuperscript{92} propose that if enough spermatozoa are present, autosomal DNA profiling should be employed as it is able to individualise the sample, however, if the cytological examination is negative, or if there is only a few spermatozoa present in the sample, Y-STR analysis should still be utilised as a haplotype can still be observed.

\textbf{4.2.5.2. Applying Y-STR Analysis to Degraded Samples}

Hall and Ballantyne\textsuperscript{62} analysed whether Y-STR typing could be used to determine the Y-haplotype of the male donor in cervicovaginal samples, which had been collected after an extended post-coital interval. This is important within the forensic science field, because the traditional differential extraction method is demonstrated to have difficulty with older samples, as the spermatozoa diminish quickly after ejaculation. A small number of spermatozoa may survive in the vaginal canal a number of days after intercourse, however, traditional methods are not sensitive enough to detect them, or utilising differential extraction may result in a loss of sperm cells, making obtaining a DNA profile impossible.\textsuperscript{62} Hall and Ballantyne\textsuperscript{62} produced two novel multiplex systems which, when the amplification products were combined, allowed a 10-loci male Y-STR profile to be typed in samples which had been collected up to four days after
intercourse. The first multiplex consisted of the following Y-STRs: DYS425, DYS390, DYS439, DYS437, and DYS385I/II and the second multiplex consisted of: DYS393, DYS389I, DYS389II, Y-GATA-A7.2, and Y-GATA-H4. The authors utilised specific PCR conditions to allow preferential amplification of a small volume of degraded male DNA, i.e. increased PCR cycles.62

In 2008, Mayntz-Press et al.95 built on the work of Hall and Ballantyne62 by assessing whether three commercial Y-STR systems: Y-PLEX™ 12 (Reliagene),96 PowerPlex® Y (Promega)97 and AmpFSTR® Yfiler™ (Applied Biosystems)98 would be able to generate Y-STR haplotypes for post-coital samples which had been collected more than three days after intercourse. Samples which had been obtained three days post-coitus were subjected to differential lysis and then profiled using the three kits above. All of the kits were able to generate full male profiles, except for the Y-PLEX™ 12, which could only generate full profiles for 66% of the samples.95 When the interval was altered to five days, the same results were obtained and, when the interval was altered to 6 days, only the PowerPlex® Y and Yfiler™ systems were able to generate partial profiles of the male donor.

4.2.5.3. Multiplex Analysis Using an Extensive Number of Markers

Butler94 introduced a novel Y-STR analysis for the multiplex amplification of 20 Y-STRs. By the time this article was published, a “minimal haplotype” and an “extended haplotype” had been proposed, by the European Y chromosome typing community, which allowed for common loci to be utilised by researchers, and then their results could be compiled in a central DNA database. The minimal haplotype includes the following nine loci: DYS19, DYS389I, DYS389II, DYS390,
DYS391, DYS392, DYS393 and DYS385, while the extended haplotype includes the addition of the YCAII marker. Using these markers alone, 74 – 90% of male individuals can be distinguished. This study by Butler uses all the loci required for the “extended haplotype”, as well as a number of other Y-STRs including: DYS437, DYS438, DYS439, DYS447, DYS448, DYS388, DYS426, GATA A7.1 (DYS460), and GATA H4. The Y chromosome consortium panel (YCC) consists of 74 male DNA samples, and two female DNA samples, which represent individuals from various world populations and this set of DNA samples were used for this study.

The results of Butler’s 20plex system were then compared with the results of the Y-PLEX™ kit (ReliaGene Technologies, New Orleans, LA), which targeted the DYS19, DYS385, DYS389II, DYS390, DYS391, and DYS393 loci. The results showed that all of the alleles that were typed were in concordance with the alleles typed using the Y-PLEX™ kit. The sensitivity studies performed for this multiplex indicate that as little as 250 pg of DNA is required for detection and the multiplex was also applied to samples containing male/female DNA mixtures to imitate casework samples. The highest ratio assessed was 1:150, and the male DNA was able to be detected in this sample. Given the extensive number of Y-STRs which are included in this multiplex, it has the potential to offer an increased discrimination power when compared to other multiplex reactions. However, this was only a preliminary study, demonstrating that a 20-plex system could be applied, and further work would need to be performed prior to applying this to real casework samples.
Hanson and Ballantyne\textsuperscript{100} then introduced a multiplex system which was able to amplify 21 loci simultaneously. They chose to avoid the Y-STRs which had been included in the “minimal haplotype”, instead focusing on 21 other Y-STRs. Using the 21-locus system: 50 pg of male DNA was enough to generate a haplotype, there was no observed cross-reactivity with female DNA, the male profile could be derived when the female cells were present in a 100-fold excess, the system was able to differentiate between two male donors, and finally, the system worked when applied to samples collected 48 hours after intercourse.\textsuperscript{100} All of these factors indicate that this multiplex system would be applicable to forensic casework samples. Hanson and Ballantyne\textsuperscript{101} then built upon their own work and published another multiplex in 2007, which targeted 14 other Y-STRs. This multiplex was demonstrated to have a better discriminatory power than other, commercially available, kits. Also, when this multiplex was combined with the AmpF\textregistered STR® Yfiler™, the entire population was able to be discriminated.\textsuperscript{101}

4.2.5.4. Y-STR Profiling Kits

A number of Y-STR kits have also been developed and validated for use on forensic casework samples. In 2004, Shewale et al.\textsuperscript{94} validated the Y-PLEX™ 12 system, which is able to detect male DNA in a male/female DNA ratio of 1:800 and can detect male DNA present in as little as 0.1 ng. The PowerPlex® Y System also amplifies 12 Y-STRs, is able to detect male cells in a ratio of 1:1200 excess female DNA, and only requires 30 pg of male DNA to produce a profile.\textsuperscript{97} Mulero et al.\textsuperscript{98} produced the AmpF\textregistered STR® Yfiler™ PCR amplification kit, which is able to simultaneously amplify 17 Y-STRs. In comparison to the other kits, Yfiler is able to generate male profiles from DNA samples below 125 pg and, importantly, male profiles can be generated from samples which
contain female cells at a ratio of 1:1000. More recently, the PowerPlex® Y23 System was introduced which is able to amplify 23 Y-STRs simultaneously. The extra Y-STRs which are included in this kit are selected for their high gene diversity values, meaning they have higher discriminatory powers. In particular, there are two rapidly mutating Y-STRs, which may be able to discriminate individuals who are related. Only 62.5 pg of male DNA is required to generate full male DNA profiles, and the male DNA can be detected when there is 125 pg of male DNA in 3000ng of female DNA. This kit was utilised by Purps et al. who found that it had much better discriminatory powers than previously published kits. The most recent kit was generated by Gopinath et al. in 2016, which uses a 27 Y-STR multiplex system to amplify DNA. Its application surpasses the other kits which are currently employed, providing better discrimination between samples.

5. LIMITATIONS OF CURRENT METHODS – NEED FOR RESEARCH

Although differential lysis is the most common technique used by forensic laboratories, none of the differential lysis techniques have been able to completely separate the male and female fractions. They are one of the most time-consuming methods which are discussed and, within forensic science, time is obviously of the essence when trying to find a perpetrator. The methods are also quite tedious, require extensive handling, are reliant on the operator’s experience, and are difficult to automate. They also require a number of washing steps, which limits the male DNA yield. However, if enough wash steps are not employed then epithelial cells will carry over into the male fraction, resulting in a mixed STR profile. The initial digestion step can also result in the lysis and subsequent loss of sperm cells, or, if the
initial lysis is not strong enough, the epithelial cells can remain undigested and result in contamination of the male fraction.\textsuperscript{61,107} Using these methods, it is also impossible to distinguish between two male donors.\textsuperscript{107} There is also very little replication of results between any of the differential extraction methods which have been discussed. This was especially the case in the studies which looked at alterations to the initial lysis buffer. Each of the methods which looked at optimising proteinase K concentration obtained different results. Given that laboratories around the world employ slightly different versions of the differential extraction method, this absence of replication could become a major issue, especially if different laboratories are trying to compare their results.

Rather than having to separate the male and female fractions, as required using the differential extraction method, Y-STR profiling targets the male DNA only, and therefore, it is not affected by an excess of epithelial cells.\textsuperscript{89,90,93} This means that the extensive washing steps required by traditional differential extraction methods, which can result in decreased sperm yield, are not required.\textsuperscript{92} It is also useful for samples whereby the perpetrator is azoospermic or vasectomized, if more than one perpetrator contributes to the sample, and also, for other samples such as saliva in contact with the females skin.\textsuperscript{86,92,93} However, this method is restricted in its applicability. The main limitation of this method is that Y-STRs are not as highly polymorphic as their autosomal DNA counterparts.\textsuperscript{90,93} This means that their power at discriminating between individuals is less than what is achieved through autosomal DNA profiling. For instance; autosomal DNA can be used to differentiate two individuals with a high statistical significance, whereas the use of Y-STRs can differentiate between individuals with
different paternal lineages, and not distinguish between immediate male relatives. From a forensic perspective, this means that if the Y haplotype of the suspect matches the Y-haplotype of a crime scene sample it does not mean that they committed the crime, it could still be any number of people, i.e. a brother, father, grandfather or cousin. Also, within areas with low gene flow, Y-chromosomes can be shared by a large portion of the population living within the same region. Therefore, Y-STR profiling is especially helpful as an exclusionary tool, however, it should only be used if autosomal DNA profiling has been ineffective. The disadvantages which have been discussed here require that techniques be introduced which are more efficient, and also less costly.

6. THE FUTURE OF SPERM CELL ISOLATION

The technique of using immunomagnetic beads (IMBs) to purify various types of cells from cell mixtures has been employed within the scientific community for an extensive period of time. It has been used within medical-based research to; isolate single tumour cells from a wide variety of cell mixtures associated with cancer, determine the serum peptidome patterns associated with breast cancer, identify disease-associated biomarkers in urine using peptidome profiling, and also to isolate basophils from human blood. It has also been employed within the food-based sciences, in combination with an enzyme-linked immunosorbent assay (ELISA) to detect and quantify proteins within drink and food products that could be harmful, or to detect the addition of adulterants. The studies which have had the greatest impact on the forensic science field have been published within the assisted-reproduction literature. These studies are the most important as they were able to effectively
target apoptotic sperm cells and remove them from semen samples. Before these studies are discussed, the principles and techniques of IMBs will be described.

6.1. Immunomagnetic Bead Separation Techniques

IMB techniques are designed to select for specific cell types within a cellular mixture by targeting an antigen, on the surface of those cells, which is specific for that cell-type only. To target that antigen, magnetic beads are conjugated with a compound, or antibody, which is able to bind with the antigen. The wide variety of magnetic beads which are used throughout the literature are readily obtained from scientific manufacturing companies, and therefore, the generation of magnetic beads is not discussed within this review. When the magnetic beads are incubated with the antibodies, labelled as “anti-antigen A antibody” in Figure 1, the antibodies form a coating around the beads as shown below. The beads are then washed and the free sites on the magnetic beads must be blocked to stop free binding of other molecules to the bead. Once the antibody-conjugated magnetic beads are incubated with an “antigen-A containing solution”, the antigen will become bound to the magnetic bead, through antigen-antibody binding, which is demonstrated in Figure 1. Solutions are then generally run through a magnetic affinity-type column made from some type of metal, which becomes magnetised once a magnetic field is applied. When the magnet is applied, the magnetic beads are attracted to the column walls while the non-IMB bound cells elute through the column. After the non-IMB-bound cells are collected, the magnetic field is removed and the antigen-A-bound beads are easily eluted from the column.
6.2. Immunomagnetic Bead Techniques in the Assisted-Reproduction Field

Within the assisted-reproduction field, the focus is generally on improving sperm quality to increase fertility or on separating the healthy and apoptotic fractions to then analyse the unhealthy cells for possible biomarkers for male infertility. Grunewald et al.\textsuperscript{118} were the first authors to use an IMB technique for the enrichment of non-apoptotic sperm cells, to optimise the fertilisation rates of men. The antigen which the authors chose to target was the cell membrane component, phosphatidylserine (PS). When spermatozoa are healthy PS is located on the inner plasma membrane, however, when the cells begin to undergo apoptosis, PS is able to translocate the membrane and it becomes exposed.\textsuperscript{119,120} PS also remains externalised on the plasma membrane after cell death\textsuperscript{121} and has a high affinity for annexin V, which is a 35-36kD phospholipid binding protein.\textsuperscript{122} Grunewald et al.\textsuperscript{118} hypothesised that magnetic beads, conjugated with annexin V, would be able to bind selectively to PS on apoptotic or dead cells but not on healthy cells, as they cannot access the PS. The apoptotic cells could then be separated from healthy cells by applying a magnet, collecting the healthy cells in the column elute, removing the magnet and then collecting the apoptotic cells in another elution.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{immunomagnetic bead binding}
\caption{Overview of IMB binding (adapted).\textsuperscript{114}}
\end{figure}
To determine whether this separation technique was effective, the two fractions were both incubated with fluorescein isothiocyanate (FITC)-conjugated anti-annexin V antibodies and then the fluorescence was analysed using flow cytometry. Incubation with these antibodies, and the resulting fluorescently-labelled IMB, is demonstrated below. The results showed that 5.2% of the healthy fraction contained fluorescent cells and 72.6% of the apoptotic fraction were fluorescent. Importantly, passage through the column only resulted in a 0.8% loss of sperm cells. The authors concluded that this method was effective for the removal of sperm in the early apoptosis stage from a semen sample.

This method was replicated by Paasch et al., whose results were almost identical to that of Grunewald et al. They found that the healthy sperm fraction contained 5.2% fluorescently-labelled spermatozoa, while 72.2% of the apoptotic sperm fraction were able to fluoresce. Paasch et al. took it one step further and used electron microscopy to visualise the healthy cell sample solution. This showed that no magnetic beads were observed in this fraction, and therefore, it was hypothesised that the fluorescent signal which is observed in the healthy fraction corresponds to nonspecific binding of the antibody. After the fractions were separated, Paasch et al. assessed the presence of activated caspases in each fraction to determine their association with the deterioration of the plasma membrane. The method has also been employed by a host of other authors to either; remove the apoptotic fraction to improve semen quality which results in higher fertilisation rates and better embryo quality, or to observe both fractions to search for biomarkers of male infertility. Given the success of this method
at targeting sperm-specific antigens to remove them from a cellular mixture, it is no surprise that this method has now been applied within the forensic science field.

6.3. IMB Techniques in Forensic Science

Although IMBs are able to target and remove apoptotic sperm cells, in the forensic science field the objective is to bind to, and remove, all sperm cells from the mixture while not removing any other cell types. For this to occur, the antigen target must be sperm-cell specific and located on the sperm cell plasma membrane surface. In the first study published in forensics, Eisenberg\textsuperscript{125} evaluated three different anti-sperm antibodies for their ability to remove sperm cells from solution, when bound with a magnetic bead. The three antibodies assessed were: MHS-10, NUH-2 and HS-21. The MHS-10 antibody reacts with the intra-acrosomal antigen SP-10 and, within primates, it is only able to bind with sperm.\textsuperscript{125} Results from Eisenberg’s\textsuperscript{125} study showed that, when bound to magnetic beads, the MHS-10 antibody was able to capture 90-95% of spermatozoa, making it the most effective antibody of the three which were tested. NUH-2 reacts with ganglioside containing disialyl I structures, and inactivates human sperm.\textsuperscript{125} This antibody was able to capture 80% of spermatozoa, and primarily bound to the tail region of the spermatozoa. HS-21 is a monoclonal antibody which is specific for mammalian sperm and reacts with an antigen on the acrosomal cap of mammalian sperm.\textsuperscript{125} This antibody was only able to capture 60% of spermatozoa from samples, making it the least effective of the three antibodies, although it was shown to primarily bind to the acrosomal cap. The main conclusion taken from this study is that, the choice of antigen can have a significant effect on the amount of
spermatozoa which can be recovered, and that selection of an antibody needs to be optimised.

6.4. Primary Antibody-Secondary Antibody IMB Technique

Interestingly, it was another six years before another article was published which used IMBs in forensics. Anslinger et al.\textsuperscript{126} did not use one of the antibodies proposed by Eisenberg,\textsuperscript{125} but instead chose to target their antibodies against the testicular isoform of the angiotensin-converting enzyme (tACE). Testicular ACE is located on the mid-piece, neck and flagellums of the sperm\textsuperscript{127} and the authors hypothesised that since this enzyme is highly-specific for sperm cells, it would be an excellent target antigen. They generated mixed samples of sperm cells and female buccal cells which were smeared onto microscope slides, fixed and dehydrated and then incubated with one of nine different mouse anti-human monoclonal antibodies which had been generated against tACE. The slides were then stained and observed using microscopy which showed that the antibodies bound only to the neck, post-acrosomal area and mid-piece of the sperm.\textsuperscript{126} Of the nine antibodies, only the three which were most successful were chosen for further application. The technique employed in this study is termed a primary antibody-secondary antibody system. In this technique, highly-specific antibodies (anti-tACE) are generated against sperm-cell specific proteins (tACE) and a secondary antibody, created against the primary antibody, is used to coat the magnetic bead. The sperm cells are thereby linked to the magnetic beads via antigen-antibody bonds and can be isolated by applying a magnetic field. The DNA was then isolated and DNA was isolated and then quantified prior to STR profiles being generated.
The total yield of DNA recovered demonstrated that the most effective antibody was able to recover almost 100% of the spermatozoa for a $10^6$ sperm cell dilution, when using optimal binding steps. Samples which contained $10^5$ sperm cells were able to produce profiles which provided a major male component, although the STR profile was mixed. Samples which contained $10^4$ or $10^3$ sperm cells also provided mixed STR profiles, however, the major component of the profile was female DNA. When the samples contained only $10^2$ or $10^1$ sperm cells, no male alleles were detected. Although these results show that this technique is only completely successful when the spermatozoa are in a concentration of $10^6$ or higher, it was able to enhance the amount of male DNA, to produce a major male component, for $10^4$ or $10^3$ sperm cells. Therefore, Anslinger et al.\textsuperscript{126} proposed that this antibody-based method for the isolation of sperm cells could be a possible alternative to the traditional methods being employed. However, the samples in this study were stored in PBS immediately after collection, rather than air drying which is typical procedure, and the female cells collected were buccal cells which means the study has a limited applicability. Given the location of the tACE antibody across the sperm cells, the authors hypothesise that if this technique was applied to dried samples, it would have a decreased success rate as the mid-piece and flagellum are lost due to degradation. Anslinger et al.\textsuperscript{126} conclude that finding the right sperm-specific antigen, located on the head of the spermatozoa, will increase both the recovery of sperm cells and also the applicability of the method.

6.5. Biotin-Avidin IMB Technique

Li et al.\textsuperscript{106} furthered progressed the work of Anslinger et al.\textsuperscript{126} by targeting the motile sperm domain-containing protein 3 (MOSPD3) of sperm cells, by binding an avidin-coated magnetic
bead to a biotin-labelled anti-MOSPD3 antibody. This antigen was selected as the target as it is distributed on all surfaces of the sperm cell including the head, neck, flagella and mid-piece. Prior to this antigen being used, Li et al.\textsuperscript{106} used a western blot to demonstrate that MOSPD3 is observed in sperm cells, but not in epithelial cells. The authors proposed that, as well as selecting an appropriate antigen, the success of the method is also dependant on the binding efficiency between the antibody and the magnetic bead. As such, they chose the avidin-biotin system for separation because this interaction is the strongest known non-covalent interaction which can occur between a protein and its ligand.\textsuperscript{128}

Male and female buccal samples were collected, and mixed samples were generated as per the Anslinger et al.\textsuperscript{126} method, however, to extend the applicability of this method, 52 vaginal swabs were also used that had been gathered from rape cases. The swabs had been stored for various amounts of time; 1 day, 3 days or 10 days. To determine whether incubation with the anti-MOSPD3 antibody was successful at selectively binding sperm cells; mixed cell suspensions were added to slides, which were dried and fixed, the anti-MOSPD3 antibody was added and then the slides were incubated with an Alexa Fluor 488-conjugated secondary antibody. As per Anslinger et al.,\textsuperscript{126} a number of washes were performed between the primary and secondary antibody incubations to ensure that any unbound anti-MOSPD3 would be removed, to stop non-specific binding. The nuclei of the cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI), which fluoresces blue, and the slides were visualised using fluorescent microscopy. Observation of the slides showed that the sperm cells fluoresced, while the epithelial cells gave no fluorescent signal.
To conduct the IMB technique, the MOSPD3 antibody was labelled with biotin (biotinylated) and the magnetic beads were coupled with avidin. Figure 2 shown below demonstrates the principle of the binding of avidin-coupled immunomagnetic beads to biotinylated anti-MOSPD3.

The mixed cell samples were first incubated with biotinylated anti-MOSPD3, then; the samples were centrifuged, the supernatant was discarded, the pellet was washed three times and was then re-suspended. The avidin-coupled magnetic beads were then added to the solution and allowed to incubate. A magnet was then applied, the non-bound cells were eluted, and the samples were washed to remove any epithelial cells. A sample was taken of the sperm cell suspension captured using the magnetic beads, prior to dilution, and the sperm cells were then eluted using release buffer. The sample was observed using microscopy which showed that each sperm cell was bound by two or more immunomagnetic beads, and the beads were located mainly in the sperm head and tail. These results indicate that this antigen may be a more suitable target for IMB-based separation techniques than the tACE selected by Anslinger et al.,\textsuperscript{126} especially when applied to older swabs.
After the cells had been isolated, DNA extraction, quantification and STR analysis was performed. When the sperm cell density was $10^5$, all 10 samples provided full STR profiles (16 loci). When decreased to $10^4$ sperm cells/mL, 8 samples were able to provide a full profile (16 loci) and 2 samples provided 13 loci. When decreased again, to $10^3$ cells/mL; 1 sample was able to provide a full profile, 2 samples were able to provide a 15 loci profile, 1 sample was able to provide a 14 loci sample, 4 samples were able to provide a 13-loci profile and 2 of the samples gave profiles of less than 13 loci. Importantly, only male DNA was observed in the STR profiles, there was no female alleles called.

When the method was applied to the dried vaginal swabs, it was shown that the type of swab used (nylon vs flocked swab) had a great impact on the recovery of cells as the samples were left to dry for a longer period of time. This was also reported by Benschop et al.,\textsuperscript{119} who compared cell elution between nylon and flocked swabs in their study. Flocked swabs gave the following rates of successful detection: 100% for 1 day, 87.5% for 3 days, and 40% for 10 days. Samples which had been collected using cotton swabs had quite different rates of successful detection: 100% after 1 day, 40% after 3 days and 16.67% after 10 days. Importantly, both types of swabs observed a decrease in successful detection as the number of days increased. The authors propose that the decrease in success as the storage time increases could be due to the loss of sperm cell tails over time, which would decrease the amount of MOSPD3 which the antibody can bind to. However, the technique is still demonstrated to be applicable to older, dried samples.
As with Anslinger et al., the authors propose that antigens which are better suited for this method could be found, and that future studies should be aimed at antigens on the sperm head. They believe that finding the most suited antigen will lead to greater success of this method and, in combination with the general improvements it has over the differential extraction method, it could be a potential alternative for the isolation of sperm cells from mixed samples. It eliminates the centrifugation steps, overnight incubation step and repeated washes which are required for the differential lysis method, which generally lead to decreased sperm cell yield. It is also more amenable for automation than the other methods which have been discussed in this review.

6.6. Single Antibody IMB Technique

Grosjean and Castella proposed that the sperm cells could be stained with a CD52 antibody coupled with a magnetic bead and then separation could be achieved using a magnetic column. Samples were again collected as per Anslinger et al., however, the swabs were stored up to 6 months at room temperature which is much longer than the times observed by Li et al. After elution of the cells from the swab, they were centrifuged to allow pelleting of all the cells and then a PBS and EDTA solution containing magnetic beads coupled with CD25 antibody was added to allow staining of the sperm cells. The solutions were then transferred into columns and a magnetic field was applied, which allowed elution of the non-sperm cells through the column while retaining the sperm cells. The non-sperm cell solution was collected and pelleted so that microscopy could be performed. The columns were washed, as per Anslinger et al. and Li et al. to ensure the removal of all epithelial cells. The columns were then removed from
the magnetic field and the sperm cells were eluted through the column. As with the non-sperm fraction, the sperm-cell elute was centrifuged to allow pelleting of the cells. The cells were then lysed and PCR analysis was performed.

Although some of the samples had been left at room temperature for up to 6 months, the results showed that the staining which was observed on fresh samples was also observed on samples which had been stored for longer. This indicates that the CD52 antigen is consistently observed, even in older, more degraded samples. However, using flow cytometry, it was shown that the epithelial cells also expressed CD52 which means that the magnetic beads would also be able to bind the epithelial cells, resulting in female DNA in the male STR profile. The authors claim that they were able to separate mixed cell samples containing diluted sperm (up to 3000x), however, this data was not shown in the article and therefore this conclusion should be treated with caution. In comparison to the methods used by Anslinger et al.\textsuperscript{126} and Li et al.,\textsuperscript{106} this method only used one incubation step for magnetic bead-antibody binding while the other two require a two-phase incubation step. If a suitable antigen-antibody complex could be found, it is possible that this technique could be applied which would allow direct binding of the magnetic bead with the antigen via only one antibody, which would reduce the time required for analysis. Although unsuccessful, this method is still important as it demonstrates one antigen which is not able to be used with the selected IMB technique and, therefore, other antigens need to be discovered. It also demonstrates that finding an antigen located on the head of the spermatozoa is not necessarily the most important factor to consider when selecting an antigen.
6.7. Single Antibody Technique 2.0

One of the more recent studies, conducted by Xu et al.\textsuperscript{107} targeted the sperm-specific antigen, A kinase anchor protein 3 (AKAP3). Prior to this antigen being used as a target, as per the study by Li et al.,\textsuperscript{106} a western blot analysis was performed which found that the AKAP3 protein was expressed in sperm cells, and not in vaginal or buccal epithelial cells. Fluorescently-labelled antibodies were also generated against AKAP3 to determine where on the sperm it is expressed. The results showed that it is expressed abundantly expressed in the head, neck, mid-piece and flagellum but is primarily on the sperm head and tail.\textsuperscript{107} Also, no fluorescence was observed on epithelial cells. This makes AKAP3 an excellent target for an IMB-based technique.

Magnetic beads were incubated with FITC-conjugated AKAP3 polyclonal antibody to generate the IMB beads. The beads were then added to the cell solution, incubated, washed and then centrifuged to ensure any unbound primary antibody was removed. Magnetic separation was then performed and the FITC-labelled antibodies were detected by flow cytometry.\textsuperscript{107} Epithelial cells were still found in the male fraction, due to the non-specific binding of epithelial cells during the column sperm-cell purification. However, the STR results showed that only male alleles were observed, for all of the 30 samples which were tested after they had been subjected to the method. Although, in comparison to the method by Li et al.,\textsuperscript{106} the STR analysis only required $>13$ loci to be classified as a full single-source STR profile. The sensitivity tests performed by Xu et al.\textsuperscript{107} showed that sperm cells could be isolated completely by this method when the ratio was as low as 1:32, but was unsuccessful when it was decreased to 1:64.
6.8. Single Antibody Technique 3.0

One of the more recent articles published was authored by Zhao et al.\textsuperscript{132} and uses another different IMB technique, as well as a different target antigen, to try to isolate the sperm cells. This technique links an anti-PH-20 antibody with the carboxylic group of the magnetic beads via amide bonds which ensures that the two remain coupled throughout the entire experiment.\textsuperscript{132}

The PH-20 antigen, which plays a role in fertilisation, is also known as the sperm adhesion molecule 1 (SPAM1) and is the target antigen for this method. This antigen was chosen as previous studies have shown that PH-20 is located on the entire head of the sperm, but is not located on the mid-piece or tail.\textsuperscript{133,134} To determine whether the anti-PH-20 antibody was specific for male cells, and where on the sperm they were binding, mock casework samples were generated and incubated with anti-PH-20 antibody IMBs. The beads were washed and then suspended in solution so that the cells could be observed using microscopy. Both optical microscopy and scanning electron microscopy were employed which demonstrated that 1) there were no epithelial cells observed in the suspension and 2) the beads bound selectively to the sperm heads.

The mock sexual assault samples were generated as per Anslinger et al.\textsuperscript{126} and vaginal epithelial cell samples were also used in parts of the study. The anti-PH-20 IMBs were generated by incubating the magnetic beads with the antibody. The IMBs were incubated with the mixed cell samples and a magnet was applied to the resulting solutions to allow separation of the male and female cells. As already described, a number of wash steps were employed to remove the epithelial cells. After washing, the magnet is removed and the sperm cells can be eluted. The
DNA was then extracted, quantified and amplified to generate STR profiles. The resultant STR profiles showed that the male fraction had alleles corresponding to both the male and female donors, meaning that epithelial cells were still present in that fraction. However, the male donor corresponded with the major component and the minor component corresponded with the female donor. This means that the technique has been able to successfully enrich the sperm cell fraction but has been unable to separate them completely. This is what was also observed by Anslinger et al.\textsuperscript{126} Zhao et al.\textsuperscript{132} reviewed the first procedure and hypothesised that broken epithelial cell DNA was able to attach to the surface of the IMB-sperm complexes which would cause carryover of female DNA into the male fraction. Therefore, they proposed an incubation step using DNAse I, after the column had been washed a number of times. The results showed that this methodology gave a single-source profile which was a match to the male donor.

To determine whether this antigen was best suited for the proposed method, the authors chose to target a number of other sperm cell antigens; sperm protein-10 (SP-10),\textsuperscript{135} a disintegrin and metalloprotease 2 (ADAM2),\textsuperscript{136,137} and JNK-associated leucine zipper protein (JLP).\textsuperscript{138} The magnetic beads were therefore coated in the corresponding antibodies; anti-SP-10, anti-ADAM2 or anti-JLP. The sensitivity of each of the methods was also determined by keeping the epithelial cell concentration constant at $10^5$/mL and the sperm concentrations were altered from $10^2$–$10^5$/mL. In contrast to the rest of the experiment, this section used vaginal swabs.

All of the methods were able to generate single-source male DNA profiles when the sperm cell concentration was $10^5$/mL and $10^4$/mL. When the concentration of sperm cells was decreased to $10^3$/mL, the anti-PH-20 IMBs were able to genotype 90% of the samples tested. In
comparison, the other three IMBs were unable to provide a single-source profile at this sperm cell concentration. When the sperm cell concentration was again decreased to $10^2$/mL, none of the methods were able to generate a single-source profile. These results show that this IMB method, combined with anti-PH-20 was the most sensitive at detecting spermatozoa, in comparison to the other antibodies tested in this study. The sensitivity of this technique, targeting PH-20, was also more sensitive than the technique used by Anslinger et al.\textsuperscript{126} Anslinger et al.\textsuperscript{126} could only produce a single-source STR profile when the concentration of spermatozoa was $10^6$, while this method could provide a profile at $10^5$/mL and $10^4$/mL and 90% of the time when the concentration was $10^3$/mL. This new method was also more sensitive that the method proposed by Li et al.\textsuperscript{106} which was able to generate a single-source profile; 100% of the time when the concentration was $10^5$/mL, 80% of the time when it was $10^4$/mL and only 10% of the time when it was decreased to $10^3$/mL. This means that, of all the papers published within the literature, this method is the most sensitive at detecting spermatozoa and therefore, the use of IMBs in forensics is heading in the right direction.

This new technique was also compared against the current standard of differential extraction. Using the sensitivity data obtained above, the epithelial cell concentration was kept constant at $10^5$/mL and the sperm cell concentration was kept at $10^3$/mL. Samples were subjected to either the differential lysis method or the anti-PH-20-IMB-based method and then STR genotyping. Treatment with the differential lysis method resulted in only 5% of samples producing a single-source profile while the anti-PH-20 IMB method was successful 90% of the time. This is compelling evidence that this method could be more effective than the current methods being employed.
The authors hypothesised that this method was more sensitive than the other due to the location of the PH-20 antigen primarily on the sperm head. The nuclear DNA within spermatozoa is located within the sperm heads, and therefore, targeting an antigen located primarily on the head of the sperm, or only on the head of the sperm, means that the area containing the DNA is targeted. The results of this study showed that the anti-PH-20 was able to bind the entire head of the sperm which means that this technique would be applicable to older samples where degradation may have caused the loss of tails or flagellums. In comparison, Anslinger et al. were unable to stain some spermatozoa which were missing flagellums and the mid-piece. The authors hypothesise that the anti-PH-20 was more sensitive than the anti-SP-10 method because, although the SP-10 protein expressed on the sperm head, it is only expressed at the equatorial region. In conclusion, this technique shows the most sensitivity to sperm cells and it is also effective at completely removing the epithelial cell fraction, and therefore, this is a very promising approach.

6.9. Bringing the Literature Together

The latest study performed in this field was only performed a few months ago, in the form of a manuscript written by Hutchinson. The work undertaken by Hutchinson was performed, as a preliminary study, to determine whether SPAM-1 (PH-20), that was first targeted by Zhao et al., could be an effective target for the primary antibody-secondary antibody system that was established by Anslinger et al. Although the method by Anslinger et al. was ineffective in comparison to the other methods, it was hypothesised that this was due to the antigen selected, rather than the isolation technique. Given that the technique targeting PH-20 was the most sensitive, this was chosen as the target antigen. The proof-of-concept study
aimed to determine whether a primary antibody (anti-SPAM1 antibody) could be successfully linked with a fluorescently tagged secondary antibody and whether this would be specific for sperm cells in a mixed cell solution. A number of other studies have used fluorescently tagged antibodies to observe the binding of the primary antibody.\textsuperscript{106,107}

Hutchinson\textsuperscript{23} also used buccal epithelial cells and semen samples to generate mixed cell samples. These were then added to sterile cotton swabs and air dried to mimic sexual assault swabs. The swabs were stored for varying amounts of time to determine whether this antigen binding can occur on older samples. To observe immunofluorescence, sterile phosphate buffer (PBS) solution was first added to a microscope slide and cells were transferred by rolling the swab through the PBS on the slide. The slides were then treated as per the method by Xu et al.,\textsuperscript{126} except that the samples were air dried, rather than dried at 37°C and the blocking buffer which was used was 10\% Normal Goat Serum (ThermoFisher) which required incubation at room temperature for 10 minutes. The slides were then incubated with the primary antibody (SPAM-1 Polyclonal Antibody, Thermo-Fisher Scientific) and then the secondary antibody (Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, ThermoFisher Scientific), as per Li et al.\textsuperscript{106} and Xu et al.\textsuperscript{107} A number of washes were performed between incubation with the primary and secondary antibodies, to ensure any unbound primary antibody is removed before addition of the secondary antibody. After incubation with both antibodies, the nuclei were stained using DAPI, as per Xu et al.\textsuperscript{107} and Li et al.\textsuperscript{106} The slides were observed using fluorescent microscopy and the appropriate filters. Nuclei which had been stained with DAPI using a filter with an excitation wavelength of 330 – 385nm, and they appear...
blue. The Alexa Fluor 488-tagged antibodies were visualised using a FITC filter and these appear green.

As with the study by Zhao et al.,\textsuperscript{132} the PH-20 antigen was found to be primarily on the sperm head. Also, it can be visualised that sperm cells which have lost their tails (after the samples had been stored for 3 days) still show antibody binding, indicating that this method is applicable for older, more degraded swabs. However, visualisation of the samples shows that, after incubation with both antibodies, both the epithelial and sperm cells fluoresce green, indicating that the secondary antibody, which is fluorescently labelled, has been able to bind to both cells types. It is possible that this has occurred due to inadequate washing of the slides after addition of the primary antibody, or secondary antibody which can both lead to non-specific binding. Hutchinson\textsuperscript{23} proposes that the secondary antibody is able to bind directly with the epithelial cells and this is what is causing their fluorescence. This is based on the idea that the secondary antibody is fluorescently labelled, and therefore, binding of the primary antibody is not required to allow fluorescence of cells. Given that the primary antibody is successfully used by Zhao et al.,\textsuperscript{132} it is probable that it is not the primary antibody which is the issue here. Given that it is now shown that the antibodies can bind after a short drying time, and that Zhao et al.\textsuperscript{132} are able to isolate sperm cells using this antibody, it is now more important than ever to determine why there is non-specific binding, how this can be fixed, and if using this antibody in combination with a secondary antibody coated magnetic bead can produce a method with a higher sensitivity than that observed by Zhao et al.\textsuperscript{132}
6.10. Study Objectives - Immunomagnetic beads

Given the observations of the study by Hutchinson,\textsuperscript{23} I propose a study which almost replicates his study, but with a few changes to attempt to determine why there was non-specific binding of the antibodies. A proof-of-concept study outline is presented which aims to 1) develop a method for the selection of sperm cells in a mixed solution using a primary antibody-secondary antibody system targeted at the SPAM1 (PH-20) antigen, and 2) assess whether SPAM1 is an effective sperm-cell antigen target for a primary antibody-secondary antibody system-based IMB. As per the studies by Li et al.,\textsuperscript{106} Zhao et al.,\textsuperscript{132} and Hutchinson,\textsuperscript{23} a fluorescent tag will be bound to the secondary antibody to assess whether the system has bound specifically to sperm cells. If the method is successful, only sperm cells will fluoresce when viewed under the microscope. It is hypothesised that the SPAM1 primary antibody-secondary antibody system that is being employed will be specific for sperm cells only. Furthermore, it is hypothesised that this will be true when observing male-only, female-only, and mixed cell samples.

6.10.1. Methodology

The first step of this study is to obtain semen and buccal cells from participants who have provided written consent. The participants will be responsible for collecting their own samples in sterile containers. The semen samples will undergo serological testing before use, to ensure the safety of those conducting the experiments. The study can be divided into two separate experiments. The first experiment uses swabs which have been produced from male-only or female-only cells, to determine whether the antibodies bind specifically to sperm cells. After confirmation of binding, the experiment will be reproduced using swabs that contain varying
concentrations of mixed male and female cells to ensure that the antibody system remains specific.

Swab Generation: For the first experiment, a number of dilutions will be generated from the semen and buccal cell samples; 1:10, 1:100 and 1:200. A 100uL aliquot will be taken from each dilution and added to six separate swabs. Another two swabs will be produced which contain neat buccal or neat semen samples. For the second experiment, six different solutions will be generated which contain varying concentrations of male and female cells. A 100uL aliquot of each different solution will be applied to separate swabs. All swabs will be generated in triplicate and left to dry at room temperature.

Generation of Microscope Slides: Sterile phosphate buffered saline (PBS) solution will be added to each swab and the swab will be rolled around the microscope extensively to transfer maximum cellular material. The slides will be prepared as described by Xu et al.\textsuperscript{107} using 10% Normal Goat Serum (ThermoFisher) as the blocking buffer, with a 10 minute incubation time. The antibody system will be applied to two of the replicate slides and the third replicate will be stained using the Christmas Tree Stain.

Application of Antibody System: SPAM1, as described by Zhao et al.\textsuperscript{132} has been chosen as the sperm-cell specific antigen that the antibody system will be targeted at. The following method has been provided by ThermoFisher staff: 1) Incubate slides with the primary antibody - SPAM1 Polyclonal Antibody (ThermoFisher), 2) Wash with PBS, 3) Incubate with a secondary antibody
coupled with a fluorescent tag - Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 conjugate (ThermoFisher), 4) Wash slides with PBS, 5) Counterstain nuclei with DAPI (ThermoFisher), 6) wash with PBS, and 7) Mount coverslips onto slides.

*Slide Examination:* Fluorescently tagging the secondary antibody with the Alexa Fluor 488 conjugate (ThermoFisher) allows the slides to be viewed under a fluorescent microscope using a FITC filter and visualise where binding has occurred. Nuclei stained with DAPI can be visualised using a fluorescent microscope with an excitation wavelength of 330 – 385nm (Mirror Unit: UMWU2, Olympus). Slides stained using the Christmas Tree Stain can be examined using bright field microscopy. The sperm cells on each slide can be counted per field of view to determine the concentration of sperm cells in each sample and the concentrations will then be compared amongst the different staining techniques.

If the study is successful then the next phase would be to coat magnetic beads with the secondary antibody and then apply the magnetic beads to a swab elution which has been treated with the primary antibody. If that is successful, then the method needs to be compared with previous IMB techniques, as well as the current differential extraction methods which are employed in laboratories.

7. CONCLUSION

This dissertation has established that the current methods which are employed by forensic DNA laboratories are notoriously inefficient at isolating male DNA from mixed cell fractions. Differential extraction is particularly poor at achieving non-contaminated fractions which can
lead to the production of mixed DNA profiles, or, what is particularly worrisome, is that it can result in the loss of sperm cell yield. Given that sexual assault samples typically have a very small starting volume of male DNA, any loss of sperm cells is likely to have a detrimental effect on the outcome of STR profiling. Therefore, differential extraction should not be recommended for use on vaginal sexual assault swabs. Although Y-STR profiling is effective at isolating the sperm cell DNA, the limited genetic diversity of the Y-STR markers means that the haplotype which is generated is not able to individualise samples, and therefore, analysis using autosomal STRs is much more effective. To solve these issues, new isolation techniques are required that can effectively separate the male and female fractions, which can then be submitted for autosomal STR analysis.

Although an enormous amount of literature has been published on novel techniques for sperm cell isolation, I have proposed that the way forward is with IMB techniques. They have been shown to be effective outside of forensic science, particularly in the assisted-reproduction field, but have also recently been applied to mock forensic casework samples where success has also been observed. In particular, a preliminary study performed by Hutchinson has shown relative success, however, there was also non-specific binding of the antibodies observed. Future studies should therefore be targeted at aiming to discover why there is non-specific binding of the antibodies, if this can be corrected, and whether this technique can be used in combination with an IMB.
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Part Two

Manuscript

Forensic Techniques for the Isolation of Sperm Cells from Mixed Cell Fractions: A Review
Abstract

The technique of isolating one specific cell type, from samples containing a mixture of cells, has been employed extensively within the scientific community. Within forensic science, techniques have been targeted at isolating sperm cells from samples containing both male and female cells. The various sperm cell isolation techniques which are employed by forensic DNA analysts will be discussed in this review, with a specific focus on differential extraction and Y-chromosome DNA profiling. Their benefit to the forensic community will be established and any alterations which have been made to the original methods, in an effort to increase their effectivities, will also be discussed. Although each of these methods have their advantages, it is ascertained by this review that they are either limited in their overall success rate, or they have a limited applicability. For these reasons, the more recent technique of using immunomagnetic beads (IMBs) to isolate sperm cells is suggested as an alternative method to those currently employed.

Keywords: DNA Analysis, forensic, differential extraction, Y-chromosome STR, immunomagnetic beads
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List of Abbreviations

ABS Australian Bureau of Statistics
ADAM2 A Disintegrin and Metalloprotease
AKAP3 A Kinase Anchor Protein 3
DNA Deoxyribonucleic Acid
DTT Dithiothreitol
IMBs Immunomagnetic beads
JLP JNK-associated Leucine Zipper Protein
LCM Laser Capture Microdissection
MOSPD3 Motile Sperm Domain-containing Protein 3
PTC Paternity Testing Corporation
SP-10 Sperm protein-10
SPRED Separation Potential Ratio for the Extraction Differential
tACE Testicular Isoform of the Angiotensin-converting Enzyme
TSI Time Since Intercourse
Y-STR Y-chromosome Short Tandem Repeat
INTRODUCTION

The term “sexual assault” refers to any unwanted, coerced, or forced sexual contact that occurs between two or more people, without the victim’s consent.\(^1\) Recent crime data published by the Australian Bureau of Statistics (ABS) indicates that from 2010 to 2016, a 22% increase in the number of reported sexual assaults has been recorded nationwide, with a 5% increase documented from 2015 to 2016 alone. The data also reveals that 82% of recorded sexual assaults are committed against women. Given the severe physical and mental trauma that sexual assault victims suffer, it is imperative that the perpetrator of the crime is arrested and convicted.\(^1,3-6\) The three main types of evidence that are required to link the perpetrator with the crime are samples from the crime scene and victim, and reference samples from the suspect.

When a victim presents themselves to hospital for forensic evidence collection following a sexual assault, the acquisition of vaginal swabs is prioritised, as the presence of semen on these swabs provides substantial evidence that sexual contact has occurred.\(^7-10\) To confirm the presence of semen on swabs: a portion of the sample is transferred to a microscope slide, the cells are histologically stained, and the slides are searched for spermatozoa.\(^11\) Once the presence of semen is confirmed through visualisation of spermatozoa, cells can be extracted from the swabs and undergo DNA profiling to assist with identification of the perpetrator.\(^12\)

Prior to submitting the sample for DNA profiling, the male fraction must be isolated from the cellular mixture. If the male fraction is not isolated, there can be one of two outcomes: 1)
there will be complications with the PCR amplification, allowing amplification of the female fraction to be favoured, and there will be no male profile observed,\textsuperscript{13} or 2) a mixed DNA profile will be generated.\textsuperscript{14} These outcomes are as a result of the large excess of epithelial cells in the sample, in comparison to the often minute amount of male DNA.

This review focuses on the available techniques for isolating sperm cells from mixed cell samples. The two main techniques employed within forensic DNA laboratories are differential lysis and Y-chromosome short tandem repeat (Y-STR) analysis, which will both be reviewed in detail. In particular, the limitations of these methods will be conveyed to the reader to establish why new techniques are required for sperm cell isolation. A multitude of techniques have been proposed in the literature, such as: Laser capture microdissection (LCM),\textsuperscript{15-17} filtration-based methods,\textsuperscript{12,18} microdevices,\textsuperscript{19-21} and flow cytometry,\textsuperscript{22} however, this review recommends immunomagnetic bead (IMB) methods as the separation techniques of the future. Using IMBs to isolate sperm cells has only recently been applied within the forensic sciences. However, it has demonstrated considerable success within the literature and will therefore be proposed as a possible alternative method to those that are currently utilised.

**CURRENT METHODS FOR ISOLATING SPERM CELLS: DIFFERENTIAL EXTRACTION**

Differential extraction is the primary method utilised by forensic DNA laboratories worldwide.\textsuperscript{10} It was first proposed by Gill et al.\textsuperscript{23} in 1985 and an outline of the critical steps has recently been summarised by Cotton and Fisher.\textsuperscript{24} The steps involved are: 1) incubation of the sample in a gentle lysis mixture which can lyse epithelial cells but will leave sperm cells
intact, 2) centrifugation to allow the sperm cells to pellet and subsequent removal of the supernatant which contains the lysed female epithelial cells, 3) a number of washing and successive centrifugation steps, 4) resuspension of the sperm cells and incubation in a secondary lysis buffer which will lyse the sperm cells, and 5) DNA purification for both isolated fractions. Following purification, the fractions can be submitted for STR analysis which results in the generation of two single-source DNA profiles.  

Typically, the initial lysis buffer consists of proteinase K, in the absence of dithiothreitol (DTT), which allows digestion of the fragile epithelial cells while the robust sperm cells remain intact due to the presence of disulphide cross-links in their nucleus. The second lysis buffer contains DTT, which will break down the disulphide cross-links in the sperm nucleus, resulting in male DNA being released. The main goals of differential extraction are to: extract the sperm cells successfully from the swab, retain them throughout the isolation process (high yield), and minimise carryover of other cell types into the sperm fraction (high purity).

The Introduction of Differential Extraction

Gill et al. successfully demonstrated that, using differential extraction, a male DNA profile could be obtained from a mixed cellular sample without showing any contaminating female DNA. This method was replicated by Gill et al. for over 100 semen-contaminated vaginal swabs, however, the success rate of the method was only 62%, indicating that it is not particularly efficient. It is proposed that this lack of success is due to ineffective separation of the male and female fractions, which resulted from the carryover of male cells into the female fraction and vice versa.
If the male fraction is contaminated with female cells, and they are similar in concentrations, a mixed STR profile will be produced. However, if female cells are present in excess of the male cells, amplification of the female cells will be favoured by the reaction, resulting in a single-source profile which corresponds to the female victim. There are a variety of ways in which male DNA can carry over into the female fraction, including: if the pellet is agitated during the washing steps, if damage occurs to the sperm cells during the repeated washing and centrifugation steps, or, if the initial lysis buffer is able to lyse sperm cells. Epithelial cells are able to carry over into the male fraction if they are not effectively lysed during the initial incubation step.

**Modifications to Differential Extraction**

Since its inception, there has been an extensive amount of literature published aiming to improve the success rate of the method. To increase the efficiency, most of the modifications target one, or more, of the issues that cause contamination of the opposing fractions. For example, when Gill et al.’s method was published, the protocol did not include any wash steps. These were introduced by Gill et al. after they discovered that female epithelial cells were found in the male fraction, even after treatment with DTT. It was hypothesised that washing the sperm cell pellet would improve elimination of epithelial cells, which was ascertained to be true. These findings were also observed by Comey et al., however, it was later established that increasing the number of wash steps decreases the sperm cell yield by damaging the cells and releasing the male DNA prematurely. This provides an indication of the delicate balancing act which is required for successful differential extraction methods.
Other modifications to the differential extraction method include altering the initial lysis buffer,\textsuperscript{13,25,35-37} and digesting epithelial cells with DNase.\textsuperscript{10,34,38} A one-step buffer\textsuperscript{30} and two kit-based methods have also been introduced.\textsuperscript{10,28,31,39,40} The most recently published article has developed a semi-quantitative calculation to compare methods and determine which has a higher separation potential.\textsuperscript{41}

\textbf{Modifications to the Initial Lysis Buffer}

The stringency of the initial lysis buffer is particularly important as it must digest the epithelial cells but leave the sperm cells intact. Modifications to the initial lysis buffer include altering the: proteinase K concentration, detergent concentration, detergent type, incubation temperature, incubation time and, the addition of a cellulase to the buffer was also investigated.\textsuperscript{13,25,35,36,37} Although all the research demonstrated that modifying the initial lysis buffer did alter the success of the method, there was little consensus between the authors about what effect each modification had. For example, Norris et al.’s\textsuperscript{36} results showed that addition of proteinase K immediately reduced the amount of sperm cells in the sample by 24%, while Hennekens et al.\textsuperscript{25} found no observable loss of sperm cells, even with a two hour incubation. The buffers utilised were different for each study, and therefore, comparing the results was difficult.
Kit-Based Methods

There are currently two kit-based methods which are utilised by forensic DNA laboratories: Differex™ (Promega Corporation, Madison, WI) and Erase Sperm Isolation Kit (Erase), which is produced by Paternity Testing Corporation (PTC) Labs.\textsuperscript{10,28}

Differex™ aims to increase the yield and purity of sperm cells by eliminating the wash steps which are required for traditional methods.\textsuperscript{28} In comparison, this method can be performed in as little as two hours, while differential extraction typically requires at least 24. According to Valgren et al.,\textsuperscript{42} the system required the addition of a number of extra steps for the results to be comparable to the Chelex-100® method, and also, the separation solution generated issues when microscopy was being performed. The authors therefore conclude that the system is not applicable for laboratories.\textsuperscript{42} In contrast, Vuichard et al.\textsuperscript{31} compared differential extraction methods currently employed by nine laboratories and found that Differex™ was the only method able to produce a full male DNA profile, with no contaminating epithelial cells. The Differex™ kit is also easy to employ, making it preferential to other methods.\textsuperscript{40} Again, as with modifications made to the initial lysis buffer, the literature provides contrasting results.

The Erase kit uses a proteinase K/detergent lysis step to elute cells from the swabs and digest the epithelial cells, the samples are then centrifuged, the supernatant is removed, and finally, a nuclease is added to remove any residual epithelial cell DNA which has remained attached to the sperm cell pellet.\textsuperscript{10} This method was compared with two traditional differential extraction methods and two Differex™ methods, which demonstrated that although the standard methods were able to collect a higher yield of male DNA 80% of the
time, Erase was able to give a full male profile 55% more often. Importantly, both kits still observed carryover of male DNA into the female fraction, which does not introduce difficulties in generating a female profile, however, it may become an issue if there is a low starting level of male DNA. Garvin et al.\textsuperscript{10} proposes that this carryover is due to male non-sperm cells being collected in the supernatant as “free DNA”. As a result, the carryover of male DNA into the female fraction is inevitable.

**Treatment of Epithelial Cells with DNase or Alkaline Lysis**

Garvin et al.\textsuperscript{34} attempted to decrease the amount of epithelial cell carry over by treating the epithelial cells with DNase. Comparison of this method with a traditional differential extraction method and Differex\textsuperscript{™} showed that this nuclease-based approach was more successful, especially at longer time since intercourse (TSI) intervals. Hudlow et al.\textsuperscript{38} applied an alkaline lysis-based method to swabs which had already been subjected to the traditional differential extraction method, which demonstrated that traditional differential extraction was unable to recover up to 50% of the sperm cells from swabs. In comparison to the traditional methods, these two methods are preferential as only a small number of pipetting steps are required, decreasing the loss of sperm cell DNA.\textsuperscript{34,38}

**Recent Methods**

Lounsbury et al.\textsuperscript{30} aimed to eliminate a number of steps of the differential extraction procedure by removing the need for a second buffer. This was achieved by adding the proteinase K directly to the elution buffer. The method demonstrated successful lysis of the epithelial cells and the sperm cell recovery was almost double that of traditional differential extraction methods. Also, as the initial cell count of the samples decreased below 1000 cells,
the one-step buffer was able to designate more loci than the traditional differential extraction method. Importantly, this method was able to recover 89% of the sperm cells, while the two-step system\textsuperscript{35} recovered 81% and the conventional differential extraction buffer could only recover 45%. Klein and Buoncristiani\textsuperscript{41} then developed a semi-quantitative method for analysing the success of differential extraction methods. The success is measured in terms of the ability of the method to recover high yield male DNA, and minimise epithelial cell carryover. The calculation is termed the Separation Potential Ratio for the Extraction Differential (“SPRED”) and it is able to compare two methods against each other by calculating their overall method efficiencies.\textsuperscript{41} Application of the calculation to three methods demonstrated that Erase had a better method efficiency than the traditional differential extraction method, however, a two initial lysis step differential extraction method had a higher separation efficiency than Erase. Importantly, the method efficiencies of each method could be calculated and compared to determine which is the most “successful”.

\textit{Limitations of Differential Extraction}

Although modifications to the differential extraction method have increased the efficiency of this method, the main limitation is that it is unable to produce non-contaminated male and female fractions. They are one of the most time-consuming methods available in the literature,\textsuperscript{43,44} and are also difficult to automate.\textsuperscript{18,22} Furthermore, there is a fine balance required when employing wash steps: if too many are performed, there will be a loss of male DNA, however, if too little are performed the epithelial cells can carry over into the male fraction.\textsuperscript{18,45} Likewise, there is a fine balance required for the initial lysis step: if it is too strong, the sperm cells will be lysed prematurely,\textsuperscript{45,46} and if it is too weak, the epithelial cells
will remain intact, leading to contamination of the male fraction.\textsuperscript{14,46} Finally, when performing this review, it was discovered that there is very little replication of results observed amongst articles. Given that laboratories around the world employ different versions of differential extraction,\textsuperscript{31} the absence of replication within the results could become an issue if laboratories were to try comparing their results.

\textbf{CURRENT METHODS FOR ISOLATING SPERM DNA: Y-STR ANALYSIS}

Y-STRs are short tandem repeat sequences, located on the Y-chromosome, which comprise of short nucleotide sequences (i.e. di-, tri- and tetranucleotides) that are repeated a number of times.\textsuperscript{47} The number of times that the nucleotides are repeated will vary amongst individuals, meaning that when samples are submitted for Y-STR analysis, the size of the fragment which is amplified will also differ between individuals. Amplification of a number of Y-STRs results in the generation of a haplotype, which consists of multiple differently-sized fragments. The number of times the nucleotides are repeated is correlated with the length of the amplified fragments. Each STR locus can then be designated an allele number, which corresponds to the number of times the STR is repeated on that allele. If the number of times the STR is repeated is different amongst individuals, then the STR is termed polymorphic. The more polymorphic a Y-STR is, the greater their usefulness for discriminating forensic stains.\textsuperscript{47} Although highly polymorphic STRs had been discovered in autosomal DNA already, it was not until 1992 that evidence was provided for polymorphic Y-STRs.
The Introduction of Y-STRs

This evidence was provided by Roewer et al., who discovered that the 27H39LR (DYS19) locus on the Y-chromosome was relatively polymorphic. He compared this locus with two autosomal STRs located on chromosome 12 and, although the Y-STR was not as polymorphic as those on the autosomal DNA, there was still polymorphisms observed. The polymorphisms were later characterised by Muller et al., Gomolka et al., and Santos et al., who all reported that different populations demonstrated considerably different allele frequencies at the DYS19 locus. Y-STRs were first used within forensic science almost immediately after they were discovered, to help exclude a suspect from a murder case. Roewer and Epplen were able to successfully amplify the DYS19 locus in a sample collected from the victim and also from reference samples. The allele designation for the suspect was different to the allele designation for the crime scene sample and therefore the suspect could be excluded. It was also reported that, even though the casework sample was degraded, the length of chromosome which was being amplified was quite small, and so amplification was still possible. In comparison, differential extraction has been shown to perform poorly for degraded samples, and therefore, this method could be more applicable for older casework samples. Although Y-STRs are not highly polymorphic, as are their autosomal counterparts, they were polymorphic enough to allow an exclusion of a suspect based on the amplification of one locus. It was proposed by Roewer et al. that if more Y-STRs could be found, and targeted in combination with this Y-STR, then the degree of discernibility provided would be much greater. As such, the search for highly polymorphic Y-STRs began.
The Continued Discovery of Y-STR Markers

From Y-STRs first being employed, to the most recently published study, hundreds of Y-STRs have been discovered. There has been an enormous amount of literature published which covers the discovery, and validation, of Y-STRs in the forensic science community. The first article that generated a Y-STR haplotype targeted four microsatellites (DYS19, DYS389-I, DYS389-II and DYS390) and discovered that the allele frequencies for each allele differed between populations. More recent analyses use large numbers of Y-STRs combined in one reaction. Prior to 2002, the European Y chromosome typing community had developed a “minimal haplotype” and an “extended haplotype” which could be used by researchers for analysis, and then the results could be uploaded to a DNA database, allowing population data to be collected across these loci. The minimal haplotype includes the following nine loci: DYS19, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393 and DYS385, while the extended haplotype includes the addition of the YCAII marker.

Butler et al. then designed a proof-of-concept study which demonstrated that 20 Y-STRs could be combined into one multiplex reaction. Hanson and Ballantyne applied this technique using a 21-loci multiplex system targeted at loci which were not included in the extended haplotype set. This reaction was sensitive enough to detect a male profile when the female cells were present in a 100-fold excess and it could also detect male donors in samples which were collected 48 hours after intercourse. It was also able to differentiate between two male donors. All of these successful applications indicate that this multiplex system could be applicable to forensic casework samples.
Hanson and Ballantyne\textsuperscript{101} then furthered their own work by generating another multiplex system targeted at 14 different Y-STRs. When compared with commercially available kits, this new multiplex demonstrated a higher discriminatory power. When the AmpF\textregistered ESTR\textsuperscript{®} Yfiler™ kit was combined with these 14 Y-STRs, 100% of the selected population was able to be discriminated.\textsuperscript{57} This provided significant evidence that increasing the number of Y-STRs included in a reaction increases the discriminatory power of the system.

In their studies, Cooper et al.\textsuperscript{55} and de Knijff et al.\textsuperscript{57} both concluded that the haplotype diversity within populations is less than haplotype diversity between populations. This means that Y-STR haplotypes are better able to discriminate between individuals who come from different populations, rather than those in the same population. However, the statistical analysis which is applied to autosomal STR profiles cannot be applied to Y-STR haplotypes. This is due to the Y-chromosome being inherited directly from father to son, without any recombination occurring within the genes, meaning that diversity is only introduced through mutations.\textsuperscript{55} To be able to apply statistical analyses to haplotypes, population databases need to be generated which are able to record the allele frequencies, at certain loci, from entire populations. Kayser et al.\textsuperscript{56} and de Knijff et al.\textsuperscript{57} both performed studies aimed at collecting population information from a large dataset, however, they were only preliminary studies and they both concluded that larger population studies need to be performed. Using seven of the microsatellites collected in the database, Y-STR analysis was able to discriminate 74-90% of European populations, and with the addition of another three Y-STRs, the discriminatory power approached 100%.\textsuperscript{56}
**Y-STR Analysis and Its Application to Forensics**

Prinz et al.\(^{58}\) were the first authors to target sperm cell isolation from mixed cell samples by generating the corresponding male haplotype. Swabs which had already been subjected to differential extraction were subjected to further cell elution, allowing the collection of DNA for Y-STR analysis. They found that differential extraction was only able to recover 50% of the sperm cells, leaving more than enough DNA in the swabs for Y-STR analysis. The analysis itself consisted of a quadruplex Y-STR system, composed of the DYS19, DYS390, DYS389I and DYS389II loci. They demonstrated that the Y-STR haplotype produced for a pure male DNA sample was exactly the same as the haplotype produced for male DNA present in a 1:2000 ratio of epithelial cells. Therefore, the analysis is a useful application for sexual assault samples. This work was furthered by Dekairelle et al.\(^{61}\) who also demonstrated that samples which had been subjected to differential extraction could still be used to generate Y-STR profiles. Their results showed that differential extraction failed in 35% of the cases they investigated, and these failed samples were then subjected to Y-STR analysis using the Y-STR pentaplex generated by Gusmao et al.\(^{60}\) and an extra four loci, meaning that nine loci were targeted in one reaction. From these samples, Dekairelle et al.\(^{61}\) were able to generate Y-STR haplotypes for 45% of the samples. This was also demonstrated by Cerri et al.\(^{59}\) who were able to generate Y-STR profiles from samples which could not be profiled using autosomal STR analysis. Although the statistical analysis cannot be applied, Y-STRs still remain useful as an exclusionary tool for suspects, narrowing the suspect pool.\(^{58}\)

**Analysing Samples with Low Sperm Cell Numbers**

Gusmao et al.\(^{60}\) demonstrated that Y-STR analysis could also be applied in sexual assault cases where the perpetrator is azoospermic. They used a pentaplex Y-STR system to analyse
two azoospermic individuals and discovered that haplotypes could still be observed for individuals whose semen contains no sperm cells. This is an important application because differential extraction has typically been unsuccessful for samples containing minimal amounts of male DNA. Furthering the work of Gusmao et al., it was demonstrated that samples which gave a negative cytology could still be amplified using Y-STRs. Y-STRs were able to be generated from 28.8% of the negative cytology samples, targeting the DYS393 and DYS389 loci separately. The reason why Y-STR information can still be generated when the sample is showing low numbers of spermatozoa, is because Y-STR analysis targets all of the male DNA, not just the sperm cells. Therefore, a haplotype can still be generated from the male non-sperm cells. Both of these articles demonstrate the applicability of Y-STRs to cold case samples, which may have already been subjected to differential extraction and gave no male profile.

**Y-STR Analysis for Samples with a Prolonged TSI**

Hall and Ballantyne were able to generate a 10 loci Y-STR profile from samples which had been collected up to four days after intercourse. This is an important application because differential extraction typically struggles to generate profiles from older samples, as the spermatozoa degrade quite rapidly after ejaculation. Mayntz-Press et al. also assessed the ability of Y-STR analysis for extended post-coital intervals, however, they used three commercially available kits: Y-PLEX™ 12 (Reliagene), PowerPlex® Y (Promega) and AmpFSTR® Yfiler™ (Applied Biosystems). They discovered that PowerPlex® Y and AmpFSTR® Yfiler™ could produce Y-STR haplotypes for samples which were collected more up to six days after intercourse. When samples were collected after six days, PowerPlex® Y and AmpFSTR® Yfiler™ were only able to generate partial profiles.
Kit-Based Methods

A number of Y-STR kits have also been developed and validated for use on forensic casework samples. The Y-PLEX™ 12 system was developed first and amplifies 12 Y-STRs in one reaction. Then the PowerPlex® Y System was validated, which also amplifies 12 Y-STRs. Mulero et al. developed the AmpF® STR Yfiler™ PCR amplification kit, which is able to simultaneously amplify 17 Y-STRs. More recent discoveries of Y-STRs with high polymorphic tendencies has led to the development of the PowerPlex® Y23 System, which is able to simultaneously amplify 23 Y-STRs. Of particular use to the forensic investigator is the inclusion of two rapidly mutating Y-STRs which can possibly discriminate between related individuals. The most recently generated kit uses one multiplex to target 27 Y-STRs. Kits which have been developed more recently have a much higher discriminatory power than those initially produced, and as more polymorphic Y-STRs are discovered, it is possible that more kits will be generated with even greater discriminatory powers.

Limitations of Y-STR Analysis

The key advantage that this technique has over differential extraction is that separation of the fractions is not required prior to analysis, meaning that male DNA is not lost during the analysis. Given that the technique only targets male DNA, it is not affected by an excess of epithelial cells, and therefore, can be applicable to sexual assault samples. It is also demonstrated that this method is applicable to samples whereby the perpetrator is azoospermic or with low cell counts, and to samples containing two male DNA profiles. However, its applicability is limited due to their reduced polymorphic capabilities, in comparison with their autosomal counterparts. For example, autosomal STR profiles are
able to confidently individualise a sample, with a high statistical probability, while haplotype information can differentiate between two males of separate populations, but not those with the same lineage. From a forensic perspective, this means that if the haplotype of a suspect matches with the casework sample then they can be included as suspects, however, if they do not match then they can be excluded. For this reason, Y-STR analysis is particularly employed in cases where an autosomal STR profile is not obtainable.

THE ISOLATION TECHNIQUE OF THE FUTURE: IMBS

Given that the two methods already discussed are those most commonly employed by DNA laboratories, it is proposed that new techniques need to be employed which are able to effectively isolate sperm cells. The technique of using IMBs to separate cell mixtures has been employed within the scientific community for an extended period of time. Studies which are most relevant, include those within the assisted-reproduction field that are able to effectively target apoptotic sperm cells in solution, and remove them from the sample. Although used within other science disciplines, IMBs have only recently been proposed as a useful forensic tool.

IMBs are designed to select for a specific cell type by targeting an antigen, on the surface of those cells, which is specific for that cell type only. The magnetic beads are coated with a compound, or antibody, that is able to bind with the antigen via various mechanisms. The concept of this method is demonstrated in Figure 1 below. The magnetic beads are first incubated with an antibody (anti-antigen A antibody) that has been generated against the cell-specific antigen, and they form a coating around the bead, as demonstrated by the
second bead. The IMBs can then be incubated with cells containing the target antigen (antigen A solution), shown in red below. The magnetic beads are then bound with the cells through antigen-antibody binding. The bead-containing solution is then run through some type of metal column with a magnetic field applied, which will allow the magnetic beads to attach to the column while the supernatant runs through and is collected. The magnetic field is then removed from the column, allowing the magnetic beads to be easily eluted from the column.

From a forensic perspective, IMBs are being used in conjunction with sperm-specific antibodies to isolate male cells from mixed samples. The earliest forensic-based study employing IMBs evaluated three different anti-sperm antibodies, bound with magnetic beads, for their ability to bind and remove sperm cells from solution. The three antibodies assessed were: MHS-10, NUH-2 and HS-21. Of these three antibodies, MHS-10 was the most effective, and was able to capture 90-95% of spermatozoa, while NUH-2 was able to recover 80% of the spermatozoa and, HS-21 was the least effective, only capturing 60% of spermatozoa. This article demonstrates that the selection of antigen, and the corresponding antibody, is priority for these studies. There was a 30-35% increase in sperm recovery based solely on altering the targeted antigen.
Anslinger et al.\textsuperscript{88} chose to target their magnetic beads at the testicular isoform of the angiotensin-converting enzyme (tACE) which is located on the mid-piece, neck and flagellums of the sperm.\textsuperscript{89} The beads and antigen were bound to each other using a primary antibody-secondary antibody system. This IMB mechanism involves: generating an antibody against the sperm cell antigen (anti-tACE), then generating an antibody against the primary antibody (secondary antibody) which is able to bind with the magnetic beads.\textsuperscript{88} Therefore, the magnetic beads are linked with the sperm cells by two antibodies. This method used samples which had been stored in PBS immediately after collection, and therefore, even though this method was successful, it is possible that applying it to casework samples would not be as effective.\textsuperscript{88} This is due to tACE being located on the mid-piece, neck and flagellums of the sperm cells, which degrade quite rapidly after ejaculation. Anslinger et al.\textsuperscript{88} proposed that any further studies conducted should target antigens located on the sperm heads as 1) this is where the DNA is located, and 2) the sperm heads are robust.

A number of other studies have been published which all target different sperm cell antigens and employ different mechanisms for binding the bead with the antibody. Prior to targeting the sperm cell antigens, they are all subjected to analyses to determine where on the sperm cell they are primarily expressed, and whether they are expressed on epithelial cells. All of the following studies found that their selected antigens were expressed only on male cells, and primarily on the sperm head.

One study targeted the motile sperm domain-containing protein 3 (MOSPD3) antigen by generating biotinylated MOSPD3 antibodies which were able to bond with avidin-coated
magnetic beads.\textsuperscript{44} This biotin-avidin system was selected for binding the antibodies with the bead as this is the strongest bond which can occur between a protein and ligand. Visualisation of the male STR profiles demonstrated that there was no carryover of female cells into the male DNA and the technique was also applicable to samples which had been stored for over 10 days.\textsuperscript{88} Importantly, it has a number of advantages over differential extraction: 1) the centrifugation steps are eliminated, 2) the overnight incubation step is eliminated, 3) there is no repeated washing required, and 4) it is more amenable for automation.

Xu et al.\textsuperscript{46} targeted the A kinase anchor protein 3 (AKAP3) antigen using a single, fluorescently-labelled antibody bound with the magnetic beads. Cells which were fluorescently-labelled were then sorted using flow cytometry. The STR results showed that the male DNA fractions were not contaminated with female DNA, and also, sensitivity tests demonstrated that the sperm cells could be isolated from mixtures containing up to 1:32 epithelial cells.\textsuperscript{46}

The most recently published article using IMBs in forensics binds an anti-PH-20 antibody to magnetic beads through the carboxylic group on the magnetic beads.\textsuperscript{90} When this technique was employed, with the addition of a DNase I wash step, it was observed that the male profiles were not contaminated with female DNA. This indicated that the method had been successful at isolating a clear male fraction, making it already more successful than any of the differential extraction methods. Zhao et al.\textsuperscript{90} also chose to investigate a number of other sperm-cell antigens, including: sperm protein-10 (SP-10),\textsuperscript{91} a disintegrin and metalloprotease 2 (ADAM2),\textsuperscript{92,93} and JNK-associated leucine zipper protein (JLP).\textsuperscript{94} However,
it was observed that the IMBS bound with the anti-PH-20 antibodies were most successful at isolating male cells. When the concentration of sperm cells was decreased to $10^3$/mL, the anti-PH-20 IMBs were still able to genotype 90% of the samples tested. Therefore, the sensitivity of this method was improved over both Anslinger et al.\textsuperscript{88} and Li et al.,\textsuperscript{44} which demonstrates that the use of IMBs in forensic science is moving in the right direction.

A proof-of-concept study conducted this year by Hutchinson,\textsuperscript{95} demonstrated that the PH-20 antigen is a suitable target for a primary antibody-secondary antibody system bound with IMBs. However, there was non-specific binding observed for the antibodies, or, there was inadequate washing of the microscope slides performed between the addition of antibodies, which resulted in fluorescence of the epithelial cells as well as the sperm cells. Importantly, it was demonstrated that the PH-20 antigen was still expressed on the sperm cells up to three days after collection, making it an excellent choice of target antigen. Future studies need to be performed which are able to determine why there is non-specific binding of the antibodies throughout the microscope slide, or whether it is simply a procedural error which needs to be corrected before the method can be applied to casework samples.

**DISCUSSION/CONCLUSIONS**

A thorough review of the literature has demonstrated that sperm cell isolation techniques which are currently employed within forensic DNA laboratories are notoriously inefficient. In particular, differential extraction methods are unable to generate fractions which are not contaminated with opposing cells without causing a severe loss of sperm cells. This damage to sperm cells can have a detrimental effect if the starting volume of male DNA is very low,
as would be observed in sexual assault samples. Y-STR analysis, on the other hand, is excellent at targeting the male fraction of the mixed cellular samples, however, its applicability is quite limited given that Y-STRs are not as highly polymorphic as autosomal profiles. It does have its place within forensic science, but it is primarily used when autosomal DNA profiling is not available, or as an exclusionary tool for eliminating suspects.

Although an extensive number of techniques have been published within the literature for isolating sperm cells, I have proposed that the way forward is with IMB techniques. They are shown to be successful at generating male STR profiles which are not contaminated with female DNA, they are sensitive, specific for male DNA, and can be used when the sample contains a large excess of epithelial cells. This makes them an optimum technique for use on sexual assault samples. Future studies should be targeted at either increasing the success of the IMB techniques which have already been proposed, or at generating entirely new techniques for targeting different antigens.
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