

7-3-2014

A Novel Method for Rapid and Selective Extraction of Male DNA from Rape Kits using Alkaline Lysis and Pressure Cycling Technology (PCT)

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

A NOVEL METHOD FOR RAPID AND SELECTIVE EXTRACTION OF MALE DNA
FROM RAPE KITS USING ALKALINE LYSIS AND PRESSURE CYCLING
TECHNOLOGY (PCT)

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

by

DEEPTHI V. NORI

2014

To: Interim Dean Michael R. Heithaus
College of Arts and Sciences

This dissertation, written by Deepthi V. Nori, and entitled A Novel Method for Rapid and Selective Extraction of Male DNA from Rape Kits using Alkaline Lysis and Pressure Cycling Technology (PCT), having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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The dissertation of Deepthi V. Nori is approved.

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Florida International University, 2014

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DEDICATION

I dedicate this thesis to my mother and husband. Their support, patience and unconditional faith in my abilities have turned every failure into a lesson and every victory into wisdom.

ACKNOWLEDGMENTS

Words are not enough to begin to appreciate every member who guided and supported me throughout this arduous process. It seems surreal when I reflect upon this journey and realize that I made it, albeit with too many relationships tested along the way. Many pats on the back and words of encouragement lit the way and I would like to take a moment to say thanks to all those people who believed in me.

You realize you are lucky when your family living away from you is reassured by the fact that you have your advisor's support in times of need. Lost in the chaos we hardly get to say thanks to one person who endured it all with you, your advisor. Thanks to Dr. Bruce McCord, who is not just an exemplary scientist but also an amazing individual. Too many times I have heaved a sigh of relief knowing he has my back. Thank you Dr. McCord, for indulging my ideas and allowing me to grow as an independent thinker.

Every member of my committee deserves a special mention for contributing tremendously to this journey. It is humbling to be in the presence of people who have numerous accomplishments but are always open to learning. Dr. Watson Lees, Dr. DeEtta Mills, Dr. Yuan Liu and Dr. Yong Cai were always ready with ideas, suggestions, support and a lot of understanding. I couldn't have asked for a better committee to guide me through this maze.

I would like to extend my thanks to Dr. George Duncan, Chris Comar and Arlene Petrosky from Broward sheriff's office (BSO) crime lab. They have generously shared their technical expertise and provided me with much needed resources to see this project to fruition. Special thanks to Dr. Nate Lawrence and Richard Schumacher from Pressure

BioSciences Inc. who have been very helpful with training me and extending technical support throughout this project.

Nothing would have been possible without my family. My father's presence and his blessings have accompanied me throughout this journey. My mother, Dr. Lalitha Devi, who is the bravest and the most resilient person I ever met, has guided me by example by showing that one should always pursue their dreams. I have tremendous appreciation for the support of my husband, Vinay, who knows every experiment I ever did and has been there patiently listening to the travails of a graduate student. This herculean task has been made bearable only through his patience and faith in me. A special mention of my brothers, Vamsi and Abhishek, who have been there to see me through all the choices I made, some questionable, and still love me. My sisters-in-law, Jyothsna and Srujana, for being the wonderful sisters I never had. Not to forget my little nephew, Shreyan, whose infectious enthusiasm inspires us all to have a good time when the going gets tough.

My marriage brought new parents into my life. My in-laws, Shyamala and Ramankutty Menon, have been great pillars of strength throughout this process. My sister-in-law, Reshma and my brother, Amit, have always made me feel like there is nothing I can't do. So I indeed have a big troop cheer leading for me from the sidelines and I would like to convey my heartfelt gratitude to every single one of them.

ABSTRACT OF THE DISSERTATION

A NOVEL METHOD FOR RAPID AND SELECTIVE EXTRACTION OF MALE DNA
FROM RAPE KITS USING ALKALINE LYSIS AND PRESSURE CYCLING
TECHNOLOGY (PCT)

by

Deepthi V. Nori

Florida International University, 2014

Miami, Florida

Professor Bruce McCord, Major Professor

There is an increasing demand for DNA analysis because of the sensitivity of the method and the ability to uniquely identify and distinguish individuals with a high degree of certainty. But this demand has led to huge backlogs in evidence lockers since the current DNA extraction protocols require long processing time. The DNA analysis procedure becomes more complicated when analyzing sexual assault casework samples where the evidence contains more than one contributor. Additional processing to separate different cell types in order to simplify the final data interpretation further contributes to the existing cumbersome protocols. The goal of the present project is to develop a rapid and efficient extraction method that permits selective digestion of mixtures.

Selective recovery of male DNA was achieved with as little as 15 minutes lysis time upon exposure to high pressure under alkaline conditions. Pressure cycling technology (PCT) is carried out in a barocycler that has a small footprint and is semi-automated. Typically less than 10% male DNA is recovered using the standard extraction protocol for rape kits, almost seven times more male DNA was recovered from swabs

using this novel method. Various parameters including instrument setting and buffer composition were optimized to achieve selective recovery of sperm DNA. Some developmental validation studies were also done to determine the efficiency of this method in processing samples exposed to various conditions that can affect the quality of the extraction and the final DNA profile.

Easy to use interface, minimal manual interference and the ability to achieve high yields with simple reagents in a relatively short time make this an ideal method for potential application in analyzing sexual assault samples.

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ACRONYMS AND ABBREVIATIONS

°C	Degree Celsius
A	Adenine
ABI	Applied Biosystems by Life Technologies
bp	Base Pair
BSA	Bovine Serum Albumin
BSO	Broward sheriff's office
C	Cytosine
ccd	Charged Couple Device
CE	Capillary Electrophoresis
CODIS	Combined DNA Index System
CST	Charge switch technology
Ct	Cycle Threshold
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double Stranded DNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EOF	Electroosmotic Flow
FBI	Federal Bureau of Investigations
FDL	Fingerprint DNA Finder kit

G	Guanine
ILS	Internal Lane Standard
Iso-C	5-methylisocytosine
Iso-G	Isoguanine
LM	Laser microdissection
LCM	Laser-capture microdissection
MgCl ₂	Magnesium Chloride
PCIA	Phenol- Chloroform Isoamyl Alcohol
PCR	Polymerase Chain Reaction
PCT	Pressure Cycling Technology
PHR	Peak Height Ratio
RFLP	Restriction Fragment Length Polymorphism
SDS	Sodium Dodecyl Sulfate
STRs	Short Tandem Repeats
T	Thymine
TAPS	3-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]propane-1-sulfonic acid
Taq	Thermus aquaticus Polymerase
TCEP	Tris (2-Carboxyethyl) phosphine, Hydrochloride
VNTR	Variable Number Tandem Repeats

CHAPTER I: FORENSIC DNA ANALYSIS

A. History and technology

Crime scene investigators abide by Edmond Locard's dictum, "every contact leaves a trace", when investigating a crime scene because physical evidence is extremely crucial in recreating events that transpired [57]. Forensic science deals with the collection and examination of evidence to gather information. The application of scientific knowledge to understand the cause of a fatality dates back to as early as the time of Julius Cesar when his physician determined that only one of his wounds was fatal [134]. One of the earliest descriptions of analysis done to determine the kind of weapon used to murder a person was given in a Chinese book titled Hsi DuanYu (the Washing Away of Wrongs) written by Song Ci in 1248 A.D. The book demonstrated the application of medical knowledge towards solving crimes [11]. Though scientific principles have been applied to criminal investigations throughout history, it was not until the last century that the discipline of forensic science took an identity of its own with fingerprint classifications, DNA profiling, toxicology, ballistics, trace evidence analysis and blood spatter analysis each carving its own niche. Development of the Henry classification system for fingerprint analysis in early 1900s and the discovery of blood typing from dried bloodstains by Leone Lattes in 1915 became useful tools for crime scene investigations. Dr. Calvin Goddard invented the comparison microscope during the 1920s that allowed for comparative analysis of bullets and shell casings leading to the development of forensic ballistics [120]. The late 1980s and 1990s saw the development of DNA profiling, and the establishment of DNA databases such as CODIS, which can be

used for comparison of DNA profiles recovered from crime scenes and suspects [25]. In part as a consequence of shows like CSI: Crime Scene Investigation, even a layman is aware of the probative value of DNA testing in criminal investigations. Following its discovery in 1984 by Sir Alec Jeffreys, DNA fingerprinting has become the most integral element of a forensic investigation [67]. Within two years of its discovery, DNA profiling was successfully used in a criminal conviction [32]. DNA profiling identifies genetic variations that make each person unique, so it is often referred to as DNA fingerprinting.

Deoxyribonucleic acid (DNA) is present in almost every nucleated cell in the body except mature red blood cells and it is present in a wide variety of samples such as blood, saliva, semen, hair, bone, teeth, and urine. The genetic material of humans is packaged into 23 pairs of chromosomes in each cell (except for sex cells). One chromosome in each pair comes from the father and the other from the mother. Sex chromosomes play an important role in forensic DNA testing because the presence of XY sex chromosomes indicates a male and the presence of XX chromosomes indicates a female.

Nucleotides, the basic building blocks of DNA, are composed of a nitrogenous base- adenine (A), guanine (G), thymine (T) or cytosine (C), a deoxyribose sugar molecule and a phosphate group [132]. The nucleotide sequence is 99.7% identical between any two individuals and the remaining 0.3% variation is used to distinguish one person from the other. Examining these polymorphic regions of the genomic DNA can generate a profile unique to that individual. DNA profiling has numerous applications ranging from criminal law, identification of human remains, determination of paternity and in diagnosis of certain genetic disorders [32].

The first breakthrough that paved way for DNA testing was the discovery of restriction fragment length polymorphism (RFLP) analysis by Dr. Ray White and Dr. Arlene Wyman [141]. They discovered that DNA molecule can be cut using restriction enzymes at specific recognition sites and then separated the fragments on the basis of size. These restriction enzymes, also called as restriction endonucleases, are harvested from bacteria and cleave the DNA molecule at a specific sequence of nucleotides. For example, the bacterium *Escherichia coli* produces an enzyme named **EcoRI** that cuts DNA wherever it encounters the sequence GAATTC [3]. The cut is made between the adjacent G and A. Since no two individuals have identical DNA, the length of the fragments produced with restriction enzymes is variable between individuals.

In 1985, Sir Alec Jeffreys at University of Leicester, UK, discovered regions of DNA where the same sequence was repeated end to end and these regions were observed to be of variable lengths between two individuals. These fragments, referred to as variable number of tandem repeats (VNTR), are large and have a repeat size composed of hundreds of nucleotides. Dr. Jeffreys developed a multi-locus probing (MLP) technique using RFLP technology, which allows visualization of more than one variable region. In the MLP method, a DNA fragment is first digested into small pieces using restriction enzymes. The digested DNA fragments are separated on the basis of their size, through a process known as electrophoresis in which agarose gel acts like a sieve and allows smaller fragments to migrate faster than larger fragments upon the application of an electric field [67]. The migration pattern is visualized through a process called Southern Blotting where the bands are transferred onto a nylon membrane and hybridized with probes. Probes are DNA sequences complementary to the VNTRs, tagged with a

radioactive label or a chemiluminescent compound. Upon exposure to X-ray film, fragments of DNA bound to the probe appear as dark bands on the film. (Figure 1). Since evidence from a crime scene typically contains DNA from more than one individual, Dr. Jeffreys developed single-locus probes to simplify the interpretation of complex patterns observed with multi-locus approach. The pattern of restricted DNA was used to identify individuals based on the unique band patterns and was referred to as DNA fingerprinting [67].

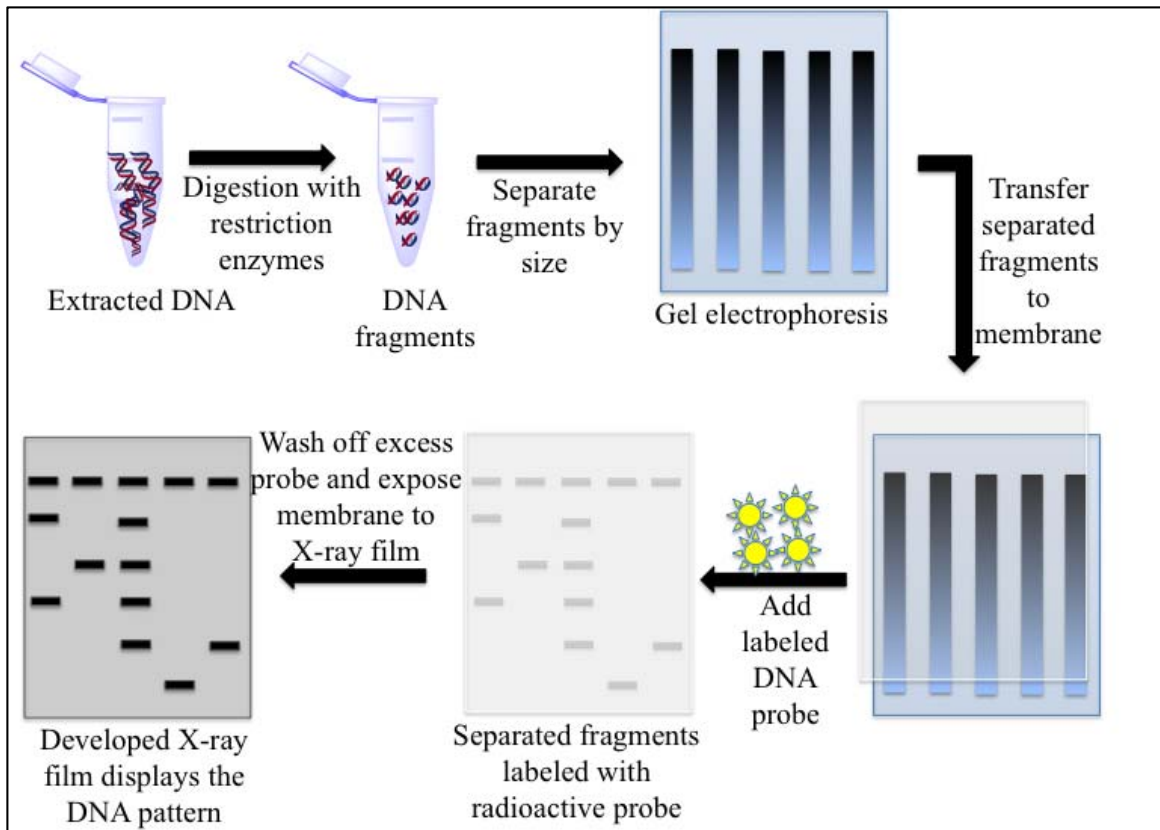


Figure 1. An overview of a DNA fingerprinting process using VNTR/RFLP analysis. DNA is digested with restriction endonucleases and the fragments are separated by electrophoresis. These fragments are transferred onto a nylon membrane and hybridized with a radioactive probe. DNA typing results appear as dark bands and are visible upon exposure to X-ray film. Adapted from How stuff works website.

Deoxyribonucleic acid (DNA) fingerprinting was used in a criminal investigation for the first time in 1986. It resulted in the exoneration of an innocent man implicated in two rape-murders that occurred in 1983 and 1986 in Leicestershire, UK and conviction of the perpetrator. Although accurate and reproducible, DNA fingerprinting has several drawbacks such as laborious protocols, use of harmful reagents and the necessity for a large amount of DNA sample [30].

Around the same time, another important discovery was taking place across the pond in the United States of America that revolutionized the field of molecular biology and forensic science by addressing all the limitations of RFLP analysis. Kary Mullis conceived the idea for polymerase chain reaction (PCR) in 1983 that led to more sensitive and rapid analysis of DNA [87]. The evidence obtained from a crime scene is usually degraded or available in small quantities, which made the use of RFLP analysis futile until the advent of PCR technology. Multiple copies of a specific DNA sequence can be obtained in a relatively short time with polymerase chain reaction. The first PCR-based forensic assay was developed to detect sequence variation at the human leukocyte antigen (HLA) locus, DQ-Alpha that could distinguish six variations in the sequence [17]. In order to improve the discrimination power, more loci were incorporated into the assay and marketed as Polymarker kit that could coamplify five other loci DNA located on human chromosomes 4, 7, 11, and 19 [117].

The next development in DNA testing came in the form of PCR amplification of variable number tandem repeat (VNTR) polymorphisms that are relatively short. Referred to as amplified fragment length polymorphism (AmpFLP), one of the popular VNTR loci used in crime laboratories was D1S80 present on chromosome 1 and

containing a 16 nucleotide sequence which is repeated between 16 and 40 times [19]. AmpFLP markers were not very popular because they are not as discriminatory as RFLP markers.

Short tandem repeats (STR), also known as microsatellites, contain repetitive sequences that are shorter than VNTRs. Each repeat unit in STR is 2-7 base pairs in length compared to VNTR that has a repeat unit in the range of 8-100 base pairs [84]. Individual STR loci are not very discriminatory but PCR allows amplification of multiple STRs in a short time. Short tandem repeat markers are excellent for forensic investigation because of their small size. A DNA profile can be generated using STR markers even when the sample is degraded, which was not possible with the larger RFLP fragments. Coupled with improved detection methods where nucleotides are labeled with fluorescent tags, STR analysis has completely replaced RFLP technology and is the method currently used for DNA profiling in crime laboratories [117].

Combined DNA Index System (CODIS) is an electronic database that contains DNA profiles of people convicted of certain crimes. Laboratories across the nation can compare DNA profiles from crime scene evidence to the DNA profiles of convicted offenders to find possible suspects. The FBI laboratory did extensive studies to choose a set of 13 STR loci that are highly discriminatory and show considerable inter-individual variation. The 13 CODIS loci are CSF1PO, FGA, TH01, TPOX, VWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11 and a sex marker, Amelogenin (Figure 2). The probability of two individuals sharing the same profile at all thirteen loci is 1 in a trillion except for identical twins [59]. Some of the commercially

available STR kits can amplify more than the 13 CODIS STR loci further improving the power of discrimination.

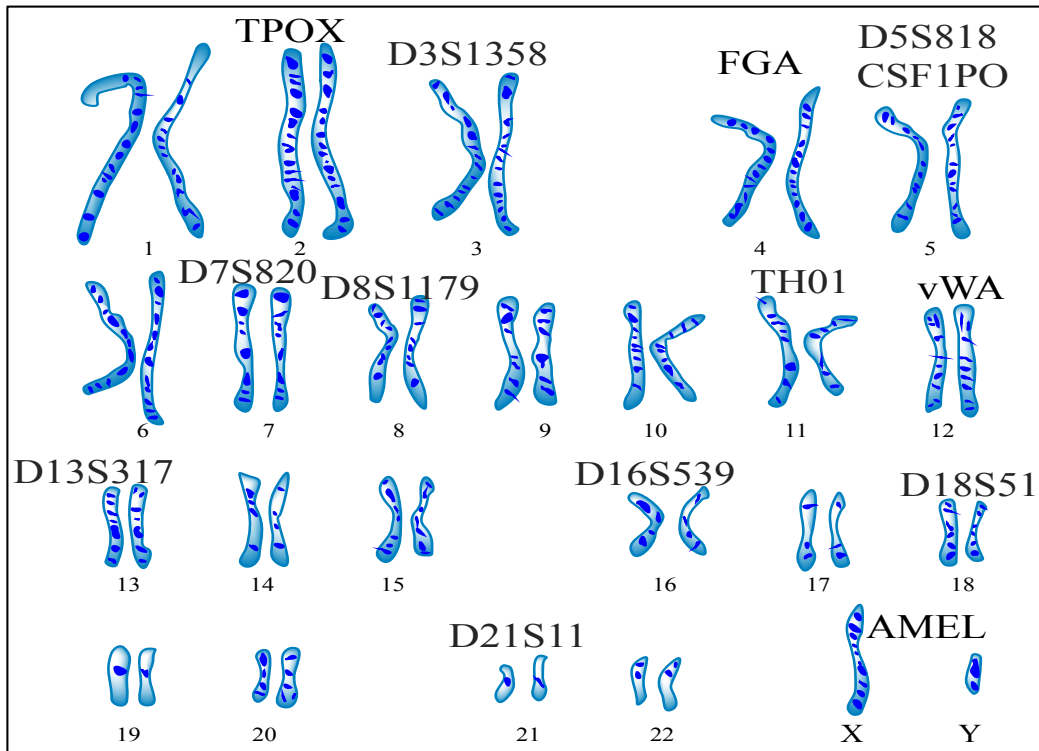


Figure 2. The chromosomal positions and nomenclature of the 13 CODIS core STR Loci and the sex marker, Amelogenin (AMEL). Source: Adapted from The National Institute of Standards and Technology (NIST) website.

B. DNA biology

B.1. Introduction

Cells are the smallest biological units of all living organisms and are the building blocks of life because all living things are composed of cells that can replicate independently. Each cell contains two important biomolecules, proteins and nucleic acids. The nucleus is contained in the cell and houses the chromosomes that are complexes of deoxyribonucleic acid and histone molecules [132]. (Figure 3)

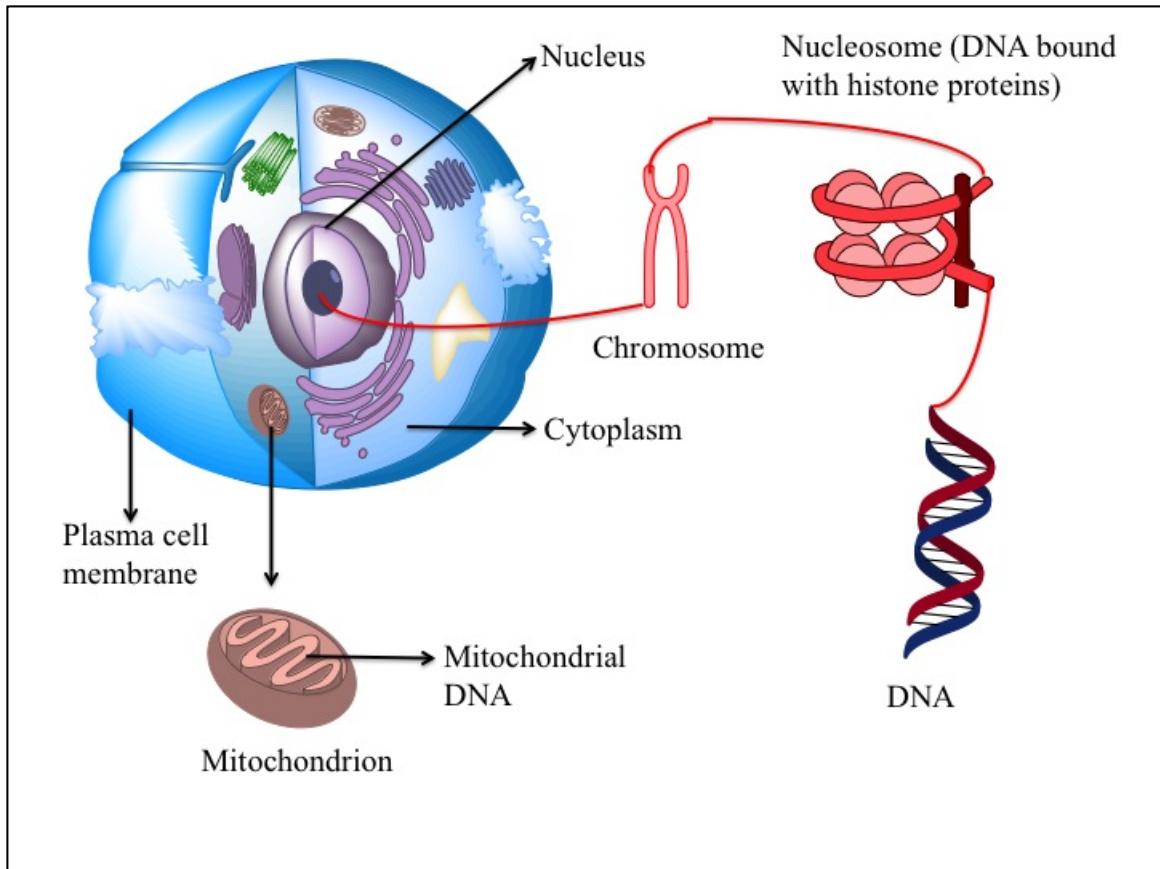


Figure 3. Structure of a cell. Nuclear DNA is present inside the nucleus of a cell. DNA and histone protein complexes called as nucleosomes form chromatin fibers that allow for compact packaging of DNA into chromosomes. Maternally transmitted mitochondrial DNA is present in mitochondria found outside the nucleus.

Deoxyribonucleic acid, or DNA, contains the genetic code required for the development of every little trait and function of a living being. The nucleic acid DNA is present in every nucleated cell in a eukaryotic organism [5]. Every person gets one half of the DNA from his mother and the other half from his father [15]. DNA is a self-replicating molecule that allows the cells to divide and carry forward this information. DNA also carries the necessary genetic information that enables protein synthesis required for sustaining any life form [5].

B.2. Structure of the DNA molecule

Deoxyribonucleic acid (DNA) is composed of nucleotide molecules, which contain one of four nitrogenous bases, adenine (A), guanine (G), thymine (T) or cytosine (C), a deoxyribose sugar, and a phosphate group. The sugar and phosphate groups form the DNA backbone whereas the variation in the arrangement of these four bases makes an individual unique [31]. The nucleotide molecule has a five-carbon ring and phosphate group attaches to the 5' carbon atom. The area surrounding the 5' end of the molecule is referred to as 5' end and the area surrounding the 3' carbon atom of the nucleotide is referred to as 3' end [5, 98]. The nucleotide molecules join together through phosphodiester bond between 5' end of one nucleotide and 3' end of the adjacent nucleotide to form a polynucleotide chain. The sugar-phosphate structure is referred to as the backbone of a DNA molecule [30, 41] (Figure 4).

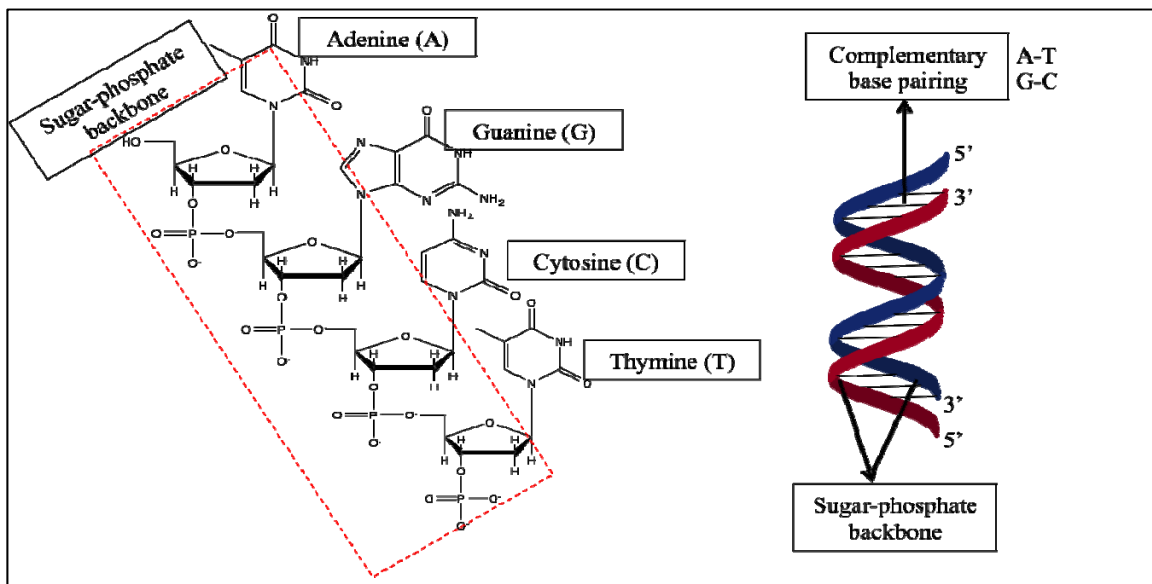


Figure 4. Sugar-phosphate backbone of the DNA molecule is formed through phosphodiester bonds between hydroxyl group on the 3' carbon atom of one sugar molecule and the phosphate group present on the 5' carbon atom on the sugar molecule of the adjacent nucleotide. The two DNA strands running in opposite directions to each other are bound together through complementary base pairing [41].

DNA exists in the form of a double helix molecule where the two polynucleotide strands are joined through complementary base pairing between the bases. Adenine (A) base pairs with thymine (T) and guanine (G) base pairs with cytosine (C). There are two hydrogen bonds between AT base pairs and three hydrogen bonds between GC base pairs due to which the latter pair requires more energy for denaturation [5, 33] (Figure 5)

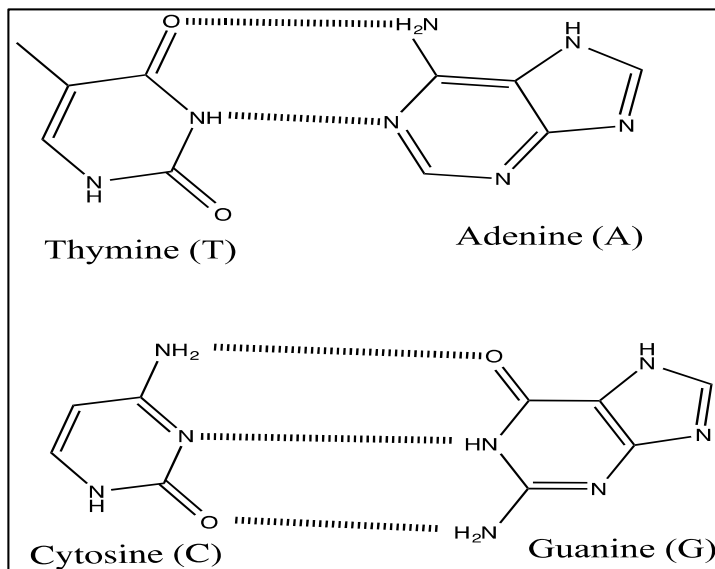


Figure 5. Structures of the four nitrogenous bases present in the DNA molecule. The two strands of DNA are held together through complementary base pairing where adenine (A) pairs with thymine (T) via two hydrogen bonds and guanine (G) pairs with cytosine (C) via three hydrogen bonds.

The two strands of DNA are anti-parallel where one strand is in 5' to 3' direction and the other strand exists in 3' to 5' direction relative to the first strand. The sequence on one strand can be determined if the sequence on the other strand is known because of the complementary base pairing [41]. The two strands of DNA can be separated by subjecting it to a variety of conditions such as elevated temperatures or chemical treatment. The process of separating the DNA strands is referred to as denaturation [5]. Denaturation is a reversible process because when the conditions return to ambient levels,

the strands come together and rehybridize in a process referred to as reannealing.

X-ray diffraction studies by Rosalind Franklin and Maurice Wilkins indicated the helical nature of the DNA molecule. The work done by Erwin Chargaff showed that the number of guanine residues is equal to the number of cytosine residues and the number of adenine residues is equal to the number of thymine residues [33]. These two observations paved way for the elucidation of the double helical structure of DNA molecule by James Watson and Francis Crick [131].

B.3. DNA arrangement- Chromosomes

Nuclear DNA is found in the nucleus of a cell and it is found in association with proteins called histones. The DNA-histone complex is packaged into 22 pairs of autosomal chromosomes and one pair of sex chromosome. There are a total of 23 pairs of chromosomes in every nucleated cell. Males inherit one copy of the X chromosome from their mother and one copy of the Y chromosome from their father and are identified by the sex chromosome pair XY. Females contain two copies of X chromosome and are identified by the sex chromosome pair XX. Forensic DNA testing for human identity examines the recovery of DNA from 22 pairs of autosomal chromosomes whereas sex chromosomes are used to identify the gender [132]. Apart from the nuclear DNA, every cell has multiple copies of mitochondrial DNA present in the cell cytoplasm. Mitochondrial DNA can also be used for human identity testing in certain scenarios.

The chromosomes consist of coding and noncoding regions. The coding regions, also called as genes, contain all the necessary information for protein synthesis. Genes consist of exons, which code for proteins and these exons are interspersed with non-

coding regions called introns [5, 132]. Most of the variations within individuals that are used for identity testing occur in the non-coding regions of the chromosome. The location of polymorphism on the chromosome is referred to as a locus and allele is the alternate form of the locus. The locus is referred to as heterozygous if the two alleles are different and homozygous if they are identical [30]. These allelic differences play a vital role in human identity testing. DNA profiling takes into account the allelic profiles at multiple loci [51].

B.4. Nomenclature for DNA markers

The DNA markers are named for their location on the chromosome or the name of the gene if they are present in the coding region of that gene. For example, the marker TH01 is found in human tyrosine hydroxylase gene located on chromosome 11. Since the repeat region of the TH01 marker is located within the first intron of the gene, the nomenclature ends in '01'. When the markers are not present in the gene, their name starts with the letter 'D' for DNA followed by chromosome location. For example, D5S818 is found on chromosome number 5 and it is a single copy sequence, which is identified by the letter 'S'. The last digits indicate the order in which the marker was discovered on that chromosome [30].

C. DNA testing

C.1. STR analysis

Extraction, quantitation, amplification, and analysis are the four steps required to generate a DNA profile using STR markers. Extraction process focuses on separating the

biological sample from the substrate it is deposited on and recover DNA present inside the cell nucleus. The amount of DNA in the extracted sample is determined in the quantitation step. Since the results from amplification and DNA typing are affected by too little or too much input DNA, it is extremely important to know the amount of DNA present in the sample. Copies of multiple STR markers are generated in the amplification step through polymerase chain reaction. The resulting products are separated by size using capillary electrophoresis (CE). ABI Prism 310 and 3130xl Genetic Analyzers are the instruments most commonly used to perform electrophoretic separations [29]. The number of repeats at each amplified region is determined with the help of an analysis software such as ABI Prism GeneMapper[®] ID software (Applied Biosystems, Foster City, CA). The combination of these repeats in all the amplified regions is referred to as a DNA profile. The DNA profile of the suspect is compared to the profile generated from reference samples. The final laboratory report describes all the evidence analyzed, the method of analysis and the loci that were amplified. The suspect is excluded if DNA profile from the evidence does not match with the reference sample. A match between the known and unknown samples is referred to as an inclusion in which case statistical analysis is done to determine the probability of a randomly selected individual in the same population having an identical genotype at all interpretable STR loci tested. [30]. Sometimes an inconclusive result is obtained because of a situation in which a partial autosomal STR profile is generated as a result of insufficient or degraded samples. Mitochondrial DNA analysis can be employed to provide additional information and discriminating power when insufficient or degraded nuclear DNA is present. Similarly Y-chromosome STR analysis plays a vital role in analyzing mixtures such as sexual assault

samples where the male component may be masked by the overwhelming quantities of female tissue [127].

C.2. Y-chromosome STR analysis

The Y-chromosome is a male-specific sex chromosome that is inherited from the father [5]. The non-recombining portion of the Y-chromosome (NRY), which comprises 95% of the Y-chromosome, remains unchanged unless a variation occurs due to mutation [32]. As a result, Y-STR profile is not as discriminatory as autosomal STR profile and all the paternal relatives exhibit identical Y-STR profile [30]. But STR markers present on the Y-chromosome can be helpful in interpreting mixtures containing large amount of female cells or azospermic or vasectomized individuals because only the male-specific STRs are amplified and the female profile is removed [32].

C.3. Mitochondrial DNA typing

Mitochondrial DNA is present outside the nucleus unlike nuclear DNA that is encased within the nuclear membrane. Mitochondrial DNA is present in all cells but only females pass it on to their offspring because mitochondrial DNA is present in the sperm tails that does not combine with the egg during fertilization. As a result, it cannot be used to distinguish between siblings and relatives from the maternal line [15].

Nuclear DNA has only two copies per cell whereas mitochondrial DNA has multiple copies, which permits DNA recovery from highly degraded samples or samples with undetectable levels of nuclear DNA [5, 105].

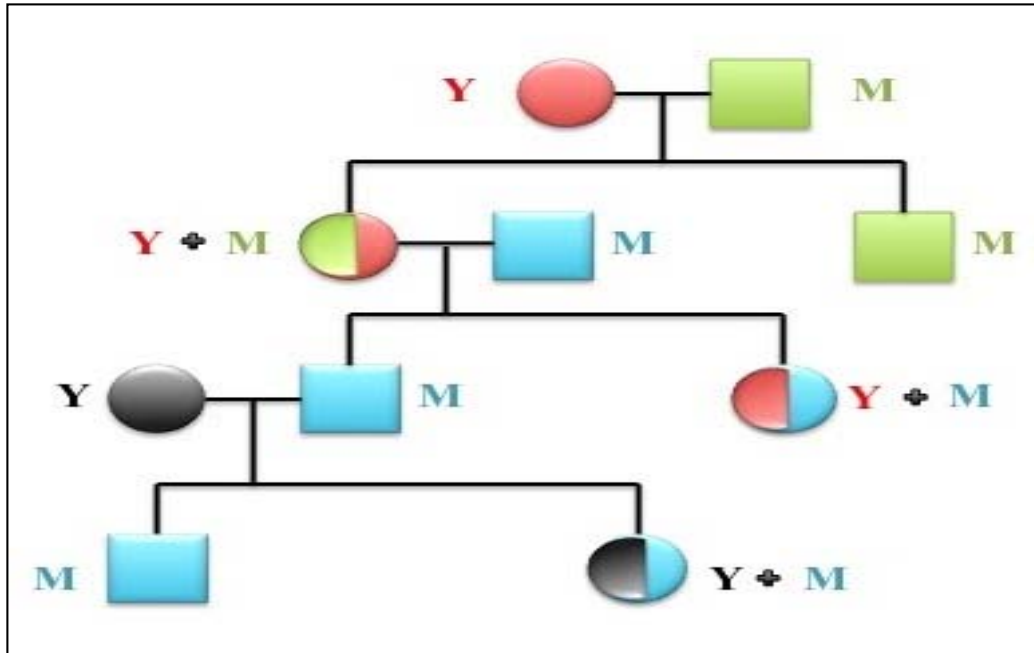


Figure 6. Inheritance pattern of mitochondrial DNA (M) and Y-chromosomal DNA (Y). Mitochondrial DNA is passed from mother to her offspring whereas Y-chromosomal DNA is inherited by the male offspring from their father. Therefore, male child inherits Y-DNA and mitochondrial DNA from father and mother respectively but passes on Y-DNA to his son. A daughter inherits mitochondrial DNA from her mother and passes this on to her son and daughter although only daughter passes this on to her progeny.

Mitochondrial DNA is very useful in the analysis of highly degraded samples or those that have insufficient nuclear material such as hair shafts, old bone samples, and teeth. It also aids in identifying unidentified remains by comparing mitochondrial DNA profile to any maternal relative [127].

CHAPTER II: EVIDENCE COLLECTION AND CHARACTERIZATION

A. Introduction

All the developments in DNA testing, which ensure the ability to generate a profile from even degraded samples, are futile if the evidence is not collected and preserved in a proper manner. The first and crucial step in a crime scene investigation is to thoroughly document the scene and collect the evidence that is not just visible to the naked eye but also examine areas that may lead to clues such as bedspreads with stains that are visible only under an alternate light source. Physical evidence plays an important role in not just convicting a person but also in acquitting the wrongfully accused [75]. Since new DNA techniques such as PCR are extremely sensitive and can replicate even trace amounts of DNA, the introduction of contaminants may interfere with the evidence [93]. A forensic scientist relies on the crime scene investigator to follow proper protocol in collecting evidence and transport it in a manner that ensures no contamination, degradation or loss of evidence. Collection and preservation techniques are contingent on the type of evidence and the substrate upon which it is present.

It is important to collect reference samples from suspects to compare with evidence collected from the crime scene, and from family members to establish kinship or identify victims [120]. The protocol and collection tools may vary between crime labs but the least invasive and most commonly used method to collect reference samples is buccal swab. A cotton swab is used to rub against the inside of an individual's cheek to collect some buccal epithelial cells. The swab is then air-dried and packaged appropriately for storage purposes [94]. It is also very important to collect control samples by collecting an unstained area of the substrate on which the evidence is found. There are some general

guidelines to bear in mind while collecting evidence for DNA testing. First and foremost, air-dry any wet stains because moisture will promote bacterial growth and cause DNA degradation. Storing biological samples in refrigerator at 4°C or in the freezer at -20°C will preserve the evidence for longer periods of time [30]. It is crucial to check for trace evidence because it may be lost during DNA analysis [57, 101]. Presumptive testing is done to give some indication as to the type of stain before proceeding with confirmatory analysis [11, 57, 60]. In keeping with the dissertation topic only evidentiary materials important for DNA testing will be discussed in this module.

B. Biological evidence

B.1. Bloodstains

One of the most commonly encountered pieces of evidence in a violent crime scene, bloodstains not only are a source of DNA but blood spatter patterns can give information on the events that transpired [76]. Red blood cells do not contain DNA but they are helpful in detecting blood because of the presence of hemoglobin. White blood cells are nucleated and are the primary source of DNA material in blood sample [11, 12]. Bloodstains present on small, movable substrates, such as bloodstained clothing can be shipped whole in a paper container after allowing the sample to dry. A sterile absorbent fabric is used to collect a wet stain present on immovable surface and packaged in a paper container [76]. Dried bloodstains are collected by either scraping with a sterile blade or by using fingerprint tape to lift it off from the substrate. When a moistened cotton-tip applicator is used to pick up the dried stain, the sample should be localized on a small area of the swab to ensure optimal recovery during testing. Double-swabbing technique is

also performed routinely where a dry swab is used following sample collection with a moist swab to ensure thorough sample collection [75]

The Hemoglobin (Hb) molecule, found in the red blood cells transports oxygen from the respiratory organs to the rest of the tissues in the body, is detected in most of the presumptive tests for blood. The ABACard[®] HemaTrace kit (Abacus diagnostics Inc., West Hills, CA) comes with test strips containing monoclonal antihuman hemoglobin antibodies which bind with the antigens of human hemoglobin and migrate through the test strip [32]. Antibody-antigen-antibody complexes form upon encountering stationary polyclonal antihuman antibodies, labeled with a pink dye, in the test area. An aggregation of these complexes results in a pink line on the test strip indicating the presence of human blood. Although highly sensitive and specific to human blood, false positives are known to occur with ferret blood and higher primates [75, 76]

Another popular presumptive test for blood is spraying the area with a solution of luminol (5-amino-2, 3-dihydro-1, 4 phthalazinedione) ($C_8H_7N_3O_2$) and hydrogen peroxide (H_2O_2), which exhibits chemiluminescence upon reacting with the iron molecule in hemoglobin. The chemiluminescence is very useful in detecting trace amounts of blood or bloodstains diluted 10 million times, and luminol was shown to not have any detrimental effect on downstream DNA typing [12]. Some of its limitations are that it can give false positives with other compounds containing metal ions such as copper, nickel, chromium and commercial bleach apart from the fact that it can obliterate any blood spatter pattern [12].

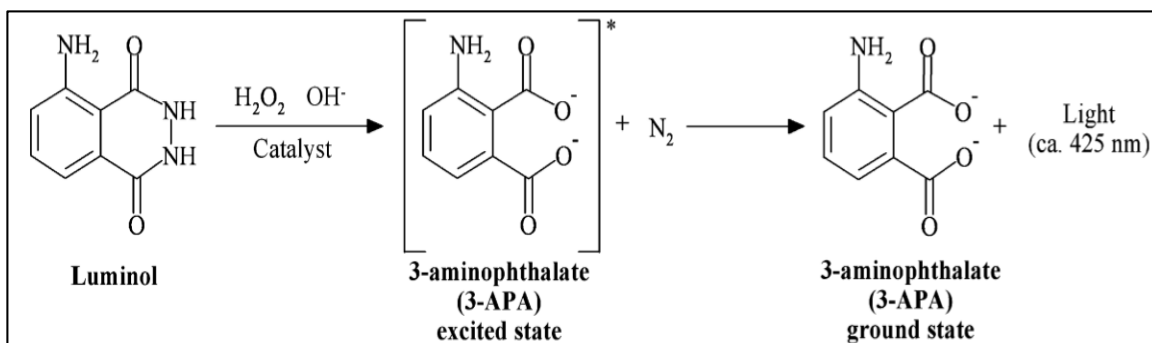


Figure 7. Mechanism of luminol chemiluminescence. In the presence of Hydrogen peroxide, luminol undergoes oxidation to form 3-aminophthalate that is in an excited state. When 3-aminophthalate comes back to the ground state, it emits light, which is the characteristic blue glow of a luminol reaction [12].

B.2. Seminal evidence

The perpetrator in a sexual assault is often male and seminal fluid is a crucial piece of evidence in such cases. It consists of seminal fluid that has enzymes that aid in stain characterization and sperm cells that contain nuclear DNA. Semen stains are most commonly found on victim's clothing, body and bedspread [105]. Semen stains are collected in the same way as blood stains ensuring that the substrate is transported as whole whenever feasible and the stain is allowed to sufficiently air-dry before packaging [124]. Alternate light source (ALS) testing at a wavelength of 450 nm can be used to aid visualization through fluorescence, because semen stains may not be visible to the naked eye upon drying. The investigator may mark this area on the substrate before sending it to the lab [77].

Presumptive testing for the presence of semen relies on the detection of acid phosphatase (AP) enzyme or prostate specific antigen (PSA) also known as p30 because of its molecular weight of 30,000 Daltons [40]. When subjected to the Brentamine spot test, the acid phosphatase enzyme, present in the seminal fluid, and produced by the

prostate gland, gives a dark purple color upon reacting with sodium alpha naphthyl phosphate and diazo blue dye solution [7]. If the substrate is large, a moistened filter paper pressed against it is subjected to the screening test. Since AP is present in low levels in other body fluids such as vaginal secretions, Brentamine spot test is strictly for screening purposes [124]. Confirmatory tests for sperm include direct visualization under a microscope using Christmas tree stain that differentially stains sperm heads and tails. It comprises of nuclear fast red, which stains the nuclei in the sperm heads red and the tails stain green from picroindigocarmine [83]. In situations such as vasectomized or azospermic individuals, identity of semen is confirmed by detecting prostate specific antigen (PSA) or p30, a glycoprotein produced by the prostate gland. Test kits for p30 detection are available from Abacus diagnostics Inc. (West Hills, CA) and they use the same principle as HemaTrace kit for blood detection except that they use anti-p30 antibodies [60].

B.3. Saliva stains

The epithelial cells, from inside the cheek, that are present in the saliva are a rich source of DNA [83]. Saliva stains are difficult to identify with naked eye and requires additional means of visualization like alternate light source (ALS) that causes them to fluoresce [83]. Saliva stains, like all body fluids, are collected in the same manner as blood and semen stains described above [76]. Saliva can be a good source of DNA evidence in cases like sexual assault where there are bite marks on the victim. Other items that need to be examined for saliva stains include drinking containers, cigarette butts, chewing gum, toothbrush, etc. [1].

Presumptive tests for detecting saliva stains are designed to detect the starch-digesting enzyme, amylase, which is present in high concentrations in saliva [69]. Since amylase is present in a variety of other body fluids, presumptive testing may be bypassed if there is limited evidentiary material available for analysis. The Phadebas[®] forensic press test is commonly used to detect saliva stains. The test strip comprises of a filter paper containing starch-dye complex that breaks down upon coming in contact with amylase enzyme and releases the blue dye [57]

B.4. Hair evidence

The hair root contains skin cells called keratinocytes that can be used for nuclear DNA testing. When hair is pulled out in a violent struggle, there is a good chance of finding the root bulb attached which can be used to generate a DNA profile for the source of the hair [84]. When hairs are visible to the naked eye, clean forceps are used to transfer them onto paper that is made into a druggist fold and placed in a paper container. A tape lifting method is employed when noisy or dark backgrounds make it difficult to visualize hairs with the naked eye. A piece of clear adhesive tape is applied to the area of interest, lifted off and affixed to a clear backing card which is packaged in a paper container. Vacuuming is also done to collect hair and fiber evidence in which the evidence is trapped in a filter attached to the vacuum. The evidence is then placed in a druggist fold and packaged in a paper envelope. One of the major limitations of this method is that there is a higher chance of contamination if the filter is not cleaned properly [104, 105].

B.5. Tissue, urine, bone and teeth

Though not as important as body fluids for DNA testing, there are specialized branches of forensic science that collect and analyze samples that can greatly aid an investigation in piecing clues together. Forensic odontology is a highly specialized field where scientists use dental x-rays, teeth impressions, and bite mark analysis in a range of caseworks encompassing identification of victims in mass disasters [91], identification of the source of bite marks [9], and identification of skeletal remains [23]. Likewise, urine analysis can give important information to a forensic toxicologist pertaining to drugs and toxins ingested by the victim or suspect [42]. Forensic anthropologists can gain information from skeletal remains such as signs of trauma, sex and age of the deceased individual that can help a criminal investigation [18].

Following established forensic practices means including proper evidence collection and preservation methods, maintaining a thorough chain of custody, and employing appropriate testing methods. These procedures involve many trained personnel and it is the responsibility of every individual involved to make sure the integrity of the evidence is not compromised.

CHAPTER III: EXTRACTION OF DNA EVIDENCE

A. Introduction

The objective of the extraction methods developed to recover DNA from a field-collected sample is threefold: 1) separate the tissue from the surface that it is deposited on, 2) lyse the cell membrane to release the organelles and 3) obtain a purified DNA extract for downstream genetic testing by removing the cellular debris and other possible inhibitors [39]. Only a small portion of the sample should be extracted and the rest of the material should be stored in a cold and dry environment to ensure that adequate sample is available for possible reanalysis. It is important to run a negative control during any experiment to ensure there is no contamination from the consumables. The negative control should be subjected to the same experimental conditions as evidentiary material. The presence of any detectable DNA is indicative of contamination, and this requires the sample to be re-extracted [50].

B. Extraction methods

B.1. Organic extraction (phenol chloroform extraction)

As the name suggests, organic extraction (phenol chloroform extraction) refers to the isolation of DNA with the aid of organic reagents. It is one of the earliest methods of extraction in which an equal volume of phenol chloroform isoamyl alcohol (25:24:1 v/v) is added to an aqueous solution of the sample [51]. Following centrifugation, the more polar DNA molecules move into the water phase, allowing recovery of a purified extract for downstream analysis.

In the organic extraction method, cell lysis primarily occurs through the addition of a stain extraction buffer containing a detergent such as sodium dodecyl sulfate (SDS), Proteinase K and a reducing agent, Dithiothreitol (DTT). Detergent and Proteinase K lyse the cell membranes and dissolve the proteins surrounding the nuclear material. Dithiothreitol (DTT) reduces disulfide bonds in the cell to further aid the release of DNA material [51, 82]. Following incubation, the substrate is removed and phenol chloroform isoamyl mixture is added to separate DNA from other cellular components. Isoamyl alcohol acts as an anti-foaming agent whereas phenol-chloroform causes partition of hydrolyzed proteins into the organic phase [82]. Ethanol precipitation or centrifugal filters are used to recover DNA from the aqueous phase [35]. Though organic extraction has been successful in isolating DNA, the use of hazardous solvents, multiple processing steps that can introduce contamination and time-consuming protocol make its use less appealing.

B.2. Chelex[®] 100 extraction

Chelex 100 (Bio-Rad Laboratories, Hercules, CA) is an ion-exchange resin composed of styrene divinylbenzene copolymer containing iminodiacetic acid groups. Nucleases, enzymes that cause DNA degradation, require a divalent cofactor such as Magnesium (Mg^{2+}). The iminodiacetate ions bind with the Magnesium cofactor and inactivate nuclease activity thus preventing DNA degradation [61]. For DNA extraction with Chelex resin, the biological sample is boiled in a 5% solution of deionized water and Chelex[®] 100 resin to lyse the cellular structure and isolate the DNA released into the supernatant [138]. Though the method has a short processing time and relatively few

transfer steps which minimizes contamination, the use of extremely high temperatures that can have a detrimental effect on degraded or low level samples is a matter of concern [61]. Moreover, single-stranded DNA isolated using this method may not be as stable as double-stranded DNA for long term storage. Finally, improper washing that may cause resin carryover into the extract can cause PCR inhibition [128, 138]

B.3. FTA™ paper

Fast Technology for Analysis of nucleic acids (FTA) was initially developed by Lee Burgoyne at Flinders University in Australia as a method of collection and storage of biological evidence [28]. The FTA paper is a cellulose-based paper saturated with reagents such as weak base, chelating agent, surfactant, and uric acid that lyse cells and denature proteins, immobilizing the DNA within the paper. If biological evidence collected on FTA paper is properly dried prior to storage, the method offers a great advantage in protecting DNA from environmental and enzymatic elements such as nucleases, ultraviolet radiation and microbes [118]. Samples spotted on FTA paper can be stored at ambient conditions for a long time. Because of their small size, FTA papers are easy to package, ship and do not take up a lot of storage space.

A small disc of the FTA paper is used for analysis after washing the paper to remove unwanted material. Further analysis does not require DNA elution and testing can be performed directly on the paper with DNA entangled in the matrix. There is no need to quantify DNA samples extracted from FTA paper before proceeding with amplification because similar-sized discs are used each time, and this gives consistent results. One major limitation of the method arises from the fact that static electricity may cause

difficulties in retaining the paper punch in the sample well leading to contamination issues or loss of sample [114].

B.4. Solid-phase extractions

In solid-phase extraction, DNA is bound to a solid-phase substance such as silica particles and unbound cellular debris is removed through multiple wash steps and centrifugation. Pure DNA bound to the silica beads is recovered with a final elution step. Recent years have seen the development of many commercially available kits such as QIAamp kits from Qiagen, Inc. (Valencia, CA) and DNA IQ™ system from Promega Corporation (Madison, WI), which are based on the principle of solid-phase extraction [45, 54].

Different QIAamp kits are available for tissue-specific DNA isolation and these mainly differ in their wash buffers. With this kit, DNA binds to the silica beads under acidic conditions and high salt concentration whereas raising the alkalinity of the solution and lowering the salt concentration releases the DNA from the silica particles [54]. A chaotropic salt solution disrupts intramolecular interactions such as hydrogen bonding leading to denaturation of macromolecules, which bind to the silica beads. An initial wash step with water removes any unbound material from the solution. A second wash step is performed with ethanol to remove the chaotropic salts followed by a rinse with water to remove any residual ethanol before eluting DNA from the beads. With increase in alkalinity the beads become more negative and DNA is eluted from the silica substrate [89]. Several robotic platforms such as BioRobot EZ1 workstation (Qiagen, Inc.,

Valencia, CA) and QIAcube system (Qiagen, Inc., Valencia, CA) have further streamlined this extraction process by allowing automation [22].

The DNA IQ™ system (Promega Corporation, Madison, WI) is similar to the Qiagen kit except that silica-coated paramagnetic beads are used to attract DNA. The DNA bound to the magnetic particles is drawn to the side of the tube and immobilized with the help of a magnetized tube rack that enables one-pot isolation as multiple wash steps can be performed in the same tube without disturbing the DNA pellet. After removing the debris present in the solution, the DNA is subjected to more wash steps and released into the elution buffer by heating to 65°C for 5 minutes [45]. One major limitation of this method is that DNA may be lost during the wash steps if any other materials interfere with the magnetic bead binding. A recent study noted the loss of DNA extracts in a major criminal investigation because the chemicals present in a presumptive test for blood prevented DNA from binding to these beads [97].

ChargeSwitch Technology® (CST®, Life Technologies, Grand Island, New York) is one of the recent developments in solid-phase DNA extractions where the surface charge of the solid phase can be changed by modifying the pH of the surrounding buffer solution. Under acidic conditions, the CST® beads have a positive charge which attracts the negatively charged DNA molecules. After removing the cellular debris with a wash step, raising the pH of the buffer solution neutralizes the surface charge on the bead, and this releases the DNA molecules bound to the beads [140]. Since CST method does not employ ethanol, organic solvents, or chaotropic salts there is less chance for carryover of inhibitory elements into the DNA extract. Moreover, minimal transfer steps and complete automation further contain contamination and sample loss.

Fingerprint DNA Finder™ (FDF kit) was recently launched to extract DNA from fingerprints that contain low levels of nucleated DNA [73, 112]. During the extraction process, proteins and other debris from cell lysis are adsorbed onto the solid phase while DNA remains in an unbound state in the solution. The principle behind FDF extraction is a reverse approach to the traditional solid-phase extraction to recover purified DNA extract. The method can be beneficial to recover DNA sample from handguns, postage stamps and other touch samples where there is a higher chance for evidentiary loss due to insufficient sample and multiple processing steps [73].

B.5. Thermostable proteinases

Proteinase K enzyme is most commonly used to hydrolyze proteins and aid in recovery of cellular components through cell lysis [51]. Proteinase K enzyme is isolated from the fungus *Tritirachium album* and is so named due to its ability to digest native keratin (hair) [37]. Proteinase K becomes inactive at a temperature above 55°C and hence requires additional reagents in the form of dithiothreitol (DTT) and sodium dodecyl sulfate (SDS) to lyse cells [112].

A modified DNA extraction procedure using a proteinase extracted from a thermophilic *Bacillus* species EA1 has been used to extract forensic evidence from a wide variety of samples including bloodstains from different substrates, swabs from drinking containers, gloves, socks and food items [86]. The sample is incubated at 75°C for 15 minutes followed by inactivation of the enzyme at 96°C with a 15-minute incubation. Exposure to such high temperature induces cell lysis and hydrolyzes proteins without the addition of other denaturing reagents.

Although DNA was extracted from most of the substrates mentioned above, cigarette butts and bloodstains on black denim failed to produce an interpretable profile, which was attributed to the possible interference from inhibitors [86]. The biggest advantage of the method is there is minimal chance of cross contamination due to lack of multiple transfer steps and the simple protocol makes it amenable for automation [79].

B.6. Alkaline lysis

An alkaline lysis method is often used in plasmid isolation from bacteria and extraction of DNA from plant tissue [16, 130]. Protein unfolding occurs due to ionization of amino acid residues in the presence of strong alkaline conditions resulting in disruption of the cell structure [72]. The alkaline lysis has been expanded to extract DNA from forensic evidence such as bloodstains and semen stains [64].

Alkaline lysis employs only two reagents, an alkaline solution such as sodium hydroxide, and a neutralization buffer. It requires less than ten minutes to lyse the tissue. The method does not give a pure DNA extract and hence should be followed up with a purification step such as phenol chloroform extraction. Alkaline lysis can be used as a one-step extraction procedure only when the sample contains cells from a single source. Because of the harsh conditions that can eventually denature the DNA molecule, it is important to optimize the exposure time to alkaline pH [64].

B.7. Differential extraction

Introduced by Peter Gill and coworkers in 1985, organic differential extraction is one of the most popular methods to separate different sources of DNA such as a mixture

of male and female cells commonly encountered in a rape kit [51]. Separating the male fraction from the female profile makes it easier to interpret the suspect's DNA profile [109, 110].

The principle behind the differential extraction protocol is based on the differences in DNA packaging in sperm and other tissues. Protamines are sperm nuclear proteins that are rich in arginine and contain cysteine residues that form disulfide bonds allowing for a denser chromatin structure [92]. The release of sperm nuclear DNA relies on the disruption of these disulfide bonds that require harsher lysis conditions compared to other cells.

Organic differential extraction is a two-step process. In the first step of digestion, vaginal epithelial cells in the mixture are lysed with Proteinase K/ Sodium Dodecyl Sulfate (SDS) solution and the sperm cells remaining in the solution are collected by centrifugation. In the second step of digestion, the sperm cells are lysed with a buffer containing Proteinase K, SDS and Dithiothreitol (DTT) as reducing agent (Figure 8). DTT is used to target the cross-linked thiol-rich proteins in the sperm nuclear membrane that makes them more resistant to organic extraction than epithelial cells in the first step of the digestion [49, 51]. Both fractions are purified separately with phenol/chloroform/isoamyl alcohol. The differential extraction protocol is employed to achieve a complete separation of two different cell types present in the mixture, and to obtain a clean male DNA profile that is not obscured by the female DNA.

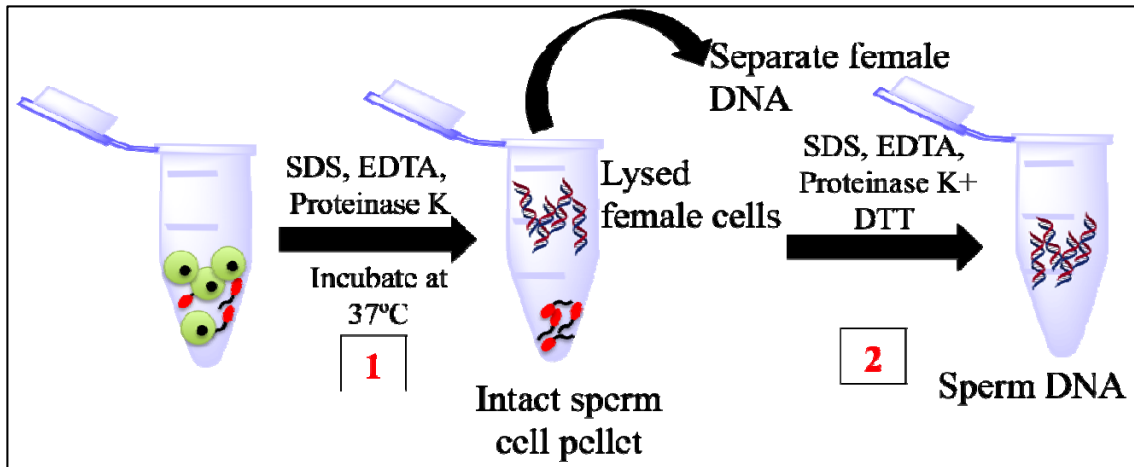


Figure 8. Organic differential extraction 1) Incubation of the mixture in a cell lysis buffer containing detergent and Proteinase K results in epithelial cell lysis. The lysed female DNA is separated from the sperm pellet through centrifugation 2) Dithiothreitol (DTT) is added in the second step to lyse the sperm pellet and recover male DNA.

Differential extraction and its modified versions are still used today to separate different cell fractions in a mixture [53, 90, 125]. Although a gold standard for mixture analysis, this method is time-consuming, technique-dependent, difficult to automate, and can result in relatively inefficient separations of female DNA from the male component [34, 64, 92]. Moreover, if the perpetrator is vasectomized or is azoospermic, differential extraction does not aid in mixture resolution due to the lack of sperm cells [32].

B.8. Laser microdissection

Laser microdissection (LM) or laser-capture microdissection (LCM) is used to selectively recover cells of interest from a sample with the aid of microscopic visualization. The LCM method has been used to successfully isolate sperm cells in sexual assault evidence [43, 107]. In order to isolate the cells, the sample is spread over a microscope slide and a thin plastic film is placed on top of it. When the cells of interest appear in the field of view an infrared laser is switched on to cut out the cells and the

plastic film captures them at that specific location. The excision process is repeated by moving the slide to capture all the target cells, which are then subjected to extraction procedures to isolate DNA. The method has raised concerns over contamination issues due to adherence of the cells to the plastic, and the possibility of heat degradation of the sample [88]. With the introduction of an ultraviolet (UV) light source during laser microdissection, the sample is spread on a polymer slide that is excised at the site of target sample and collected into a tube without coming into contact with other paraphernalia that can introduce contamination [121].

Laser capture microdissection has proved to be more effective than differential extraction in recovering sperm DNA from mixtures [43]. The biggest disadvantage is that laser capture of cells is not cost-effective and the downstream extraction protocol should involve fewer processing steps to conserve the low sample volume.

Nucleic acid extraction is one of the most challenging and critical steps in sample processing and continuous advancements in the field prove that there is still room to improve the extraction procedure which requires a method that is rapid, reliable, easy to use, cost-effective and gives high yields.

CHAPTER IV. AMPLIFICATION: POLYMERASE CHAIN REACTION (PCR)

The description of polymerase chain reaction in 1985 by Kary Mullis has opened new doors in evidence analysis. By generating multiple identical copies of a particular DNA sequence in a test tube with simple reagents, it is possible to obtain a viable DNA profile even with trace amounts of sample. Polymerase chain reaction (PCR) is used for identifying genetic disorders, DNA fingerprinting, paternity testing, genetic engineering, and to study evolution. Kary Mullis was awarded Nobel Prize in 1993 for his significant contribution [32].

A. PCR process

Polymerase chain reaction (PCR) involves three basic steps that are repeated for 30 to 40 cycles. Since the target DNA is duplicated, approximately double the amount of DNA is present at the end of each cycle if the reaction proceeds at 100% efficiency [32]. The three stages of a PCR reaction, denaturation, annealing, and extension, occur at different temperatures. The PCR reaction is carried out in a thermal cycler, an automated instrument used to rapidly heat and cool the reaction mixture. As the name suggests, the first step involves denaturation of the double-stranded DNA molecule by heating the mixture to more than 90° C. The high temperature breaks the hydrogen bonds between the nucleotides of the two DNA strands, resulting in two single-stranded DNA molecules. During the annealing step of PCR, temperature is lowered to 40-60° C to allow binding of primers to the complementary sequence on the template DNA and target the DNA to be amplified. Annealing temperature depends on the melting temperature of the primers. The annealing step prepares the reaction for multiplication in the third step by identifying

the region to be amplified. The temperature is raised to approximately 72° C during extension, the third and final step of PCR. The DNA polymerase enzyme activity is optimal at this temperature, and it extends the primers annealed to the DNA strands through the addition of deoxynucleotide triphosphates (dNTPs) in a manner complementary to the target DNA sequence [38, 39] (Figure 9).

At the end of one cycle, two single-stranded DNA molecules are amplified to form two double-stranded DNA molecules and this is repeated with every cycle resulting in an exponential accumulation of the template. The number of copies at the end of a reaction is approximately 2^n where n is the number of amplification cycles. As a result, a 32-cycle reaction will give rise to approximately a billion copies of the original template ($2^{32} = 1,073,741,842$) [32].

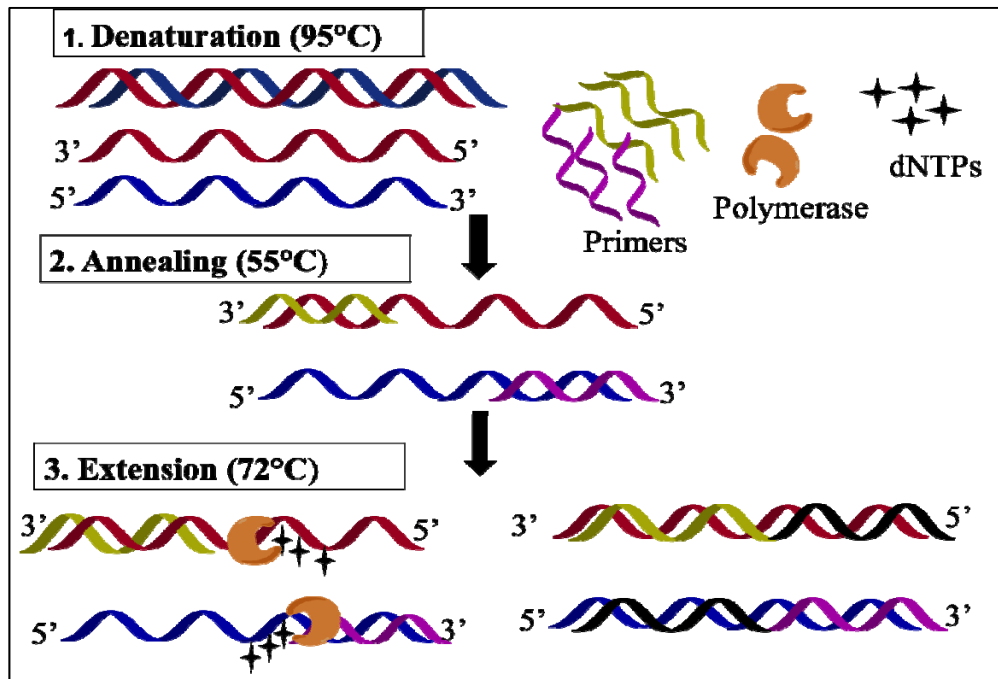


Figure 9. An overview of the PCR process. The target DNA sequence is heat-denatured in the first step to form single-strand DNA molecules. During annealing, primers are added to each of these DNA strands by complementary base pairing. DNA polymerase binds to the primers and adds nucleotides to extend and amplify the template molecule during the final step of the PCR cycle.

B. PCR components

A PCR reaction requires just a few simple components to drive the reaction forward. These components are added together to achieve optimal final concentrations and the rest of the volume is made up with deionized water. Low reaction volumes can lead to pipetting inaccuracies and sample evaporation, while excess volume may require a longer cycling time in order to achieve thermal equilibrium [38]. The PCR components include template DNA, DNA polymerase, primer pair, buffer and deoxynucleotide triphosphates (dNTPs) containing the four bases- adenine (A), guanine (G), thymine (T) and cytosine (C).

The highly sensitive PCR technology will amplify any DNA extracted from other sources of contamination apart from the target sequence. Therefore it is important to have a high quality, uncontaminated template. It is important to amplify an optimal amount of DNA to avoid artifacts in DNA typing such as allelic drop out and drop in, associated with either too little or an overload of sample respectively [31].

The location for PCR amplification on the template DNA is identified by primers, which are short DNA sequences that are complementary to the target DNA. Since the primers bind to the template strand through complementary base pairing, some prior knowledge of the DNA sequence that needs to be amplified is required [45]. Another important consideration during primer design is the prevention of the formation of secondary structures such as hairpins and primer dimers. The secondary structures will reduce amplification efficiency by hindering the annealing of primers to the template strand [122].

Polymerase enzymes bind to the primer sequence and extend the primer strand through the addition of nucleotide molecules present in the reaction mix. Initial PCR studies used the Klenow fragment, a portion of a polymerase derived from *E. coli*, to amplify the target DNA sequence [87]. Because the Klenow fragment was inactivated during the denaturation step of PCR, the reaction mix required enzyme replenishment at the end of every temperature cycle which proved to be labor-intensive until the discovery of the thermophilic bacteria, *Thermus aquaticus*. The *Taq* polymerase derived from this bacterium is stable at 95°C, the denaturation temperature that enabled the automation of the PCR process [65]. Processivity, fidelity and persistence are the three important aspects of a polymerase enzyme. Processivity refers to the rate at which it copies a DNA strand, fidelity is the ability to incorporate the correct nucleotides and persistence indicates the stability of the enzyme at high temperatures. The effect of low fidelity of *Taq* polymerase has negligible effect on PCR amplification because of the short amplicon size. Hence *Taq* polymerase still enjoys immense popularity in PCR applications [62].

A mixture containing equal concentrations of the four deoxynucleotide triphosphates (dNTP) is added to the PCR reaction to supply the basic building blocks required for DNA synthesis. These are incorporated into the DNA strand by polymerase enzyme [65].

Buffer solution is optimized for the polymerase being used. It maintains a stable environment to achieve optimum activity. Some of the buffers contain MgCl₂ that provides the Magnesium cofactor required by DNA polymerases. Dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), and glycerol are some of the additives that are commonly encountered in a PCR setup [81]. These chemicals are believed to aid in

driving the reaction forward by relaxing the secondary structure of the DNA strand, increasing the processivity of an enzyme, and preventing non-specific attachment of the polymerase enzyme [38].

Table 1. A list of typical PCR components [20]

Reagent	Concentration
Tris-HCl, pH 8.3	10 mmol/L to 50 mmol/L
Magnesium chloride	1.2 mmol/L to 2.5 mmol/L
Potassium chloride	50 mmol/L
Deoxynucleotide triphosphates (dNTPs)	0.2 mmol/L each
DNA polymerase	0.5-5 U
Bovine serum albumin (BSA)	100 µg/mL
Primers	0.1 µmol/L to 1.0 µmol/L
Template DNA	1 ng to 10 ng

C. Reaction controls

Three types of controls- positive controls, reagent blanks and negative controls, are used to ensure that no other variables are affecting the results. The positive control is used to assess whether all the reaction components and parameters are working effectively. It consists of a known DNA sequence of good quality that is added to the reaction mix. The DNA sequence in the positive control will amplify if all the PCR reaction proceeds as planned and all reagents are input properly. A negative control is used to rule out cross-contamination between samples, from personnel handling the samples, from unsterilized consumables, and from contaminated reagents. A negative

control contains all the components of a reaction mix except for the template DNA. The presence of amplified peaks in the negative control indicates the presence of contaminating DNA. An extraction control that does not have any template DNA is recommended to further rule out the presence of any exogenous DNA in the sample that was recovered during the extraction step. This control is subjected to all the steps involved in DNA extraction followed by PCR amplification and should not produce any amplified DNA if the reaction is not compromised [65].

D. Thermal cyclers

Thermal cyclers are the instruments used to rapidly heat and cool the reaction during the PCR process. Thermal cyclers mostly differ in the number of samples that can be amplified at once, the size of the reaction tube and the time taken to reach the target temperature. The different parameters that can be adjusted on a thermal cycler are temperature at each step of the amplification process, number of amplification of cycles and ramp speeds.

One of the first thermal cyclers required the addition of a drop of mineral oil on top of the sample to prevent condensation during the reaction. Heated lids were introduced in the newer models, which prevents condensation in the sample tube [65]. GeneAmp[®] PCR system 9700 (Applied Biosystems, Foster city, CA) is an example of a commonly used thermal cycler (Figure 10). The GeneAmp[®] PCR system 9700 system is fully programmable, has a maximum ramp speed of 4°C per second and can process up to 96 samples simultaneously. Newer models of thermal cyclers have been designed to reduce the experimental time which is achieved through increasing the heating and

cooling rates, installing thermal blocks containing special coating and providing specialized tubes that permit faster heat transfer to the sample [2].



Figure 10. GeneAmp[®] PCR system 9700 thermal cycler Source: Life Technologies

E. PCR inhibitors

As a consequence of the nature of evidence encountered at a crime scene, there is potential for many elements to interfere with the quality of any analysis. The effect of inhibitors on PCR and the measures to overcome it has been the subject of many studies [39, 46, 93]. Inhibitors may be introduced into the evidence during sample collection. For example, hemoglobin in blood, humic acid found in soil, and collagen in the bone, are all known to interfere with PCR amplification. Improper handling of evidence and inefficient pre-amplification steps can also introduce inhibitors into the sample [32, 138]. For example, phenol in organic extraction is notorious for interfering with post-extraction analysis. Presence of trace phenol from improper wash steps can inhibit DNA amplification and result in loss of evidence [39].

Inhibitors usually interfere with the PCR process by directly interacting with the DNA molecule or by targeting the DNA polymerase enzyme. Among the various measures employed to avoid PCR inhibition, the most effective approach is to avoid co-extraction of the inhibitor with DNA [4]. For example, when stains are present on denim jeans, swabbing the area may be more beneficial than processing the whole cutting, because of the inhibitory effects of indigo dye present in denim. Alternatively, the DNA sample may be diluted in order to reduce the inhibitor concentration [4, 32]. Another approach is to add additional DNA polymerase enzyme so that enough polymerase is available for PCR reaction after the inhibitor molecules bind some of it [32]. The analysis of internal positive control (IPC) amplification and PCR efficiency following real-time PCR analysis are excellent tools to monitor inhibitors [93, 119]

F. Real-time PCR

Real-time PCR, when applied to DNA quantification, is useful because instead of only determining the amount of DNA in a sample, this method can also predict how the DNA will respond during subsequent PCR analysis conditions. In addition to determining the quantity of human DNA present, real-time PCR techniques can also be used to detect the presence of inhibitors of the PCR reaction by analyzing amplification efficiency [95]. Determining the amount of DNA present in the sample aids in optimizing the input DNA levels during STR analysis. Newer quantification kits offer the ability to determine the total amount of human DNA and male-specific DNA in a sample at the same time. Quantifying the amount of male specific DNA in a sample is especially useful for Y-STR analysis in samples overwhelmed by female DNA.

Real-time PCR monitors the amplification process and detects the fluorescence signals as the reaction progresses. The amount of DNA amplified is detected through accumulation of fluorescent signals with increasing amount of DNA product or through fluorescence quenching where the fluorescent probe is displaced with product amplification. There are three stages in real-time PCR that define this process- the exponential phase, the linear phase and the plateau phase (Figure 11). If the reaction is proceeding at 100% efficiency, amount of DNA will double at the end of every cycle during the exponential phase. In the linear phase, the exact doubling of the copies will not occur and the reaction starts to slow down as a result of crowding and depletion of the reagents [38, 32]. The reaction finally plateaus off in the final phase after the reaction components are used up. The point at which the fluorescence intensity exceeds the background noise is called the threshold and cycle threshold (Ct) is the cycle at which the reaction reaches this point.

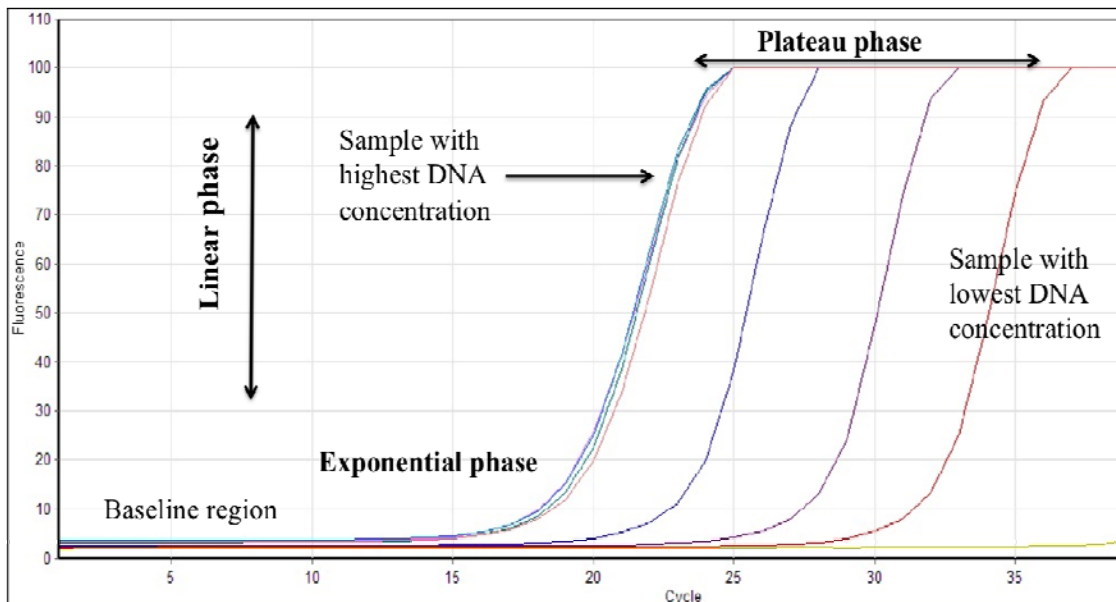


Figure 11. An overview of real-time PCR process. The initial phase is the exponential phase where a large amount of reagents move the reaction forward and plateau phase occurs upon depletion of the reagents.

Real-time PCR analysis makes use of different chemistries to perform fluorescence-based quantification [38]. The DNA binding dyes bind to a double-stranded DNA molecule and emit fluorescence. The amount of fluorescence increases with increasing amount of DNA. The dye Sybr® green I is a very popular intercalating dye that is widely used in real-time PCR analysis [50]. Since these dyes are not sequence-specific and can bind to any double-stranded DNA, they can be used for a wide variety of assays. At the same time, this very feature is a disadvantage because the dye suffers from lack of specificity and cannot be used for multiplex reactions [139].

Hydrolysis probes are sequence-specific and the fluorescence intensity is proportional to the amount of DNA in the reaction. TaqMan® probes are the most popular hydrolysis probes for real-time PCR. TaqMan® probes rely on 5'-3' exonuclease activity of Taq polymerase enzyme to release fluorescence quenchers [62]. Sybr green emits fluorescence upon hybridization whereas TaqMan® probes emit fluorescence upon hydrolysis [80]. Fluorescence is quenched through fluorescence resonance energy transfer (FRET) when the fluorophore present on 5' end is in close proximity to the quencher present on the 3' end of the probe. Upon binding to the complementary sequence on the template DNA, polymerase enzyme hydrolyzes the probe and releases the fluorophore resulting in fluorescence [58]. Quantifiler® Duo (ABI, Foster City, CA) is the most popular kit that uses TaqMan® probes.

The Plexor® HY system (Promega Corporation, Wisconsin, MA) system detects DNA amplification on the basis of fluorescence quenching. It is capable of simultaneous determination of both autosomal DNA and human male-specific (Y) DNA concentration.

It also has an internal positive control (IPC) that detects any inhibition in the samples.

Plexor chemistry utilizes two modified bases that are complementary to each other— isoguanine (iso-dG) and 5'-methylisocytosine (iso-dC). One of the two primers contains iso-dC and is labeled with a fluorescent tag on the 5' end. When the deoxynucleotides mixture containing iso-dG modified with the quencher molecule, Dabcyl, is added to the reaction, Dabcyl-iso-dG gets incorporated on the opposite side of iso-dC nucleotide and this causes fluorescent quenching. As a result, fluorescence signal decreases with increase in DNA concentration (Figure 12).

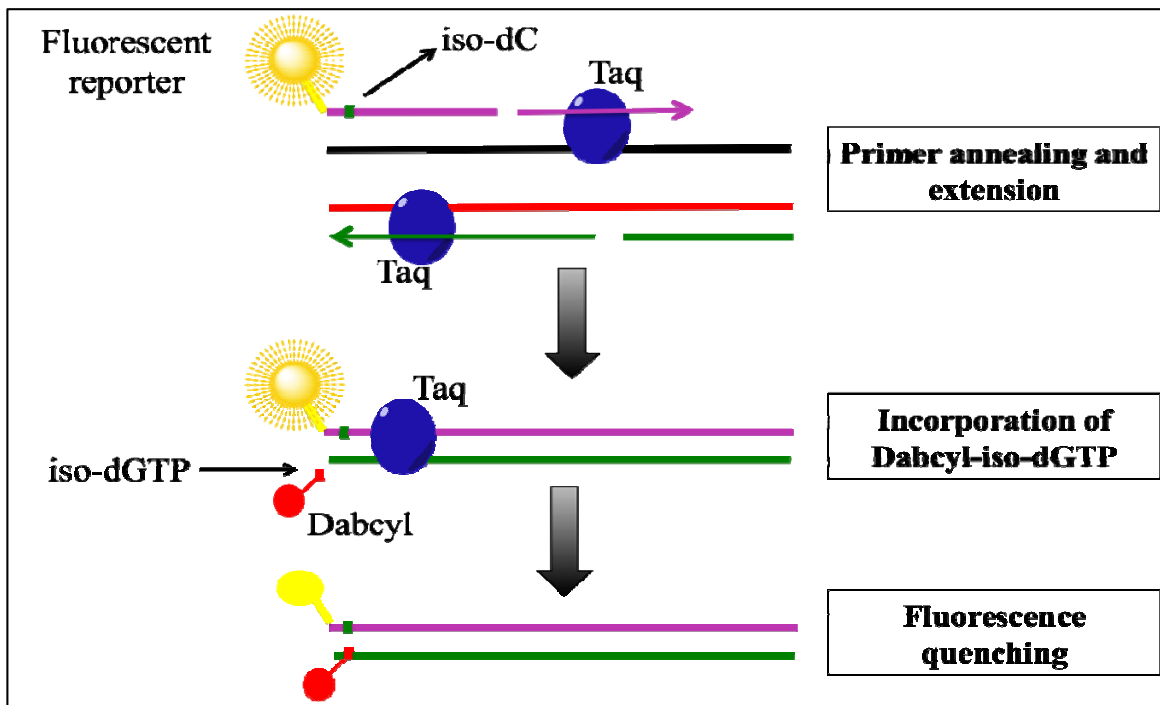


Figure 12. Schematic representation of the Plexor[®] real-time PCR process. Fluorescence quenching occurs when the modified nucleotide containing the quencher molecule is incorporated opposite the complementary nucleotide tagged with the fluorescent reporter. As a result, a decrease in fluorescence signal is observed as the amplification takes place. Adapted from Promega Corporation website

Real-time PCR is highly sensitive and detects very low quantities of DNA. For example, Plexor[®] HY system can detect DNA levels down to 6.4 pg/ μ L. High

throughput, rapid analysis time, and minimal sample handling are some of the biggest advantages of real-time PCR. Multiple probes with different fluorescent labels can be used to perform multiplexing [74].

CHAPTER V. SHORT TANDEM REPEATS (STR)

A. STR: Basics

Microsatellites or short tandem repeats (STR) are a type of variable number tandem repeat consisting of repetitive sequences of DNA that are 2-7 base pairs long. Because of their small size, there are several advantages that make STR testing the method of choice for DNA profiling in today's DNA labs [19]. More than 20,000 microsatellites have been discovered in the last 20 years suggesting their potential usefulness. Preferential amplification is less of a problem because both the copies, one inherited from each parent, are similar in size. Secondly, forensic samples are often degraded or contaminated with more than one contributor, which calls for a method that is highly discriminatory and can amplify even small, degraded targets [32]. Lastly, their small size makes it easier to distinguish them from other amplified loci present during electrophoresis thereby simplifying the analysis and interpretation of the data [103].

Primers can be designed to amplify the target microsatellites by using the sequence of the conserved regions flanking the microsatellite and designing complementary primer sequences to bind to those regions. The number of nucleotides present in one repeat unit defines an STR locus. For example, a tetranucleotide repeat such as (AGAT)_n has four nucleotides in each repeat unit. There are different types of STRs determined by the repeat unit pattern. Simple repeats contain repeat units of equal length and sequence, compound repeats have more than one simple repeat whereas complex repeats have units of variable length and sequence. Some regions of STR have incomplete alleles and these are called microvariants. For example, the HUMTH01 locus has an AATG

tetranucleotide repeats but one of its alleles has 9 copies of the tetranucleotide repeat and the last copy has only 3 nucleotides thus giving it the designation allele 9.3 [32]

Tetranucleotide repeats are commonly used in forensic testing because they produce lower percentage of stutter products compared to smaller repeats. Stutter products have one repeat unit more or less than the true allele because of slippage of template strand from the primer sequence [129]. It is also easier to resolve tetranucleotide repeats that are four base pairs apart than shorter repeat units by electrophoresis. Currently, 13 core tetranucleotide STR loci have been established by the FBI for identification of individuals through the national database, CODIS [25, 103]. Apart from these autosomal STR loci, The Scientific Working Group for DNA Analysis Methods (SWGDM) has established 11 core Y-STR loci [32]. These aid in identification of male contributors in sexual assault cases, in which the female component overwhelms the male cells. They also help identify more than one male contributor to the sample. Though not as discriminatory as autosomal loci, these can be used to determine paternal lineage or identify victims in mass disasters by establishing match with the male relatives [95, 127].

B. Commercially available STR kits

It is time-consuming and expensive to design PCR primers and validate a multiplex kit. Moreover, using a commercially available kit provides a standardized platform that allows for data sharing with other jurisdictions. These kits contain all the components necessary for amplification and separation of STR markers. This includes primers, allelic ladder, and master mix containing polymerase, buffer and dNTPs.

The first commercially available STR kit was developed in 1994 and was able to amplify three loci simultaneously, CSF1PO, TPOX and TH01. By 1997, 13 core STR loci were chosen to be included in the CODIS national database, which made a random match probability rare (1 in a trillion) [32]. The loci included were TPOX, D3S1358, FGA, CSF1PO, D5S818, D7S820, D8S1179, TH01, VWA, D13S317, D16S539, D18S51, D21S11 and AMEL. Out of these 13 loci, FGA, D18S51, and D21S11 offer the greatest amount of variation between individuals whereas TPOX, CSF1PO, and TH01 are the least polymorphic [32, 103].

TPOX, CSF1PO, D5S818, D13S317, D16S539 are simple repeats where all the repeats are of identical length and sequence. TH01, D18S51, D7S820 are simple repeats containing microvariants and the rest of the loci are compound repeats with non-consensus alleles except for D21S11 which is a complex motif containing repeats of variable length or sequence [32].

At the European Network of Forensic Science Institute (ENFSI) meeting five additional loci were included in European STR kit that also contains seven STR loci from European Standard Set (ESS). These twelve STR markers are TH01, vWA, FGA, D8S1179, D18S51, D21S11, D3S1358, D12S391, D1S1656, D2S441, D10S1248, and D22S1045.

To improve database sharing with other countries and improve discrimination of the multiplex kits, the FBI formed the CODIS Core Loci Working Group that published a to expand the CODIS STR loci set used in the United States and many other countries. D12S391, D1S1656, D2S441, D10S1249, D2S1338, D19S433, Penta E and male-specific DYS391 were the additional loci suggested to expand this core set [56].

C. Allelic ladders

Allelic ladders consist of all the alleles that can be present at a particular locus. They are constructed by co-amplifying PCR products from various individuals in a population such that all possible variations are represented at that particular locus. It is important to use the same primers as those used for testing unknown samples so that the alleles in the unknown sample can align accurately with the ladder, which allows for more reliable genotyping [73]. Data analysis software helps in allele designation by comparing the size of the fragments of unknown alleles to the size of the alleles in the ladder. If an allele has the same color fluorescent label and is within half a base pair of the ladder, it is designated as being that allele [31].

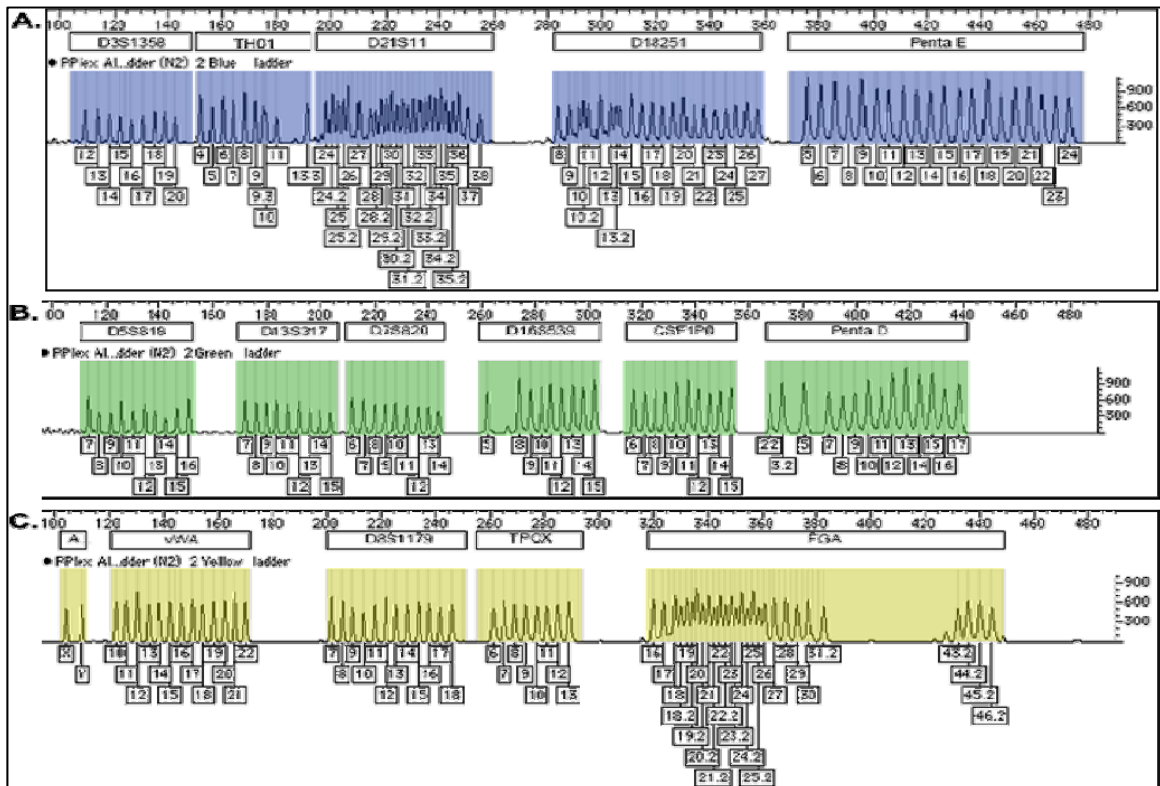


Figure 13. An electropherogram of PowerPlex® 16 ladder showing all the alleles at all loci [20]

Each manufacturer of STR kits provides allelic ladders for accurate genotyping. Allelic ladder can be obtained by diluting and re-amplifying the original ladder with the same set of primers. Allelic ladder is also important to adjust for any differences such as mobility shifts that arise from different instruments and variations in environmental conditions [30].

CHAPTER VI. CAPILLARY ELECTROPHORESIS: INSTRUMENTATION AND ANALYSIS

A. Introduction

Multiplex amplification reactions produce multiple fragments that are labeled with different fluorescent tags and are variable in length. Capillary electrophoresis (CE) is the preferred method of choice to separate these fragments that are then analyzed by fluorescence detection. Capillary electrophoresis (CE) can also be used in other forensic applications such as in drug analysis, gun shot residue analysis and explosive analysis. The instrument comprises of a glass capillary, two buffer vials and two electrodes connected to a power supply.

The silica capillary is very thin with an internal diameter of 50 μm and 36 cm or 50 cm long. Capillaries as long as 80 cm are used when higher resolution is required. The capillary is filled with a viscous polymer medium, which separates the DNA fragments on the basis of their size. The larger fragments move slowly through the sieve-like matrix compared to the smaller fragments. The negatively charged DNA fragments move towards the positive electrode upon applying voltage. Runs are faster with CE than slab gel electrophoresis because of the application of higher electric fields to the sample. A laser present near the end of the capillary illuminates the DNA fragments as they pass the laser window. The fluorescence emitted by the dyes attached to the DNA fragments is plotted as relative fluorescence intensity as they pass the detector.

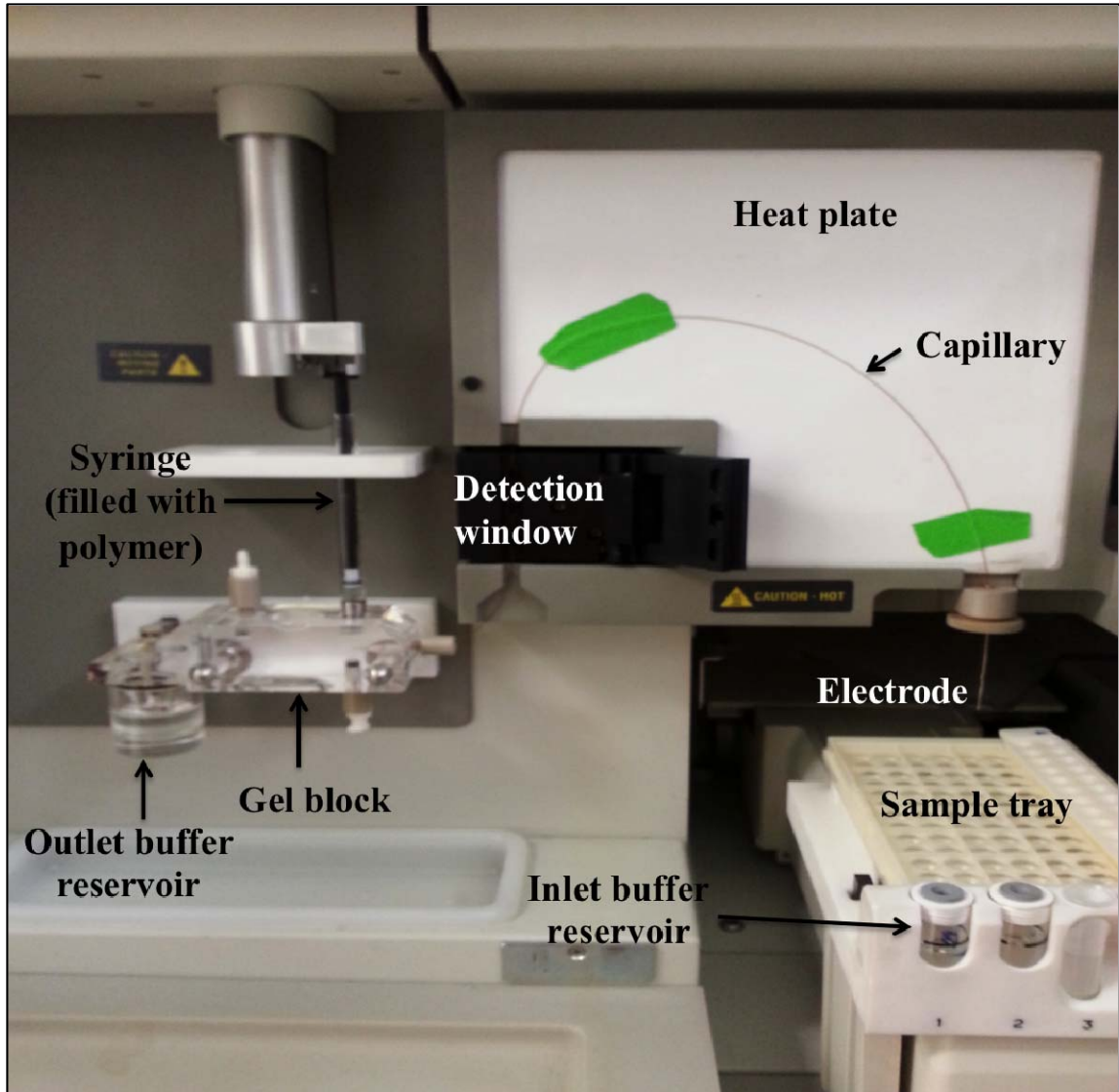


Figure 14. ABI PRISM® 310 Genetic Analyzer is used for fragment separation using capillary electrophoresis. This instrument has a mobile sample tray that presents sample during injection. Under the application of electric field, negatively charged DNA molecules move through the polymer-filled capillary towards the anode. A laser in the detection window activates the fluorescent end-labels and these fluorescence signals are collected and converted into peaks for data interpretation. Adapted from Life Technologies.

B. Electrokinetic injection

The most popular way to introduce the DNA sample into the capillary is known as electrokinetic injection. When the capillary is immersed into the sample tube and a voltage is applied, the negatively charged DNA molecules are pulled into the medium

upon application of a positive charge. The amount of DNA that enters a CE column is dependent on the electric field applied (E), the injection time (t), electrophoretic mobility (μ_{EP}), electroosmotic flow (μ_{EOF}) the concentration of DNA in the sample, the area of the capillary opening (πr^2), the ionic strength of the sample (λ_{sample}) and the ionic strength of the buffer (λ_{buffer}) (Equation 1) [32, 102].

Equation 1. DNA injection

$$[DNA_{injected}] = \frac{Et(\pi r^2)(\mu_{EP} + \mu_{EOF})[DNA_{sample}](\lambda_{buffer})}{\lambda_{sample}}$$

As indicated by the equation, modifying the injection time and voltage can control the amount of sample injected. Since the amount of DNA injected is inversely proportional to the ionic strength of the sample, presence of competing ions such as chloride ions (Cl⁻) will result in poor injection. When a sample of low conductivity is introduced into a highly conductive environment, a phenomenon called sample stacking occurs in which the analyte is concentrated in the form of a band at the interface between the low conductive and high conductive environments, which improves the injection sensitivity [32].

Under the influence of an applied voltage, the rate at which the ionic species migrate towards an electrode is directly proportional to the electric field (E) and electrophoretic mobility (μ_{EP}). The following equation for electrophoretic mobility indicates that the mobility of the ions is affected by charge-to-size ratio where net charge (q) is directly proportional and the radius of the ion (r) is inversely proportional to the

mobility. The electrophoretic mobility increases with a decrease in the frictional forces (f) in the buffer that is directly related to the viscosity of the medium (η) and the size of the analyte. (Equation 2) [134]

Equation 2. Electrophoretic mobility

$$\mu_{EP} = \frac{q}{f} = \frac{q}{6\pi\eta r}$$

C. Sample preparation

The PCR products are mixed with deionized formamide to denature the double-stranded DNA molecules and reduce sample conductivity. Formamide of low conductivity must be used because formamide degradation produces negatively charged by-products that can compete with the DNA molecules, and may be preferentially injected into the CE system [26]. The conductivity of the formamide should be below 100 $\mu\text{S}/\text{cm}$ for optimum performance [32]. Water can also be used instead of formamide followed by heat denaturation at 95°C and snap cooling to 4°C to denature the DNA molecule but there is a possibility of the DNA strands reannealing [14].

D. Sample separation

Once voltage is applied, DNA fragments migrate through the capillary where smaller fragments move faster compared to the larger fragments. There are three important variables that affect the efficiency of sample separation in a CE system- capillary, sieving matrix and buffer.

D.1. Capillary

Joule heating refers to the heat that is generated when electric current flows through another resistive medium. The separation efficiency will be affected due to temperature gradients across the capillary if the capillary walls do not dissipate this heat efficiently [134]. Most of the CE systems in forensics use a fused silica capillary that is 47 cm long with an internal diameter of 50 μm . Fused silica capillaries allow for efficient heat dissipation and the small internal diameter allows application of high electric fields and enables fast separations. Most of the capillaries have a polyimide coating that offer durability. A small optical window is created by burning a small portion of this coating in order to facilitate laser excitation and fluorescence detection of the fragments as they pass the detection window [32, 142].

Electroosmotic flow (EOF) will affect the separation when uncoated silica capillaries are used. The hydroxyl groups on the silanol molecules undergo ionization at a pH above 5 and these negative ions attract the positive ions from the buffer solution. An electrical double layer is created with the inner layer remaining stationary and the outer, more mobile layer starts moving towards the negative electrode upon application of electric field. [32]. The EOF can be suppressed by coating the inner walls of the capillary with the polymers in the matrix.

D.2. Buffer

Buffer composition plays a critical role on the efficiency of sample separation. The components of a buffer are directly responsible for the ionic strength, denaturing

capability, solubility of the DNA and solution pH, which affect the quality of the injection. EOF is highly sensitive to the pH of the buffer solution.

Equation 3. Electroosmotic flow (EOF)

$$\mu_{EOF} = \frac{\epsilon \zeta}{4\pi\eta} E$$

where ϵ is the buffer's dielectric constant, η is the viscosity of the buffer, E is the field strength and ζ is the zeta potential at the interface of the capillary and the buffer solution. The pH of the buffer plays a critical role in the degree of ionization of the silanol groups. At a high pH, the silanol groups on the capillary walls become fully ionized resulting in strong zeta potential and increased electroosmotic mobility. The ionic strength of the buffer shares an inverse relationship with the rate of electroosmotic flow. Therefore, increasing the buffer concentration aids in containing the EOF [102, 132].

The most commonly used buffer for CE separations in forensic DNA analysis is 100mM 3-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]propane-1-sulfonic acid (TAPS), 1mM ethylenediaminetetraacetic acid (EDTA) adjusted to a pH of 8 with sodium hydroxide [31, 32]. Poor resolution will occur if the buffer concentration is too high or too low causing excessive heating or long separation times respectively. Since CE separations are carried out at a temperature of 60°C, the buffer components should be stable at high temperatures.

D.3. Polymer

The polymer in the capillary acts as a sieve for the migrating DNA molecules. There are two popular models that describe the movement of DNA molecules through the

matrix- Ogston sieving and reptation. Ogston sieving predicts that DNA moves as a spherical molecule through a polymer solution as spherical molecules with a radius of gyration. The fragments having radius of gyration smaller than the pore size move freely whereas the movement of molecules with a radius of gyration larger than the pore size is hindered. Reptation theory predicts that the large DNA fragments unwind and migrate through the pores in a snake like manner [19, 30]. Polymer solutions containing 4% or 6% dimethyl polyacrylamide and urea are routinely used in CE separations. The role of urea is to ensure that the DNA molecule remains in its denatured state. The 4% polymer is used for routine STR typing whereas the 6% polymer is used in DNA sequencing which requires a higher resolution [32].

E. Fluorescence detection

During the amplification process, primers labeled with fluorescent tags bind to the DNA strands. The fluorescence from these tags is detected as the fragments pass the detection window. Argon-ion laser at 488nm is commonly used to excite multiple fluorescence dyes. The emission spectrum is recorded using a charge coupled device (CCD) camera. Spectral calibration is performed using matrix standards that allow peak distribution into their respective lanes [30].

F. Data interpretation

An internal lane standard (ILS) contains known DNA fragment sizes and this is used to determine the size of the alleles using either a local southern method or global southern method fit [32]. These two methods differ in that the local method uses an

interpolation method by using two size peaks before and after the unknown whereas the latter uses all size data available. Genemapper® ID software (Applied Biosystems, Valencia, CA) is used to perform data analysis including allele designation. Once the size of the alleles is determined, the user creates virtual bins that define the allele size range. Alleles are called by comparing them to the alleles of known sizes in the ladder [111]. The DNA profile thus generated is compared to known suspects or uploaded into the national database for a match.

CHAPTER VII. PRESSURE CYCLING TECHNOLOGY

A. Introduction

Pressure cycling technology sample preparation system (PCT SPS) is a novel method that employs cycles of hydrostatic pressure between ambient and high levels to induce mechanical stress on cells resulting in compromised cellular integrity [12, 37]. Cell lysis and efficient recovery of the cellular components dictates the success of downstream applications. Pressure cycling technology (PCT) has been used successfully in a variety of applications in molecular biology. Smejkal et al. successfully extracted protein from *Escherichia coli* using PCT [74]. It has also been used in the extraction of biomolecules such as DNA and RNA from animal and plant tissues, insects, and microbes [31] (Table 2). The advantage of pressure cycling is its ability to produce a highly efficient extraction and disruption of cell nuclei from a wide variety of substrates.

Most of the extraction methods in forensic biology rely on a combination of physical and chemical extraction protocols to achieve maximum yields. Achieving maximum DNA yields from a sample is especially challenging because the evidence collected from a crime scene is mostly present on a substrate, and recovery from the substrate is inefficient when the material is an absorbent. Long incubation times, contamination issues, health hazards from the use of chemicals and the combination of treatments that are too harsh on the biomolecules of interest are some of the biggest disadvantages associated with the current extraction methods. Pressure cycling technology sample preparation system (PCT SPS) was developed to rapidly and

efficiently release cellular contents from biological samples using alternate cycles of high and ambient pressure in a safe and controlled environment.

Table 2. A list of different kinds of tissues processed using PCT for various downstream applications [77]

Sample group	Lysed material	Extracted biomolecules	Downstream applications
Animal tissue	<u>Soft</u> - liver, brain, pancreas, spleen, kidney, lung	DNA, RNA, Proteins	PCR, RT-PCR, ELISA, SDS-PAGE, Western blot
	<u>Hard</u> - tail, heart, intestine, skeletal muscle	DNA, RNA	PCR, RT-PCR
Plant tissue	<u>Soft</u> - corn sprouts, leaves, grape skin	DNA, RNA, Proteins	PCR, RT-PCR, SDS-PAGE
	<u>Hard</u> - stem, pine needle, grape seed	DNA	PCR
Insects, small organisms	Mosquito, fruit fly, mealworm, tick	DNA, RNA	PCR, RT-PCR
Microbes	Yeast, <i>C. elegans</i>	DNA, RNA, Proteins	PCR, RT-PCR, SDS-PAGE
	mycobacteria, bacteria/ spores, soil	DNA, RNA	PCR

B. Instrumentation

B.1. Barocycler

The Barocycler™ NEP2320 (Pressure BioSciences Inc., Boston, MA) is a commercially available instrument that is equipped with a hydrostatic pressure chamber that can withstand high pressures up to 45000 psi. Both solid tissue and liquid samples can be processed using this instrument offering versatility. The Barocycler is a lightweight, portable instrument that can easily fit on a bench top. It comes equipped with

a microprocessor and a keypad that is used to input different user-controlled variables such as pressure and number of cycles. The instrument is connected to a compressor that creates high pressure. The Barocycler™ NEP2320 is a smaller version compared to NEP3229 that can hold three PULSE™ tubes [37].

B.2. PULSE™ tubes (Pressure Used to Lyse Samples for Extraction)

PULSE tubes are single-use tubes that are specially designed to withstand high pressures generated to cause cell lysis. There are two types of PULSE tubes- FT500 comes with an integrated perforated lysis disk, which is used for processing tissue samples that require some homogenization whereas the FT500-ND is not equipped with this lysis disk and is more suited for processing liquid samples.

The FT500 PULSE tubes are open-ended on both sides and are assembled by placing the sample on the lysis disk and sealing with ram on one end. Ram is made up of polypropylene and is responsible for transmitting pressure from the chamber to the sample. Lysis buffer is added to the fluid retention chamber that lies on the other side of the lysis disk and is sealed with a screw cap. A PULSE tube thus assembled is placed into the pressure chamber and subjected to cycles of ambient and high pressure. When there is a difference in pressure between the chamber and the contents of the tube, the ram moves up and pushed the sample through the lysis disk. The ram recedes when the pressure is released and this action causes the sample, along with lysis buffer, to be pulled through the disk. This process is repeated with multiple cycles of high and ambient pressure resulting in tissue homogenization and weak biomolecular interactions that ultimately lead to cell lysis.

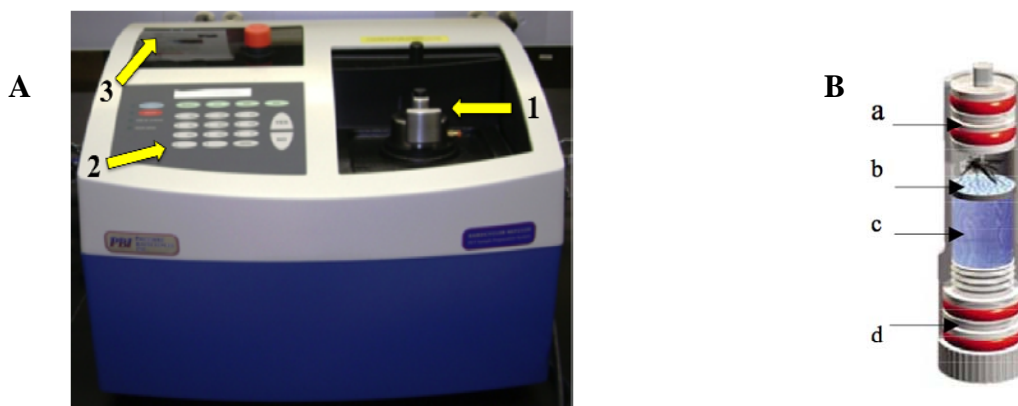


Figure 15. A. Barocycler[®] NEP 2320 1) Pressure chamber in which sample is suspended 2) Keypad to input pressure, number of cycles and holding time 3) Temperature and pressure is displayed on the screen during the treatment. B. PULSE tube (FT500) a. Ram b. Lysis disk c. Fluid retention chamber d. Screw cap [116]

B.3. PCT MicroTubes and MicroCaps

Up to 48 samples can be processed in a barocycler when working with sample volumes less than 150 μL . This can be achieved by switching to PCT MicroTubes, which are designed for use with smaller sample volumes. PCT MicroCaps are designed for use with MicroTubes and three kinds of caps, designated 50, 100, and 150, are available depending on the final sample volume. MicroCaps are designed to form a tight seal and prevent air bubble formation that may lead to foaming and sample loss. They have an indentation that can be used to excise material from slab gels and transfer it to the sample tube for PCT processing. The PCT MicroTube cartridge is designed to hold the MicroTubes in the pressure chamber. These tubes are constructed with a fluoropolymer, fluorinated ethylene propylene (FEP) that can withstand high temperatures and has a non-absorbent surface that allows for complete sample recovery. PCT MicroCaps are made from polytetrafluoroethylene (PTFE) that can maintain integrity under extreme pressure changes.

B.4. User-controlled variables

There are four main parameters that can be varied to achieve an optimum environment for cellular disruption. The applied pressure, exposure time, and number of cycles can all be adjusted using a programmable interface. Furthermore, connecting an external circulating water bath can control the temperature of the pressure chamber.

The Barocycler can withstand pressures up to 45000 pounds per square inch (psi) and samples can be exposed to target high pressure or ambient pressure anywhere from 1 to 99 seconds. Cycling between high pressure and ambient pressure can be repeated up to 99 times. The microprocessor is capable of saving up to 99 user-defined protocols [47, 55, 77].

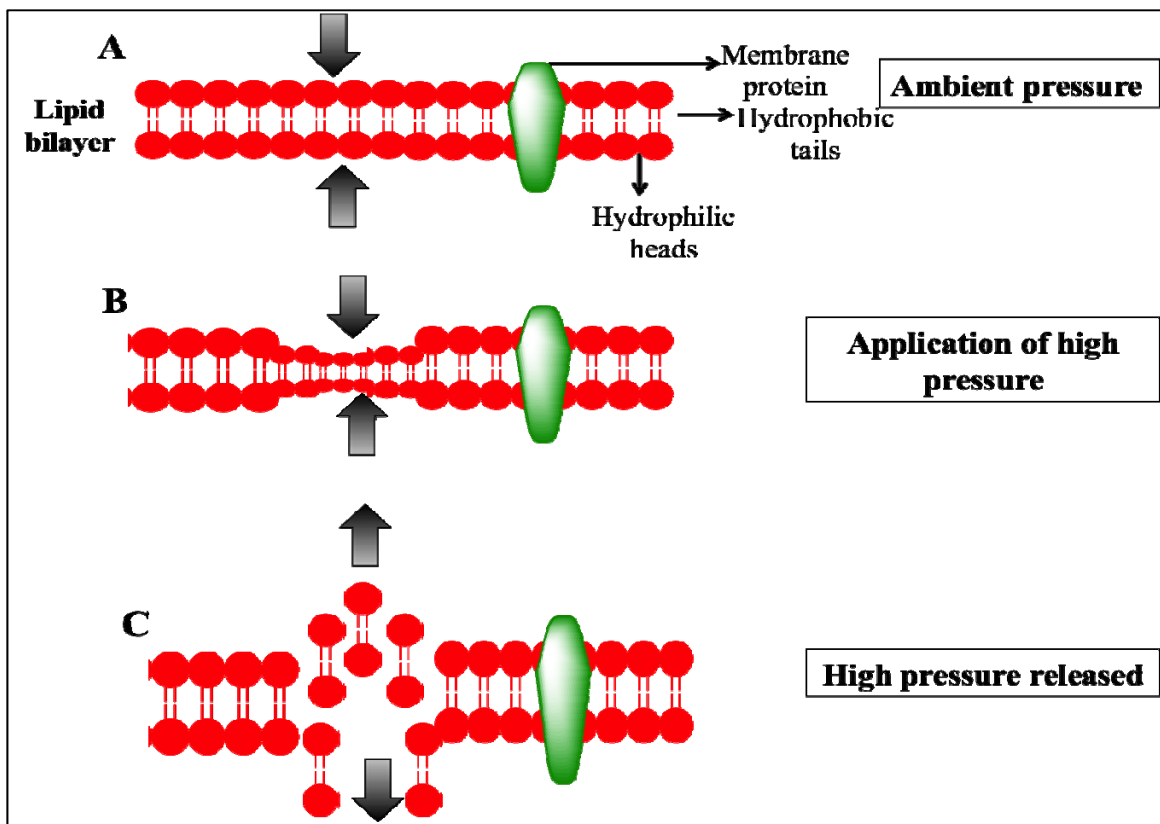


Figure 16. Mechanism of PCT extraction. Rapid cycles of high and ambient pressures cause disruption of lipid bilayer and cell lysis. Adapted from Gross et al. [55].

C. Mechanism

Application of alternating cycles of hydrostatic pressure between ambient and high levels causes disruption of biomolecular interactions. When high pressure is applied, the lipid bilayer is compressed and release of this pressure results in destabilization of the cell membrane, which leads to compromised cellular integrity and subsequent release of cellular components. (Figure 16)

D. Advantages and limitations

The Barocycler is easy to use, requires minimal training, semi-automated and can be used along with commercially available extraction kits to further increase the yields. Minimal sample handling and a closed pressure chamber limit contamination and exposure to harmful reagents. One of the biggest limitations to pressure cycling is low sample throughput when using the large PULSE™ tubes because Barocycler NEP 2320 is capable of processing only one sample at a time.

CHAPTER VIII. THE ROLE OF PRESSURE CYCLING TECHNOLOGY IN DIFFERENTIAL EXTRACTION

A. Introduction

Separating the sources of DNA from different contributors to a stain reduces the difficulty associated with mixture analysis and data interpretation. This is especially important in sexual assault cases where the samples often consist of mixtures of body fluids from the victim and suspect. The processing and interpretation of such mixed DNA samples has long been recognized as a bottleneck in forensic DNA analysis [63, 126]. The examination of physical evidence submitted in such cases can be tedious and time-consuming. As a result, subsequent DNA analysis and interpretation can be challenging especially if the evidence left behind by a male suspect is overwhelmed by the female component.

Organic differential extraction is one of the most popular methods to separate different sources of DNA encountered in a rape kit [51]. Although modified versions of this method are still being used in crime laboratories around the world, several limitations such as inefficient recoveries, incomplete separation of male and female cells and time-consuming protocols have led to numerous studies to achieve better separation. Chen et al. employed nylon mesh filters to physically separate smaller sperm cells from the much larger epithelial cells [34]. The method using nylon filters was not applied to dried stains, and the authors suggested that there is a possibility for folded cells to pass through the filters. Flow cytometry studies have demonstrated that the sperm cells can be isolated using flow cytometry when the cells are tagged by immuno-staining and other nuclear staining material [109, 110]. Another new approach to achieve differential recovery is

isolation of single sperm cells by laser microdissection [43]. The LCM method involves staining the cells and using a laser beam fitted with an optical microscope to cut out the cells of interest from smears on microscopic slides. The microdissection technique has not been tested for mixtures with overwhelming female component or rehydrated old stains that might not be effectively stained. More recently microchip-based sorting of non-lysed cells has been employed to separate the sperm cells and epithelial cells followed by DNA extraction from each cell type [63]. The study did not address the use of old and dried seminal material on different substrates routinely encountered in sexual assault cases. The presence of other cell types in mixtures, which might impede the flow of cells down the microchannels, was also not explored.

Among the commercial efforts to improve the processing of such samples includes the Differex™ System, a commercial kit for differential extraction available from Promega, which involves the use of a special separation solution and the use of a spin basket. The principle behind this kit is similar to the organic differential extraction where the procedure begins with a Proteinase K digestion to lyse non-sperm cells. The separation solution effectively separates the sperm from any soluble DNA and cell debris in the sample. The DNA IQ™ Lysis Buffer containing DTT is next added to the epithelial and sperm fractions. This buffer effectively lyses the sperm without the need for further Proteinase K digestion. DNA can then be purified from each fraction using a magnetic separation system which can be automated, helping to reduce sample processing time and avoid the use of organic solvents [45]. Though systems such as these have made it easier to handle sexual assault evidence, they still require a fairly complex two-step extraction procedure to obtain a clean male DNA fraction.

Up to this point, the application of pressure cycling technology in the lysis of sperm cells or epithelial cells has not been studied. The present thesis was aimed at evaluating the effect of pressure on the differential recovery of sperm and vaginal epithelial cells resulting from sexual assault. The effect of different parameters and the buffer compositions were studied to determine the optimal conditions for cell lysis. The variables that produced differential lysis for one cell type over another were further explored to determine the potential of this application in processing mixtures and separating fractions of sperm and epithelial cells. The overall goal of this study is to develop a reliable and efficient method to selectively recover sperm DNA from sexual assault evidence using pressure-based extraction.

B. Materials and Methods

B.1. Sample preparation

Sperm cells and vaginal epithelial cells were collected from volunteers according to the protocol approved by the institutional review board (IRB) of Florida International University. Semen sample was collected by masturbation and ejaculation into a sterile collection tube. The sample was allowed to liquefy at room temperature before diluting it with 1X PBS buffer (pH 7.5) (Fisher Scientific, Fair Lawn, NJ) to obtain a final concentration of $1-1.5 \times 10^6$ cells/mL. Vaginal swabs collected from healthy female volunteers were incubated in 1X PBS buffer with gentle agitation for 2 hours on Adams Nutator (Clay Adams, Parsipanny, NJ). Following incubation, the swab was transferred to a spin basket and the samples were subjected to micro centrifugation at 13,000 rpm for

10 minutes to pellet the epithelial cells. The pellet was washed and diluted in 1X PBS buffer to a density of $1-1.5 \times 10^6$ cells/mL.

B.2. Cell count

Cell density (cells/mL) was measured using Neubauer-improved disposable C-chip hemocytometers (INCYTO, Fisher Scientific, Fair Lawn, NJ). The hemocytometer has a large grid that is divided into nine squares with each square having a surface area of 1 mm^2 and a depth of 0.1 mm thus representing a total volume of 10^{-4} cm^3 . Since 1 cm^3 is equivalent to approximately 1 mL , the product of average cell count per square and 10^4 gives the final concentration in cells/mL (Figure 17). Cell count in the four large corner squares was performed after injecting $10 \text{ }\mu\text{L}$ sample into the injection port and visualizing it under $40\times$ magnification with a light microscope (Micromaster Model E, Fisher Scientific, NJ, USA) [51].

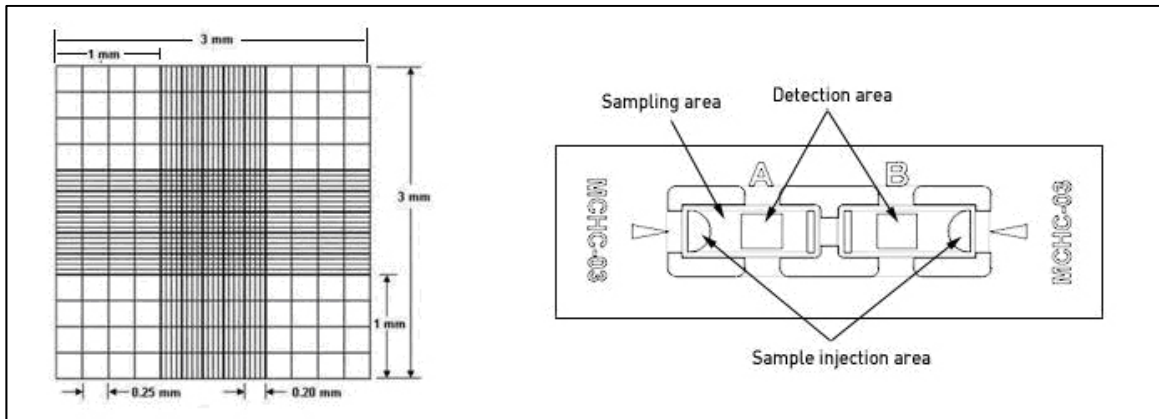


Figure 17. INCYTO C-Chip disposable hemocytometer used for cell count. Sample is injected into the sampling area and the large grid present in the detection area is used to count the number of cells present in the sample [70]

B.3. Cell staining

A common dye-exclusion method involving trypan blue 0.4% solution (MP Biomedicals, LLC, Solon, OH) was used to determine cell viability in samples subjected to pressure treatment. The cell suspension was mixed with an equal volume of trypan blue 0.4% solution and incubated for 2-3 minutes at room temperature [6]. A drop of this mixture was placed on a clean microscope slide and covered with a coverslip before visualization with a light microscope to perform a simple, preliminary determination of cell lysis.

B.4. Pressure cycling technology sample preparation system (PCT SPS)

A Barocycler[®] NEP 2320 (Pressure BioSciences Inc., South Easton, MA) was used to perform pressure cycling between ambient and high pressure to induce cell lysis by exposing the sample to rapid pressure changes. PULSE tubes and MicroTubes are single-use sample containers that effectively transmit this hydrostatic pressure to the sample resulting in pressure-induced cell lysis and the subsequent release of biomolecules. The effect of different pressure parameters on sperm and epithelial cells was studied by transferring a known volume of sperm cells or epithelial cells suspended in 1X PBS buffer into MicroTubes or PULSE tubes and exposing the samples to pressures ranging from 10,000 psi to 45,000 psi using 5-60 pressure pulses at room temperature. The temperature of the pressure chamber was also raised to 60°C by connecting it to a Endocal RTE-110 water circulator (Neslab Instruments, Inc., Newington, NH) to determine the effect of pressure treatment at increased temperature on DNA recovery from sperm cells and vaginal epithelial cells.

For mixture studies, sperm cells and epithelial cells were added to a sterile cotton swab (Puritan Medical Products Co., Guilford, ME) in the ratio of 1:4 and air dried for 1 hour at room temperature. The swab cutting was placed in a PULSE tube and 1X PBS buffer was added to completely submerge the substrate. The PULSE tube was then transferred to the pressure chamber and exposed to 60 alternating cycles of ambient pressure and high pressure at 45000 psi with each cycle lasting 30 seconds. After pressure treatment, the sample was transferred into a 2 mL micro centrifuge tube and the swab was transferred to a spin basket (DNA IQ, Promega corp., Madison, WI) placed in the same tube followed by centrifugation at 13000 rpm for 10 minutes. The swab was discarded along with the spin basket and the DNA was purified using phenol chloroform isoamyl alcohol (25:24:1 v/v).

B.5. Study of buffer composition

Tween-20 (Sigma-Aldrich, St. Louis, MO), Dodecyl sulfate sodium salt, 99% (SDS) (Acros Organics, Fair Lawn, NJ) or N-Lauroyl sarcosine sodium salt (Sarkosyl) (MP Biomedicals) was added to the sample buffer to a final concentration of 2% v/v or 2% w/v. A stock solution of 0.5 M Tris (2-carboxyethyl) phosphine (TCEP) (Gold Biotechnology, St.Louis, MO) was prepared by adding 1.4 g of TCEP to 10 mL of HPLC grade water (Fisher Scientific, Fair Lawn, NJ) and this solution was added to the sample buffer to a final concentration of 10 mM, 20 mM, 30 mM, 40 mM and 50 mM TCEP. A stock solution of 1M Dithiothreitol (DTT) (Promega Corp., Madison, WI) was prepared by adding 1.54 g of DTT to 10 mL of water and this was added to the buffer to a final concentration of 20 mM, 40 mM, 60 mM, 80 mM and 100 mM DTT. A stock solution of

500 µg/mL of cellulase enzyme derived from *Aspergillus niger* (TCI America, Portland, OR) was prepared by adding 5 mg to 10 mL of water and this was added to the buffer to a final concentration of 10 µg/mL, 50 µg/mL and 100 µg/mL.

B.6. Calculating DNA recovery efficiency

Since pressure cycle technology has not been previously applied to rape kits, it was necessary to determine the efficiency of this treatment on DNA recovery. For comparison of relative efficiency, standard organic extraction was performed to determine the amount of DNA in each cell type [31]. The quantity of DNA recovered from pressure treatment was plotted as percent recovery of DNA obtained from organic extraction. Organic extraction was performed by incubating the samples in stain extraction buffer (10 mM Tris, 100mM NaCl, 10 mM EDTA, 2% SDS, 39 mM DTT) and proteinase K (20 mg/ml) at 56°C for 2-4 hours followed by phenol-chloroform-isoamyl alcohol purification and ethanol precipitation.

B.7. Nucleic acid purification

An equal volume of phenol chloroform isoamyl alcohol (25:24:1 v/v) (Sigma-Aldrich, St. Louis, MO) and 80 µL of 3 M sodium acetate solution were added to the extracted samples. After vortexing for 10 seconds, the samples were centrifuged at 13000 rpm for 10 minutes and the aqueous layer was transferred to a 1.5 mL micro centrifuge tube. Double the volume of absolute ethanol and 40 µL of 3 M sodium acetate solution was added to the aqueous phase followed by gentle agitation and overnight incubation at 4°C (or 1-2 hour incubation at -20°C). The samples were centrifuged at 13000 rpm for 10

minutes and the DNA pellet was washed with 1 mL of 70% ethanol. After air-drying, the pellet was resuspended in water or 1X Tis-EDTA (TE) buffer (Sigma-Aldrich, St.Louis, MO) and incubated in a water bath at 56°C for 15 minutes.

B.8. DNA quantification

Amplification and quantitation of DNA extracted with pressure cycling technology was performed to establish a correlation between the pressure treatment and the amount of DNA recovered. Plexor® HY system (Promega Corp., Madison, WI) was used for simultaneous quantification of total human DNA and male human DNA. The Plexor HY® standard curve was made from a dilution series of male genomic DNA standard provided by the manufacturer. Sample preparation was performed according to the manufacturer's protocols using 10 µL Plexor® HY 2X Master Mix, 7 µL amplification-grade water, 1 µL Plexor® HY 20X Primer/IPC Mix and 2 µL DNA sample to make a total reaction volume of 20 µL. The samples were amplified on Rotor-Gene 6000 (Corbett, Australia) using the following conditions: initial hold for 2 min at 95°C followed by 40 cycles at 95 °C for 10 s and 60 °C for 45 s.

B.9. Short tandem repeat (STR) analysis

The quality of the DNA recovered from pressure treatment was assessed by performing STR analysis using PowerPlex® 16 HS system (Promega Corp., Madison, WI) according to the manufacturer's protocol. The PowerPlex® 16 HS System co-amplifies sixteen loci including Amelogenin marker. Three different colored fluorescent labels are used for detection. Fluorescein-labeled primers are used for the detecting Penta

E, D18S51, D21S11, TH01 and D3S1358 loci; primers for FGA, TPOX, D8S1179, vWA and Amelogenin loci are labeled with carboxy- tetramethylrhodamine (TMR); and one primer for each of the Penta D, CSF1PO, D16S539, D7S820, D13S317 and D5S818 loci is labeled with 6-carboxy-4',5'-dichloro- 2',7'-dimethoxy-fluorescein (JOE).

Autosomal quantification data from real-time PCR analysis was used to normalize DNA input into PowerPlex[®] 16 HS reactions where a total of 1 ng of DNA, or less for samples with lower yields, was amplified using GeneAmp[®] 9700 thermal cycler. After amplification, samples were prepared for STR analysis by adding 1 µL of sample to a mixture of 9.5 µL of Hi-Di[™] formamide (Applied Biosystems, Foster city, CA) and 0.5 µL of Internal Lane Standard 600 (Promega Corp., Madison, WI). Separation and detection of STR amplification products were performed using an ABI Prism[™] 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Initial fragment sizing and allele calling was performed using GeneMapper[®] ID v.3.2 (Applied Biosystems, Foster City, CA). The percentage of male DNA was calculated using relative fluorescence units (RFU) as $[(2 \times Y \text{ rfu}) / (X \text{ rfu} + Y \text{ rfu})] \times 100\%$ and the percentage of female DNA was calculated as $[(X \text{ rfu} - Y \text{ rfu}) / (X \text{ rfu} + Y \text{ rfu})] \times 100\%$ [13].

C. Results and Discussion

C.1. Microscopic examination

Trypan blue dye exclusion assay was used to verify if cells are undergoing lysis when exposed to pressure treatment. This assay is based on the ability of viable cells with intact cell membrane to exclude this dye, compared to a compromised cell membrane that readily allows for this dye to leak into the cytoplasm of the cell [6]. Our results indicate

that upon interaction with trypan blue stain both vaginal epithelial cells and sperm cells, exposed to 60 cycles of pressure at 35000 psi, appeared blue due to a compromised cell membrane thus indicating that they are vulnerable to pressure-induced lysis (Figure 18).

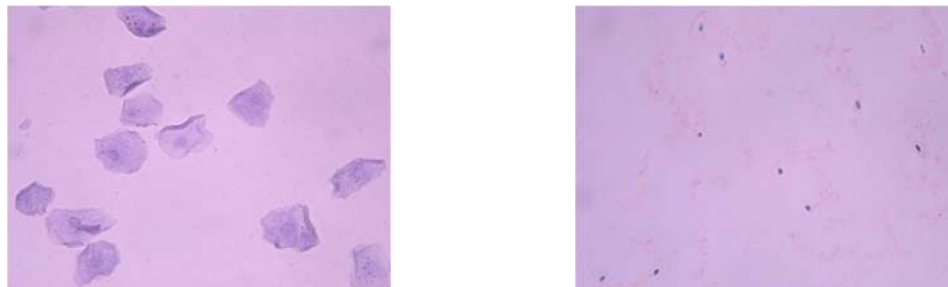


Figure 18. Trypan blue staining of vaginal epithelial cells (left) and sperm cells (right) treated with pressure cycling at 35000 psi resulted in the uptake of dye by the cells indicating a compromised cell membrane.

C.2. Determination of optimum pressure parameters

In the initial studies, individual cell types were subjected to pressure treatment under varying conditions of pressure and number of cycles to determine the optimum parameters to facilitate differential digestion and high recovery. To perform the pressure experiments, samples were suspended in 1X PBS buffer and subjected to pressure pulses from 5000 to 45000 using 5-60 total pulses. No significant improvement in DNA recovery was observed with more than 60 pressure cycles. When operated at a 60-cycle pulse rate, optimal recovery of DNA from sperm cells was observed at a pressure of 20,000 psi while epithelial cells produced their best recovery at 25,000 psi. Best selectivity was observed at 15000 psi with three times more recovery of epithelial DNA. Overall extraction efficiency was very low with only $5\% \pm 0.6$ of epithelial DNA and $3\% \pm 0.5$ of sperm DNA recovered with 60 cycles of pressure at 45000 psi, which necessitates the application of simultaneous chemical digestion (Figure 19).

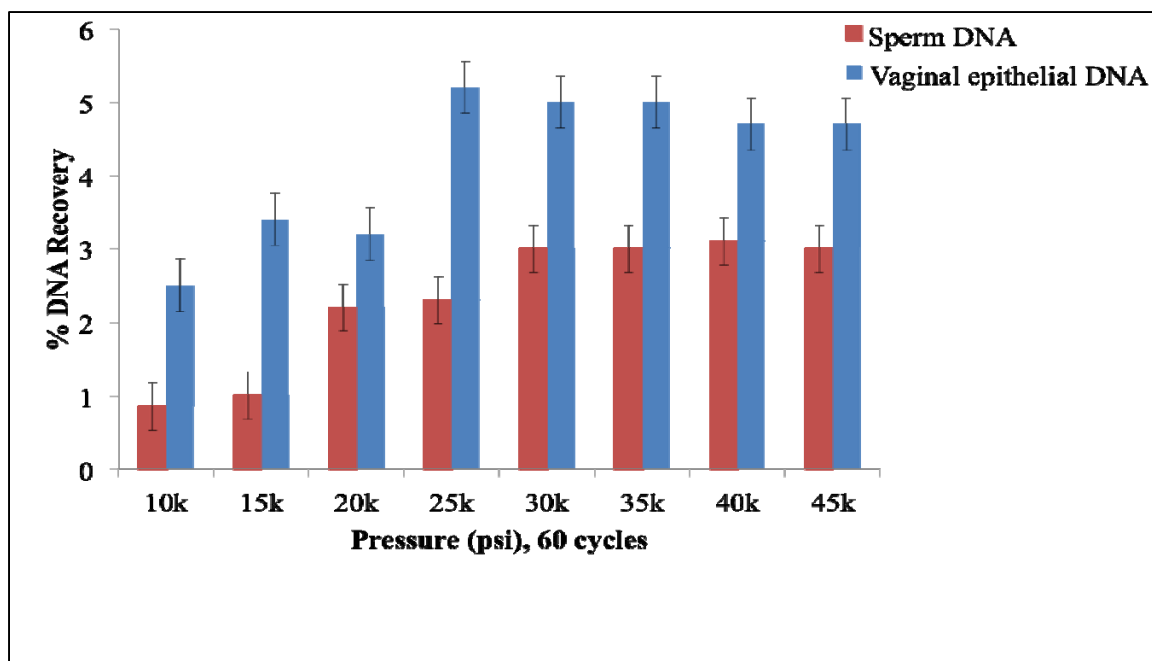


Figure 19. The effect of increasing pressure on DNA recovery from sperm cells and vaginal epithelial cells indicates overall low yields in the absence of chemical digestion (n=3 ± standard error)

C.3. Improving differential lysis and developing selective recovery of sperm DNA

The next step was to develop a procedure for selective extraction of DNA from sperm and improve DNA recovery efficiency. To do this the effects of three types of stress-temperature, detergents and reducing agent, was examined at 45000 psi and 60 cycles. In these experiments, sperm cells and epithelial cells were treated independently of one another to determine how they react before developing a protocol for selective extraction of mixtures.

The barocycler was connected to an external circulating water bath, and the samples were subjected to a high pressure of 45000 psi at 40° C, 50° C and 60° C. An increase in temperature resulted in 4-fold enrichment (16%±1) of vaginal epithelial DNA compared to pressure cycling at room temperature (3%±1) whereas no change was observed in sperm DNA yield. Detergents are a common component in extraction buffers

due to their ability to solubilize the lipid membrane and aid cell lysis. The effect of ionic and non-ionic detergents on epithelial and sperm cell lysis was studied to determine if they can be used for selective recovery when coupled with pressure cycling. Buffer solutions containing 2% (v/v) Tween-20, 2% (w/v) sodium dodecyl sulfate (SDS) or 2% (w/v) sarkosyl were examined by applying pressure treatment at 45000 psi for 60 cycles. Tween-20 did not improve DNA yields but SDS and sarkosyl produced higher epithelial DNA yields compared to sperm DNA. Sarkosyl treatment resulted in $14\% \pm 1$ female DNA recovery compared to $3\% \pm 2$ sperm DNA recovery indicating that selective recovery of female DNA is possible when samples are extracted with pressure-induced mechanical stress in the presence of detergents. Dithiothreitol (DTT) is a reducing agent commonly used in the organic differential extraction protocol to target sperm cells and extract male DNA. Protamines are sperm nuclear proteins that are rich in arginine and contain cysteine residues that form disulfide bonds. The disulfide bonds crosslink the protamines in the sperm nucleus [92]. Reducing agents such as DTT reduce disulfide linkages in protamines resulting in decondensation of sperm nuclei. A 10% increase in the quantity of DNA extracted from sperm cells was observed in the presence of 20 mM Dithiothreitol (DTT) with twice the amount of sperm DNA recovered compared to epithelial cells.

The overall results Figure 20, demonstrated that depending on the buffer conditions, differential lysis of epithelial cells or sperm cells is possible. In particular, increasing temperature or addition of detergent aided in the recovery of female cells, while the addition of reducing agents permitted selective recovery of sperm cells.

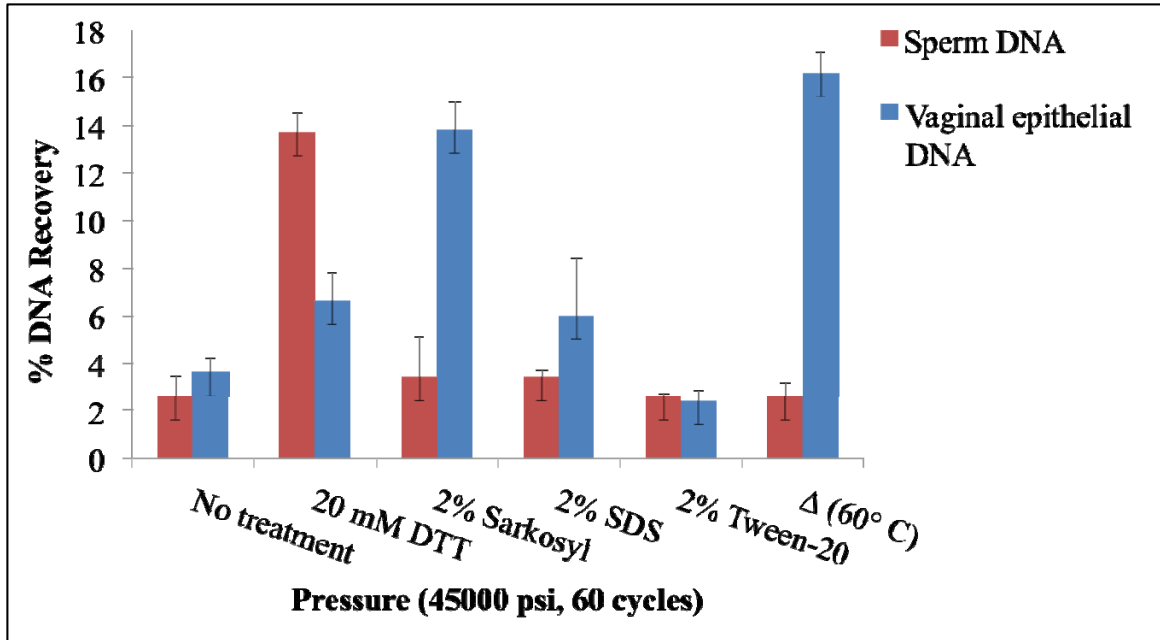


Figure 20. The effect of chemical digestion on DNA recovery with pressure treatment at 45000 psi for 60 cycles indicated relatively higher sperm DNA recovery in the presence of a reducing agent (DTT) and selective female DNA recovery in the presence of high temperature and a detergent (Sarkosyl). (n=3 ± standard error)

Further exploration of the effects of DTT concentration and pressure was performed to determine if DNA recovery from sperm cells could be further improved. Sperm cells and vaginal epithelial cells were treated with 40 mM, 60 mM, 80 mM or 100 mM DTT and extracted with 60 cycles of 45000 psi pressure. The results from figure 21 demonstrate that sperm DNA recovery could be improved to 73%±4 at 40mM DTT but the selectivity dropped with almost 47% ±12 of epithelial DNA recovery (Figure 21). As a result we evaluated tris (2-carboxyethyl) phosphine (TCEP), a stronger reducing agent, under the assumption that the key issue for selective extraction was the disruption of the disulfide bonds in the sperm cells [78].

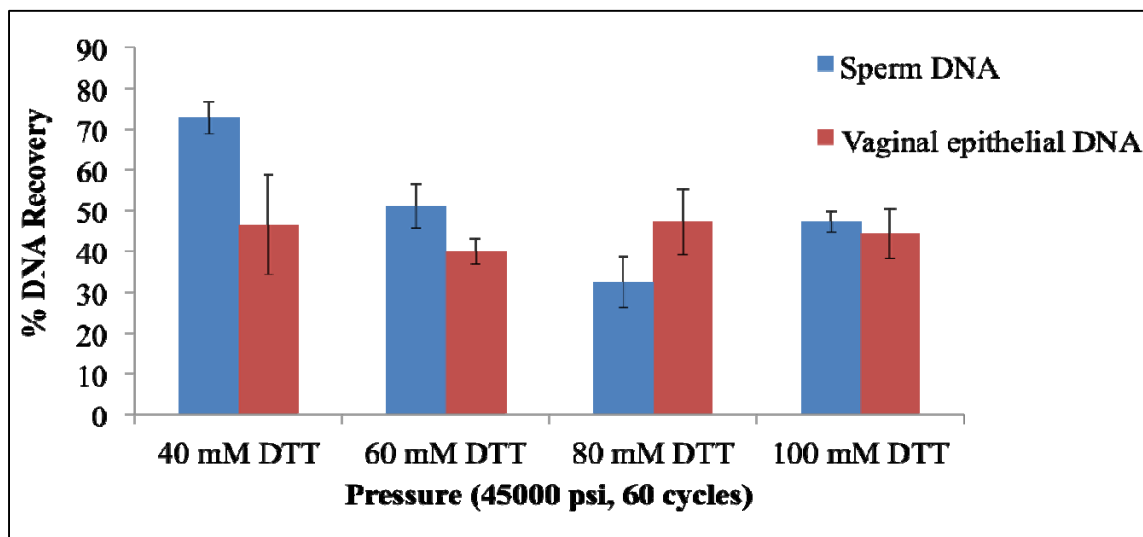


Figure 21. The effect of increasing concentration of dithiothreitol (DTT) indicates an increase in DNA yields but overall loss of selectivity between sperm and epithelial cell lysis. (n=3 ± standard error)

TCEP is a stronger reducing agent than DTT and is commonly used to break disulfide bonds within and between proteins. Moreover, properties such as high stability over a wide pH range and its odorless nature have made its use popular in biochemical applications [49, 78].

Sperm cells and vaginal epithelial cells were suspended in buffer containing 10 mM, 20 mM, 30 mM, 40 mM or 50 mM concentration of TCEP and treated with 45000 psi pressure for 60 cycles. The results Figure 22, showed a significant increase in selectivity when TCEP was added with a relatively high percentage of the sperm cells being lysed when compared to the epithelial cells. At 20 mM TCEP, nearly 60% of sperm cells were lysed with less than 20% epithelial DNA recovery. Overall compared to treatment by reduction with DTT, TCEP resulted in an improved selectivity for sperm and epithelial cell lysis with maximum sperm DNA recovery (58%±4) at 20 mM TCEP and an optimal selectivity (9/1 sperm/epithelial) at 40 mM TCEP.

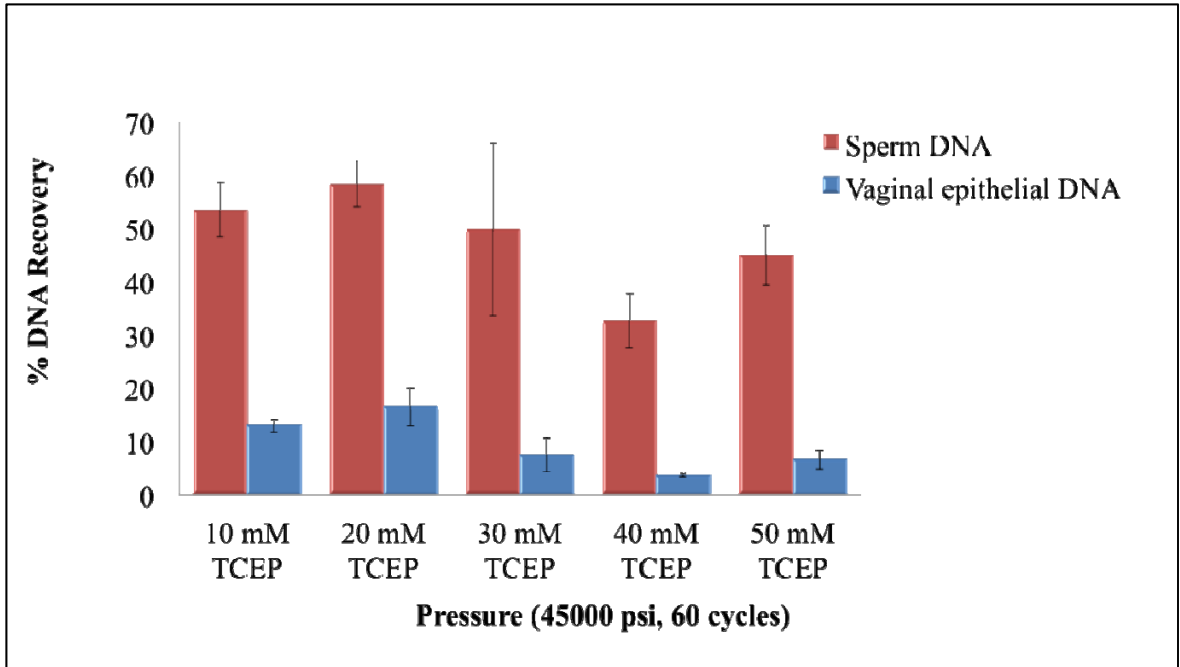


Figure 22. The effect of a stronger reducing agent (Tris (2-carboxyethyl) phosphine) on DNA recovery, with pressure treatment at 45000 psi for 60 cycles, indicates a significant increase in sperm DNA yields with minimal digestion of vaginal epithelial cells. (n=3 ± standard error)

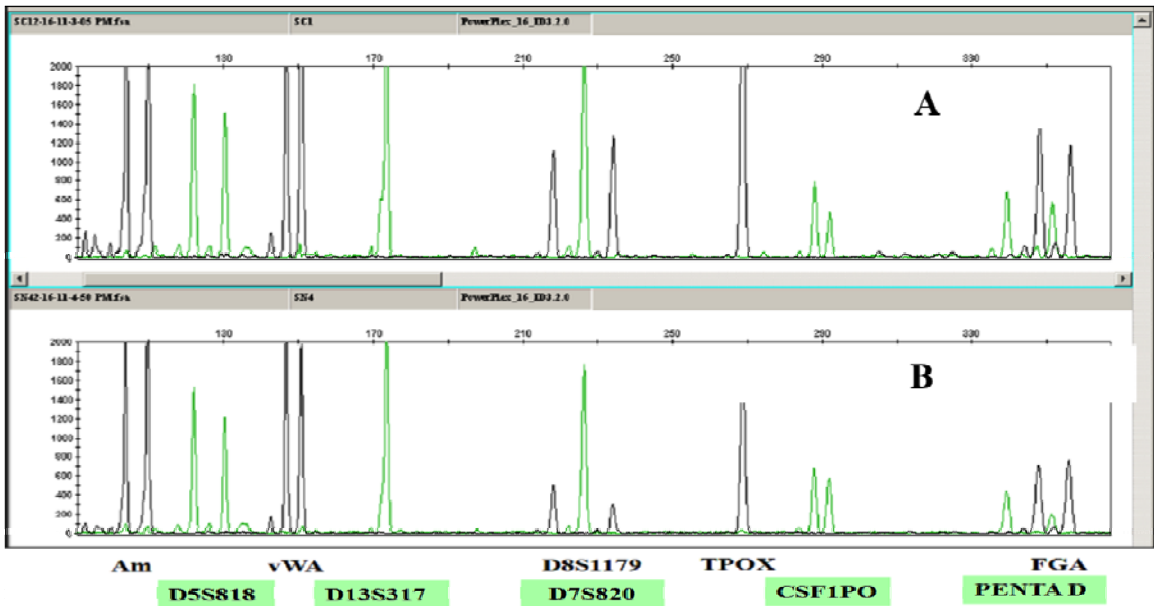


Figure 23. Electropherogram of DNA products extracted with PCT indicates good quality of genomic DNA A. Sperm control B. Sperm DNA- 20 mM TCEP + PCT (25K psi)

C.4. Application of pressure treatment to mixtures

Since preliminary studies using pressure cycling technology indicated that it was possible to selectively recover more sperm DNA than female epithelial DNA when using reducing agents such as TCEP and DTT, pressure treatment was next applied to mixtures of sperm and epithelial cells in the presence of TCEP and DTT. In order to maximize DNA recovery without compromising the selectivity of digestion, mixtures of sperm and epithelial cells were suspended in a 1X phosphate buffered saline (PBS) buffer with a final concentration of 40 mM TCEP and 20 mM DTT. Addition of DTT resulted in 13% increase in sperm DNA recovery compared to TCEP treatment alone (32%±5) while retaining a selectivity factor of 2/1 sperm to epithelial cells. (Figure 24). An increase in digestion time by exposing the samples to a maximum number of 99 pressure cycles did not improve the yields.

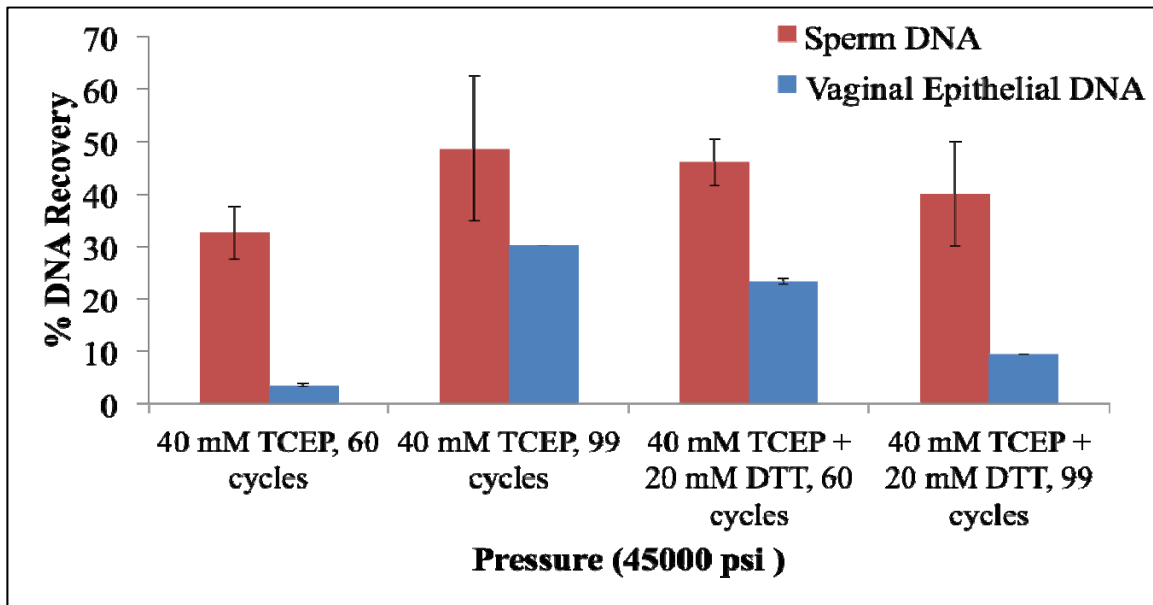


Figure 24. The effect of pressure treatment at 45000 psi on DNA recovery from a mixture in the presence of TCEP and DTT indicates a 13% increase in yields compared to TCEP treatment alone. No increase in yield was observed when the number of pressure cycles was increased to 99 cycles. (n=3 ± standard error)

For the next step, the effect of pressure treatment on DNA recovery from cotton swabs was evaluated since cotton swabs are routinely used for sample collection. Organic differential extraction is traditionally used for the extraction of rape kits from swabs in which vaginal epithelial cells are initially lysed and removed from the mixture prior to the digestion of sperm cells with DTT treatment. . Many studies have reported inefficient sperm DNA recoveries with organic differential extraction protocol leading to numerous studies to improve cell elution and subsequent DNA recovery [64, 78, 125]. Voorhees et al. demonstrated a slight increase in cell recovery by using cellulase enzyme to digest the cellulose fibers of the cotton swab [125]. Addition of detergents during incubation period [90], and use of alternate collection tools such as nylon flocked swabs [13] have also been shown to improve the recovery of intact cells from the cotton substrate. To determine the effect of PCT treatment on DNA recovery from swabs, mock samples were created by adding sperm cells and epithelial cells in the ratio of 1:4 to the swab and air-drying at room temperature for 1-2 hours. Prior to extraction with pressure treatment, the swab was incubated in a water bath at 42°C for 2 hours to loosen the cellular material from the matrix. Following incubation, TCEP was added to the buffer to a final concentration of 20 mM to maximize the DNA recovery and subjected to 60 cycles of pressure treatment at 45000 psi. The overall results Figure 8, demonstrate that the DNA recoveries dropped to less than 5% when pressure treatment was applied to swabs incubated in 1X PBS buffer. Based on the assumption that inefficient cell recovery from cotton swabs may lead to a drop in DNA recovery, treated cotton swabs were incubated in cellulase enzyme at 10 µg/mL, 50 µg/mL or 100 µg/mL concentrations for 2 hours at 42°C. This treatment did not result in any significant improvement in the yields with an

overall recovery of only 6-7% of sperm DNA. Benschop et al. demonstrated a 6-fold improvement in the recovery of intact cells from nylon-flocked swabs compared to cotton swabs [8]. Pressure treatment of nylon flocked swabs containing a mixture of sperm cells and epithelial cells was not efficient and resulted in nearly 98% loss in sperm and epithelial DNA recovery as demonstrated in figure 28. Due to poor yields, a 1% solution of SDS detergent was included in the incubation buffer in an attempt to improve recovery of cells, however the overall recovery remained around 6% for sperm DNA.

Dithiothreitol (DTT) is commonly used for sperm cell lysis and in previous results it produced an improvement in sperm DNA recovery with pressure treatment. When swab cuttings were incubated in 1X PBS buffer containing a final concentration of 20 mM DTT for 2 hours at 42°C followed by pressure treatment (45000 psi for 60 cycles) in the presence of 20 mM TCEP, a nearly 10% increase in sperm DNA yield (from 4.3%±1 to 14%±4) occurred compared to incubation in 1X PBS buffer (pH 7.4) alone. The PCT treatment did not have a significant impact on epithelial cell lysis, which showed an overall recovery of 2.3%±0.4. (Figure 25)

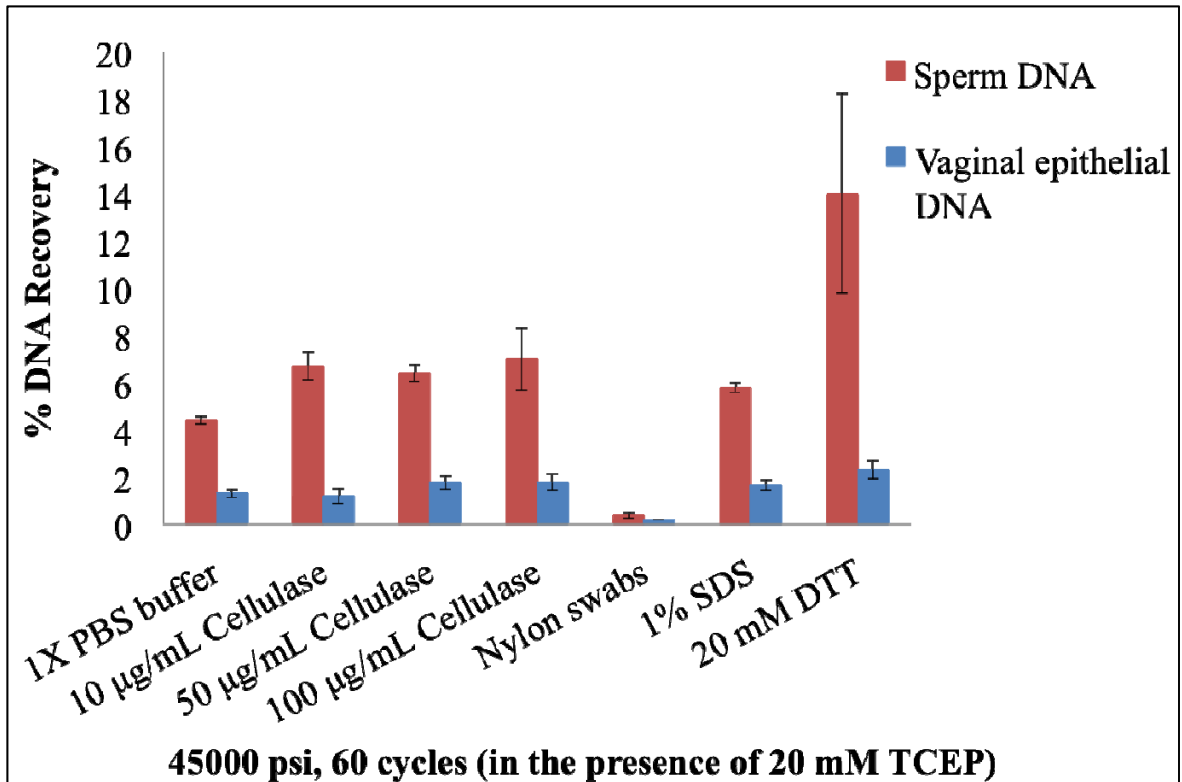


Figure 25. The effect of various treatments on DNA recovery from a swab with pressure treatment at 45000 psi for 60 cycles indicates that the best yields and selective recovery of sperm DNA was observed with a buffer containing TCEP and DTT although the overall yields dropped due to inefficient sample recovery from a cotton swab. (n=3 ± standard error)

Analysis of STR data for samples extracted with DTT and TCEP revealed that a full autosomal STR profile could be obtained for the male contributor while allelic drop out was observed at the Penta E and D7S820 loci for the female contributor (Figure 26). A mixed profile was expected considering that the swab had four times more female cells. Although DTT treatment gave the best recoveries with 14% sperm DNA recovered from the sample, the overall recovery was still low. In addition, the selectivity of this extraction procedure was low resulting in a mixed DNA profile in the purified DNA extract. To estimate the male contribution in the extracted sample, the peak height ratio at

the amelogenin (AMEL) locus was used, and the profile indicated male DNA enrichment with $59\% \pm 4$ of the total DNA recovered as male and $41\% \pm 4$ as female.

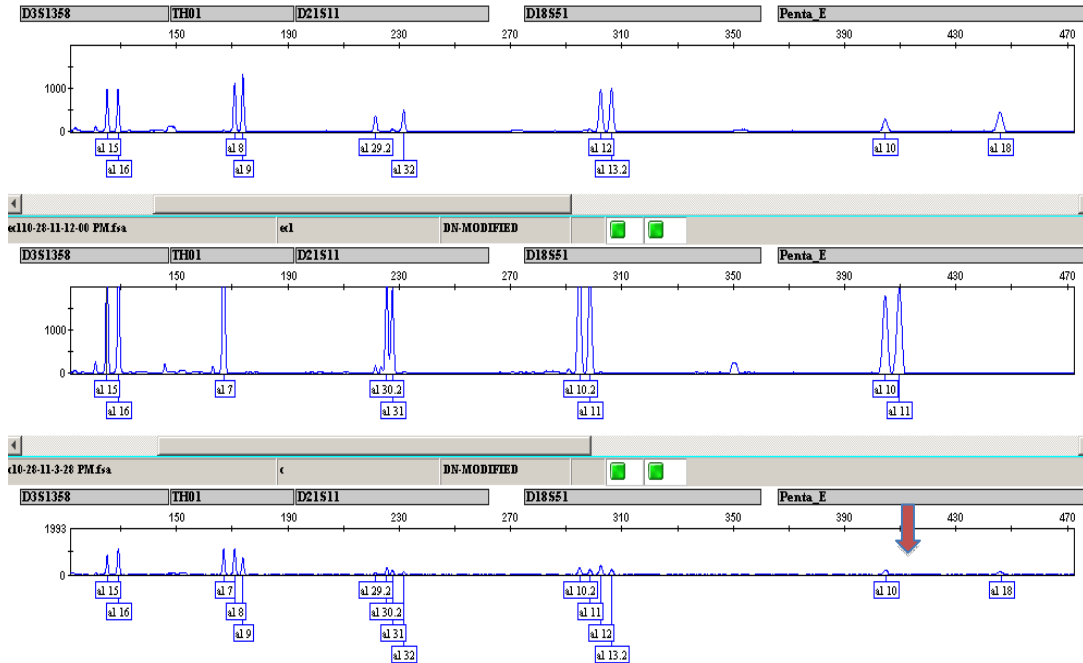


Figure 26. PowerPlex 16 HS amplification products of sperm DNA control (top panel) Epithelial DNA control (middle) DNA recovered from pressure treatment of a mixed stain on a swab, in the presence of 20 mM TCEP following incubation at 42°C for 2 hours in 20 mM DTT (bottom panel). The arrow indicates the loss of female allele at Penta E locus.

D. Concluding remarks

The data indicates less than 5% DNA recovery occurs from both male and female cells when pressure cycling technology was used as a stand-alone treatment for cell lysis. In the presence of reducing agents such as DTT and TCEP, almost 60-70% sperm DNA was recovered using a pressure treatment at 45000 psi for 60 cycles. Treatment with TCEP further improved selectivity with nine times more sperm DNA recovered compared to epithelial DNA. These results demonstrate the potential of this technology in

analyzing samples from sexual assault casework that often contain mixtures of sperm and vaginal epithelial cells.

When the study was conducted on samples deposited on a cotton swab, the overall recoveries dropped to less than 5% with TCEP treatment at high pressure of 45000 psi. This is a significant loss in recoveries that may be attributed to the inefficient sample elution from the cotton matrix. The best yields from cotton swabs were obtained when the swabs were incubated in a DTT solution, but the overall recoveries were still low at $14\% \pm 4$ and selectivity was compromised. The study demonstrates that pressure cycling technology improves DNA yields from liquid samples but requires additional treatments to obtain optimal yields when the sample is present on a cotton substrate.

Lastly these results suggest that a selective extraction of female cells should be possible through the combined use of detergents, pressure and elevated temperatures.

CHAPTER IX. ALKALINE LYSIS: IMPROVE DNA RECOVERY FROM SWABS

A. Introduction

There is a demand for protocols to recover DNA more quickly and efficiently from substrates. Due to the high discriminatory power of DNA evidence, it has become routine to collect biological samples as part of any crime scene investigation. Unfortunately, time-consuming protocols create backlogs resulting in thousands of samples remaining to be processed.

The major contributing factors to this problem are cumbersome extraction procedures and long incubation times necessary to successfully recover samples from the substrates on which evidence is deposited. Studies have reported inefficient sperm DNA recoveries from cotton swabs [22, 90, 125]. Considering the fact that cotton swabs are the most common collection tools, and that sometimes there is little to no evidence left behind in a crime scene, it is a matter of utmost importance to develop methods that can rapidly and efficiently recover evidence from the substrate.

Most of the methods utilized to process cotton swabs recover intact cells through incubation prior to subjecting the cell suspension to lysis conditions. The goal of this study was to develop a method using alkaline buffer conditions to lyse cells directly from the swab and improve DNA yields. In order to optimize differential recovery of sperm and epithelial DNA encountered in rape kits, varying concentrations of sodium hydroxide and the effects of different incubation temperatures and high pressures in the presence of alkaline conditions were studied.

B. Materials and Methods

B.1 Sample preparation

Vaginal epithelial cells and semen samples were collected from volunteers in accordance with protocols approved by the Institutional Review Board (IRB) of Florida International University. The samples were suspended in 1X PBS buffer (pH 7.4) (Fisher Scientific, NJ) and diluted to approximately 1 million cells per milliliter. The cell count was performed using a disposable hemocytometer (INCYTO C-Chip, Covington, Georgia, USA) [70]. Equal volumes of epithelial cells and sperm cells were added to a cotton swab and air dried at room temperature. Post-coital samples obtained from healthy volunteers were stored at -20° C until further use.

The total amount of DNA in a known volume of sample (60,000-70,000 cells) was determined by extracting the samples using an organic extraction method that involved incubating the samples at 56°C for 2-4 hours in a lysis buffer (10 mM Tris, 100mM NaCl, 10 mM EDTA, 2% SDS, 39 mM DTT) containing proteinase K solution (20 mg/ml). Following incubation, the samples were purified using phenol chloroform isoamyl alcohol and ethanol precipitation. The extracted samples were quantified with real-time PCR analysis using Plexor® HY system (Promega Corp., Madison, WI). In order to determine the efficiency of DNA recovery, the amount of DNA recovered from subsequent experiments during method development was calculated as percent recovery compared to DNA extracted from neat sperm or epithelial samples using organic extraction.

B.2 DNA extraction

Alkaline lysis

Hudlow et al. developed an alkaline-based differential extraction method that when combined with DNase digestion generated a purified sperm fraction [64]. Upon applying this method and quantifying the extracted DNA, it was observed that there was a significant loss of sperm DNA prior to DNase digestion. This led us to initiate a study to determine the effect of alkaline lysis on sperm cells and vaginal epithelial cells without DNase digestion at different temperatures and concentrations of sodium hydroxide.

Sodium hydroxide crystals (Fisher Scientific, NJ) were dissolved in molecular biology grade water (Fisher Scientific, NJ) to achieve concentrations of 0.2 N, 0.4 N, 0.6 N, 0.8 N and 1 N NaOH concentration. In order to maximize DNA recovery from cotton swabs, three different incubation temperatures (75°, 85°, and 95° C) and two different incubation times (2 minutes and 5 minutes) were studied for each concentration of sodium hydroxide. A cotton swab containing an equal quantity of sperm cells and epithelial cells was suspended in 400 µL of a specific concentration of sodium hydroxide and exposed to either 75°, 85°, or 95° C for 2 or 5 minutes.

Pressure Cycling Technology (PCT)

A Barocycler® NEP 2320 (Pressure BioSciences Inc., South Easton, MA) was used to generate cycles of high pressure and ambient pressure to apply mechanical stress on the cells and cause lysis. After determining the effect of alkaline conditions on sperm cells and epithelial cells, different parameters were examined including the pressure intensity and number of cycles in an effort to increase extraction selectivity. PULSE™

tubes (Pressure Biosystems Inc., South Easton, MA) are specially designed tubes that are able to withstand high pressures. To perform pressurized extraction, swabs containing mixtures of sperm cells and vaginal epithelial cells were transferred to PULSE™ tubes containing 0.4 N NaOH solution (the optimum concentration determined by alkaline lysis studies) and exposed to 10000 psi, 20000 psi or 45000 psi to determine the optimum pressure necessary to achieve selective lysis of female cells. The efficiency of the pressure treatment in the presence of increasing number of cycles was studied by varying the cycle number between 10, 20 and 60 cycles. During each pressure cycle, the holding time of the sample at ambient pressure (T1) and target pressure (T2) was 15 seconds each.

B.3 Post-extraction purification

Following extraction, the samples were neutralized with 2M Tris (pH 7.5). Then the swabs were transferred to DNA IQ™ Spin Baskets (Promega Corp., Madison, WI) and centrifuged at 13000 rpm for 5 minutes. Extracted samples were purified by adding an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma-Aldrich, St.Louis, MO), and precipitated with 3 M sodium acetate and 95% ethanol. Following precipitation, the pellet was washed with 70% ethanol, air-dried and re-suspended in 1X Tris-EDTA buffer (pH 8.0) (Fisher Scientific, NJ).

B.4 Quantification

The extracted DNA was quantified using real-time PCR assay with a commercially available kit, the Plexor® HY system (Promega Corp., Madison, WI) on Rotor-Gene 6000

(Qiagen, Inc., Valencia, CA). The system can simultaneously quantify autosomal DNA and male DNA. By calculating the Autosomal/Y ratio, the amount of male DNA and female DNA recovered from the mixture could be determined. The percent recovery of each cell type was determined by comparing the resultant quantity of DNA with samples containing a single cell type that have been directly extracted with proteinase K (20 mg/mL) based organic extraction. All the samples were analyzed in triplicate and the Plexor[®] HY analysis was performed following the manufacturer's instructions [96].

B.5 Short tandem repeat (STR) analysis

The quality of the DNA recovered from alkaline lysis and pressure treatment was assessed by performing STR analysis using PowerPlex[®] 16 HS system (Promega Corp., Madison, WI) according to manufacturer's protocols (PowerPlex[®] 16 HS system, Technical Manual# TMD022, Promega Corp., Madison, WI). Quantification data was used to normalize the input DNA for STR analysis. 1 ng of sample was amplified using GeneAmp[®] 9700 thermal cyclers and 1 μ L of the amplified products were separated using ABI Prism[™] 310 genetic analyzer (Applied Biosystems, Foster City, CA). Data analysis was performed using GeneMapper[®] ID v3.2 (Applied Biosystems, Foster City, CA).

C. Results and Discussion

C.1 Effect of alkaline lysis on DNA recovery from swab

The goal of the alkaline lysis studies was to determine parameters to maximize sperm DNA recovery from a cotton swab while maintaining sufficient selectivity to enable differential lysis of mixtures. The effect of varying concentrations of NaOH at

different temperatures and incubation times was studied, and it was observed that incubating the swab in 0.4 N NaOH at 95°C for 5 minutes resulted in the recovery of 2.5 times more sperm DNA than epithelial DNA (Figure 27). Moreover, depending on NaOH concentration and temperature, either sperm DNA or epithelial DNA exhibited relatively higher recoveries from mixtures

Though cotton swabs have been traditionally used to collect evidentiary material, recovery of biological samples is poor from this matrix. Many methods have been reported previously to improve cell recovery from cotton swabs but they involve 2-4 hours incubation time [34, 125, 126]. In cases where there are mixtures present, all the cellular material must be eluted prior to the extraction step, before differential lysis can take place. Initial results demonstrated that by incubating the swab at high temperature in alkaline conditions, almost all the cellular DNA can be extracted from the substrate in as little as 5 minutes. Furthermore, differential extraction of epithelial and sperm cells can be performed directly off of the swab without prior elution, by adjusting the concentration of base and the incubation parameters.

The results show that at any given temperature, maximum DNA recovery from sperm cells is produced at 0.4-0.6 N NaOH, whereas 0.2 N NaOH produces optimal recovery of epithelial DNA (Table 3). The best selectivity and reproducibility for the removal of sperm cells from the swabs was achieved by increasing the incubation time to 5 minutes in the presence of 0.4 N NaOH. The removal of sperm was further improved by increasing the temperature to 95°C, with the result that 99%±1 of the sperm DNA was recovered from controlled mixtures.

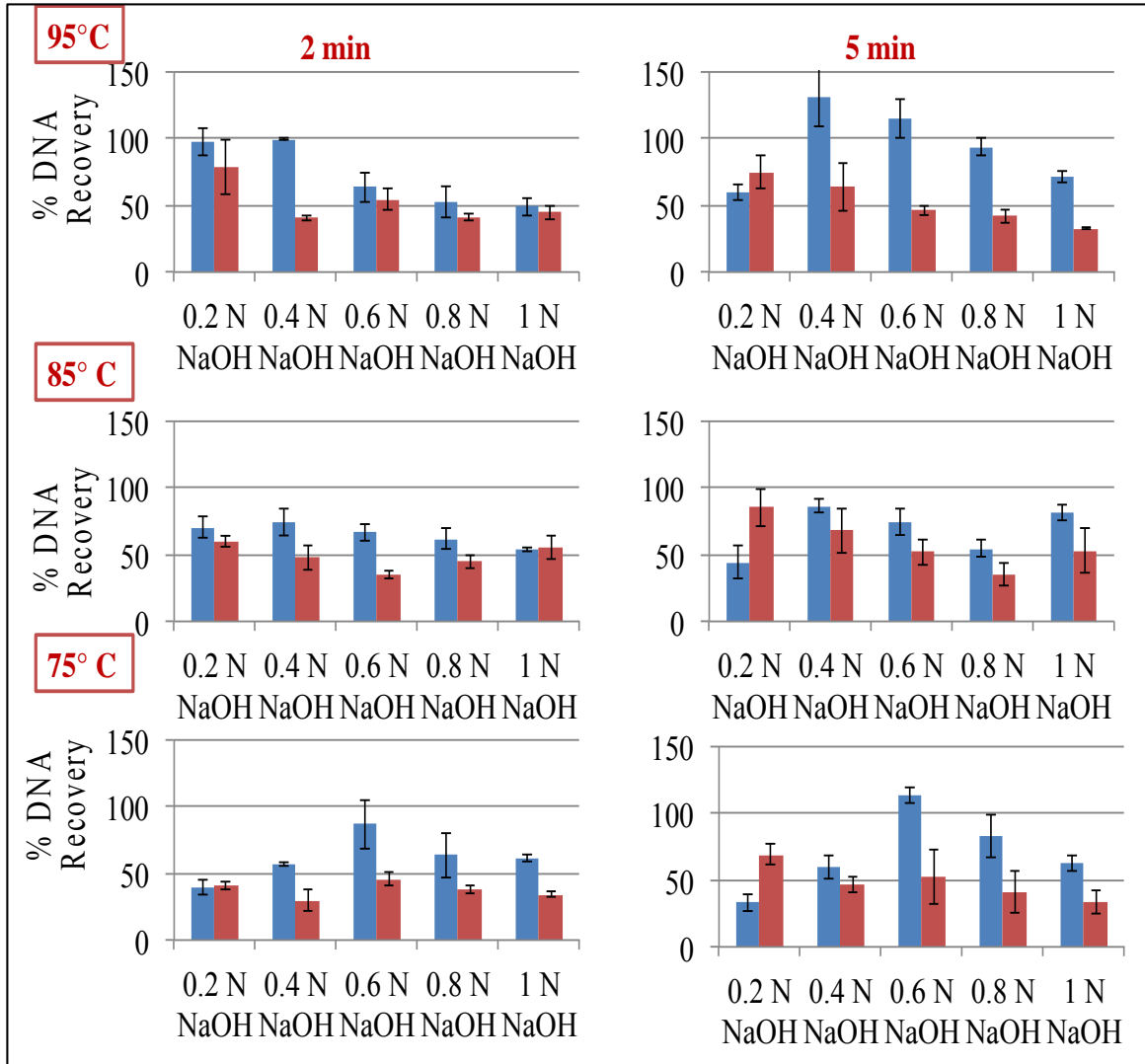


Figure 27. Effect of alkaline lysis treatment on sperm and epithelial DNA recovery. At a concentration of 0.4 N NaOH and incubation at 95°C, twice the amount of sperm DNA was recovered compared to epithelial DNA. (n=3 ± standard error)

Lower temperatures and lower concentrations of base were more conducive for the selective recovery of DNA from female epithelial cells. Overall results are shown in

Table 3.

Table 3. Amount of DNA recovered from mixtures in the presence of alkaline conditions at high temperatures.

Concentration of NaOH solution (N)	Incubation temperature (°C)	Incubation time (Minutes)	Sperm DNA recovered (%)	Vaginal epithelial DNA recovered (%)
0.2	95	2	60 ± 6	75 ± 12
0.2	95	5	98 ± 10	78 ± 21
0.4	95	2	*131 ± 22	64 ± 18
<u>0.4</u>	<u>95</u>	<u>5</u>	<u>99 ± 1.0</u>	<u>41 ± 2</u>
0.6	95	2	*115 ± 15	46 ± 4
0.6	95	5	64 ± 11	54 ± 8
0.8	95	2	94 ± 7	42 ± 5
0.8	95	5	52 ± 12	41 ± 3
1	95	2	71 ± 4	33 ± 1
1	95	5	49 ± 7	45 ± 5
0.2	85	2	44 ± 13	85 ± 14
0.2	85	5	70 ± 8	60 ± 4
0.4	85	2	86 ± 5	68 ± 17
0.4	85	5	75 ± 10	47 ± 9
0.6	85	2	75 ± 10	52 ± 10
0.6	85	5	66 ± 6	35 ± 3
0.8	85	2	55 ± 6	35 ± 9
0.8	85	5	62 ± 8	45 ± 5
1	85	2	81 ± 6	53 ± 17
1	85	5	54 ± 2	55 ± 9
0.2	75	2	33 ± 6	69 ± 7
0.2	75	5	40 ± 6	41 ± 3
0.4	75	2	60 ± 9	46 ± 6
0.4	75	5	57 ± 1	30 ± 8
0.6	75	2	*113 ± 6	52 ± 21
0.6	75	5	87 ± 19	46 ± 6
0.8	75	2	83 ± 16	41 ± 16
0.8	75	5	64 ± 17	38 ± 3
1	75	2	62 ± 6	34 ± 9
1	75	5	61 ± 2	34 ± 2

1. DNA recovery values represent the percent recovery of DNA in each fraction compared to the total amount of DNA extracted using organic extraction of neat samples and are expressed as the mean (n=3) \pm standard error
2. Bold and underlined parameters represent the optimized alkaline conditions for selective sperm DNA recovery from mixtures.
3. *Values with asterisk (*) indicate that more DNA was recovered with the experimental parameters compared to the organic extraction of a similar amount of neat semen sample.*

C.2 Optimization of PCT parameters

Although the results from the alkaline lysis studies showed that 0.4 N NaOH gave the best recovery and selectivity for sperm DNA from mixtures, there was still some DNA recovery from epithelial cell lysis that resulted in a mixed profile. In order to minimize epithelial cell lysis during sperm DNA recovery, a pressure-based extraction step was introduced before incubating the swab at high temperature. In order to do this, it was important to determine the effect of pressure treatment on the swabs in the presence of 0.4 N NaOH.

The results indicated that $104 \pm 6\%$ recovery of epithelial DNA occurred at 20,000 psi with a minimum of 10 cycles of pressure in the presence of 0.4 N NaOH (Table 4). Furthermore, this mild pressure treatment did not have a significant impact on sperm cell lysis, thus enabling the development of a two-step differential extraction protocol. Another upside of this treatment was that compared to the current extraction methods which can take up to 2-4 hours to remove cells from a swab, the total time required to remove epithelial DNA from the swab using the pressure cycling procedure under alkaline conditions was only 5 minutes. It should be noted that semen also contains non-spermatogenic cells such as epithelial and inflammatory cell types that may be

susceptible to digestion under same conditions as vaginal epithelial cells. Thus the slight recovery of male DNA seen during PCT treatment may be due to the degradation of the non sperm cells present in semen.

Table 4. Effect of pressure treatment on DNA recovery from mixtures in the presence of 0.4 N NaOH solution

Pressure (psi)	Number of cycles	Sperm recovered (%)	DNA recovered (%)	Vaginal epithelial DNA recovered (%)
10,000	10	8± 1		93± 3
20,000	10	25± 13		104± 6
20,000	20	17± 3		110± 20
20,000	60	43± 9		46± 13
45,000	20	16 ± 3		58± 5
45,000	60	26 ± 6		54± 3

Note: DNA recovery values represent the percent recovery of DNA in each fraction compared to the total amount of DNA extracted using organic extraction of neat samples and are expressed as the mean (n=3) ± standard error

C.3 Development of a two-step protocol for differential extraction

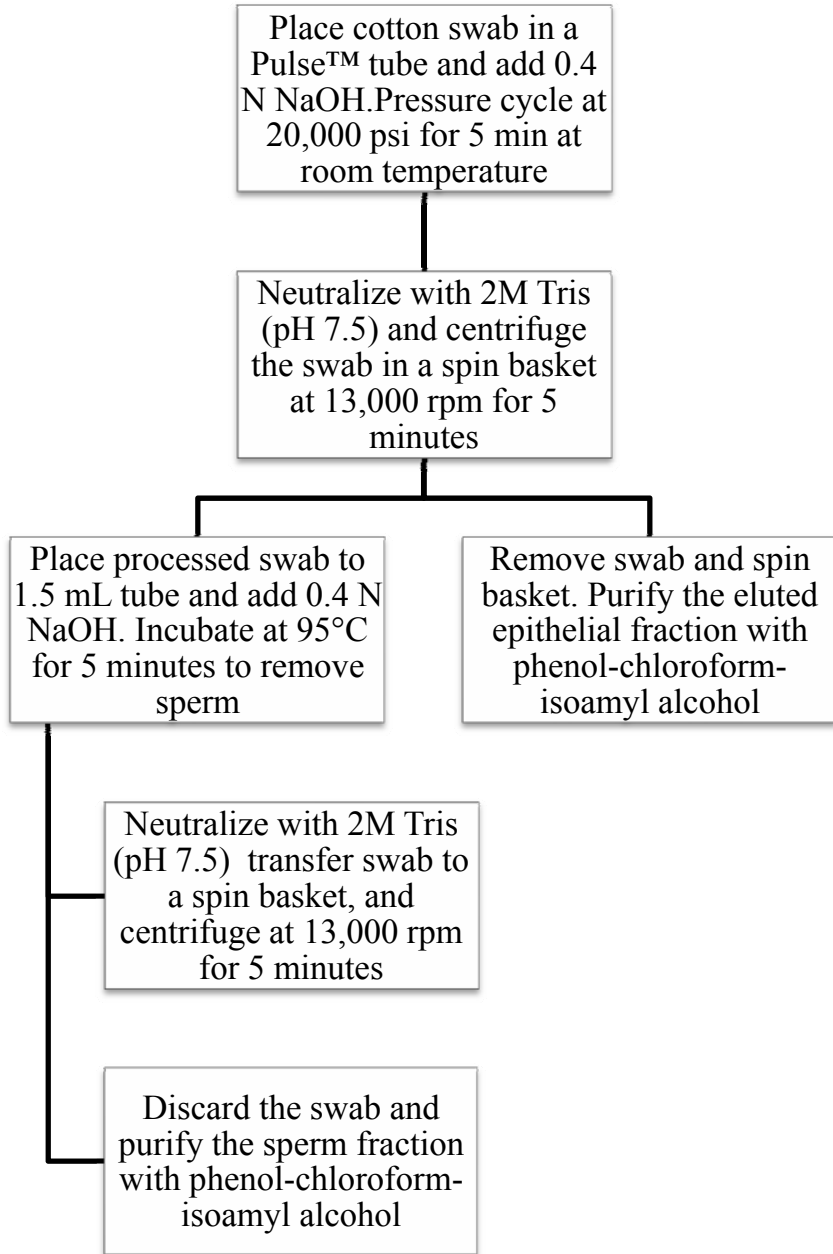
To achieve complete separation of both cell fractions and obtain a clean DNA profile, a two-step method was developed in which swabs were first placed in 0.4 N NaOH and exposed to pressure cycling for 5 min to lyse and remove the epithelial DNA. The cells remaining on the swab (sperm fraction) were then subjected to alkaline lysis at 95°C for 5 min. To perform this process, the swabs were first treated with 20,000 psi pressure for 10-20 cycles in the presence of 0.4 N NaOH solution. Following pressure treatment, the sample was immediately neutralized with 2 M Tris (pH 7.5) and the swab was transferred to a spin basket placed in 2.0 mL tube and centrifuged at 13,000 rpm for 5 minutes. DNA was purified with phenol-chloroform-isoamyl alcohol (25:24:1)

followed by ethanol precipitation (epithelial fraction). The swab was transferred to a 1.5 mL tube containing 0.4 N NaOH solution and the sample (sperm fraction) was incubated at 95°C for 5 minutes. Following incubation, the sample was neutralized with 2 M Tris (pH 7.5) and the swab was transferred to a spin basket for centrifugation at 13,000 rpm for 5 minutes. The swab was discarded and DNA from the sperm fraction was purified using phenol-chloroform-isoamyl alcohol and ethanol precipitation (Figure 28).

The results indicate complete female DNA recovery from mixture deposited on a cotton swab following pressure treatment at 20,000 psi with only 10 pressure cycles lasting 5 minutes. A small amount of sperm DNA was also recovered following this pressure treatment under alkaline conditions ($17\% \pm 3$). When this swab was exposed to high temperature under alkaline conditions in the second step, $63\% \pm 4$ of sperm DNA was recovered (Figure 29). There was no female epithelial DNA recovery in the final sperm fraction.

Short tandem repeat (STR) analysis of both epithelial and sperm fractions revealed that the male and female components were successfully separated from the mixture. The STR profile of the sperm fraction indicates $100\% \pm 2$ contribution by the male donor. (Figure 30) (Table 4). Comparative analysis of the genotypes of the mixture, neat sperm and epithelial samples with both the extracted fractions showed that this two-step protocol resulted in a clean male and female DNA profiles that are identical to the profiles generated with organic extraction of neat samples.

Figure 28. Flowchart depicting the protocol for differential extraction of mixtures using alkaline lysis and pressure cycling technology



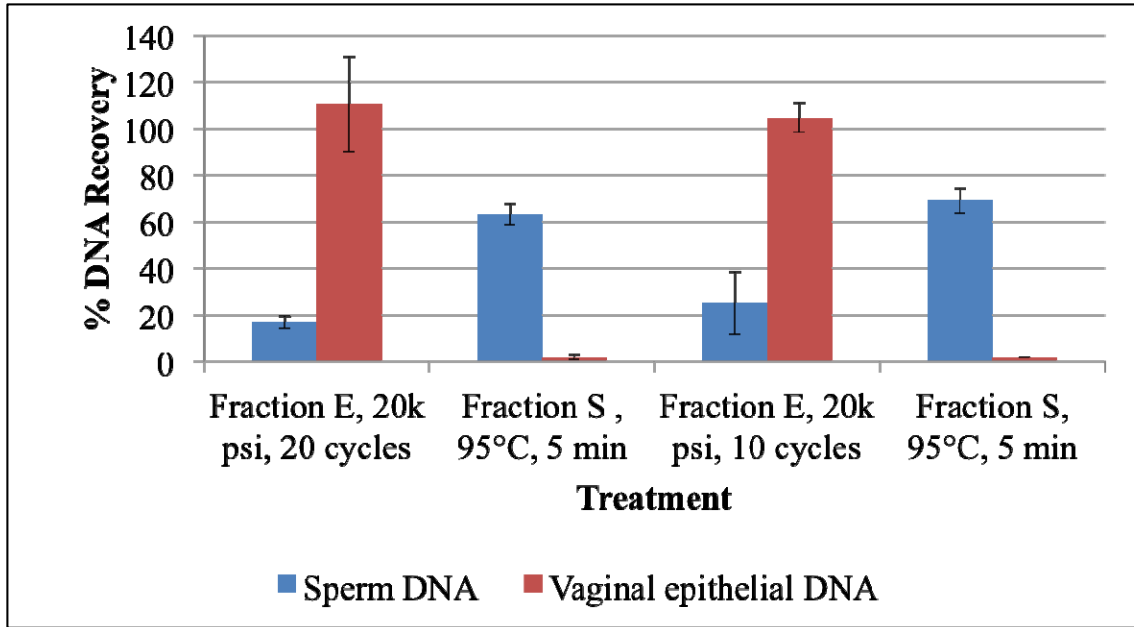
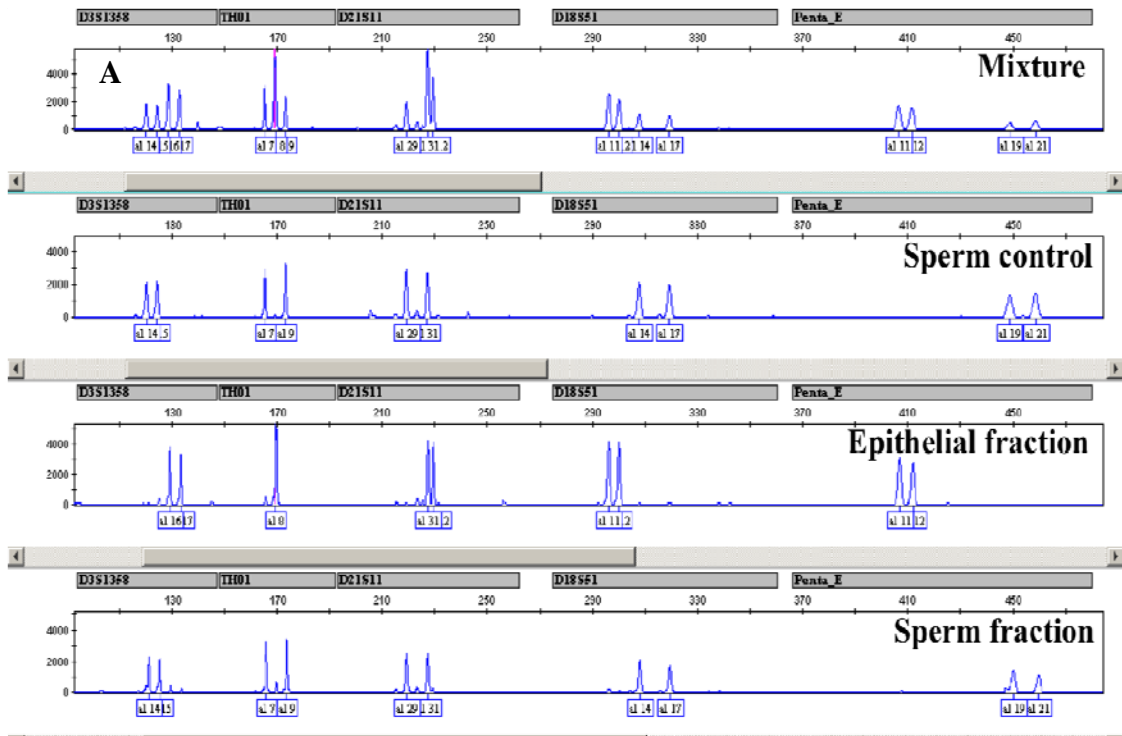


Figure 29. The effect of alkaline lysis and pressure cycling technology on DNA recovery from mixtures. The results indicate the recovery of female DNA (fraction E) with minimal sperm lysis after pressure treatment. More sperm DNA was recovered in the second step following exposure to high temperature under alkaline conditions (fraction S). (n=3 ± standard error)



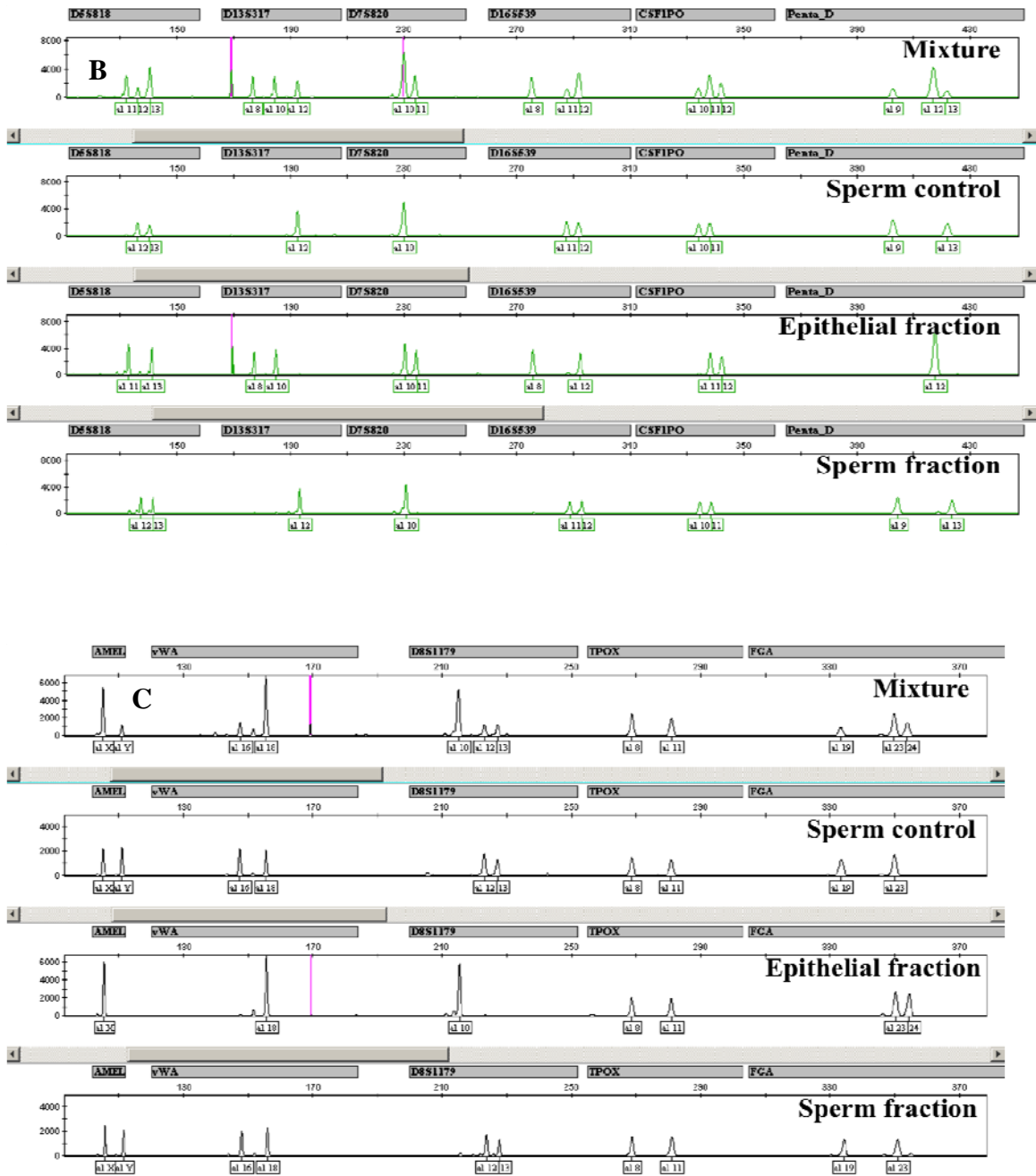


Figure 30. Powerplex® 16 HS products of mixture, sperm control, epithelial fraction (post-PCT purified fraction) and sperm fraction (post-alkaline lysis treatment). Electropherogram shows that the DNA profile obtained from sperm fraction is identical to sperm control at all loci.

A) Carboxy-tetramethylrhodamine (TMR) labeled loci

B) Fluorescein (FL)-labeled loci

C) 6-carboxy-4', 5'-dichloro-2', 7'-dimethoxy-fluorescein (JOE)-labeled loci

Table 5. Comparison of the female and male genotypes with mixture and fractions obtained from PCT treatment (Fraction E) and alkaline lysis (Fraction S). The results indicate that both male and female components were successfully separated from the mixture.

	Mixture	Epithelial	Fraction E	Sperm	Fraction S
Locus		control		control	
Penta E	11, 12, 19, 21	11, 12	11, 12	19, 21	19, 21
D18S51	11, 12, 14, 17	11, 12	11, 12	14, 17	14, 17
D21S11	29, 31, 31.2	31, 31.2	31, 31.2	29, 31	29, 31
TH01	7, 8, 9	8, 8	8, 8	7, 9	7, 9
D3S1358	14, 15, 16, 17	16, 17	16, 17	14, 15	14, 15
FGA	19, 23, 24	23, 24	23, 24	19, 23	19, 23
TPOX	8, 11	8, 11	8, 11	8, 11	8, 11
D8S1179	10, 12, 13	10, 10	10, 10	12, 13	12, 13
vWA	16, 18	18, 18	18, 18	16, 18	16, 18
Amelogenin	X, Y	X, X	X, X	X, Y	X, Y
Penta D	9, 12, 13	12, 12	12, 12	9, 13	9, 13
CSF1PO	10, 11, 12	11, 12	11, 12	10, 11	10, 11
D16S539	8, 11, 12	8, 12	8, 12	11, 12	11, 12
D7S820	10, 11	10, 11	10, 11	10, 10	10, 10
D13S317	8, 10, 12	8, 10	8, 10	12, 12	12, 12
D5S818	11, 12, 13	11, 13	11, 13	12, 13	12, 13

In the differential extraction protocol used by Broward Sheriff's Office (BSO) crime lab, BioRobot EZ1 workstation (Qiagen, Inc., Valencia, CA) is used to purify the extracted samples. This method gives better yields than standard organic differential extraction but it employs long incubation times to recover sample from the substrate. In the first step, the 1X TNE buffer (10 mM Tris, 1 mM EDTA Na₂·2H₂O, 1 M NaCl) containing 1% SDS and proteinase K (20 mg/mL) is added to the swab and incubated in a water bath at 56°C for 2 hours with intermittent mixing at 900 rpm. The supernatant is separated to recover female DNA with EZ1 purification and the swab is incubated at 56°C for 2 hours in 1X TNE buffer containing proteinase K (20 mg/mL) and 1M DTT. This fraction is purified with EZ1 trace protocol to recover sperm DNA. Since BSO method has been extensively validated and gives a clean separation, STR profiles obtained from differential digestion using alkaline lysis and pressure cycling technology was compared to the former. The results indicate that the genotypes of female and male fractions obtained with alkaline lysis and pressure-based extraction were identical to those obtained with the BSO protocol. (Figure 31)

Since the two-step extraction protocol using alkaline conditions and pressure cycling technology gave comparable results with a validated method, another study was performed to compare yields from this method to organic differential extraction and a commercially available kit used for mixture separation. These results indicate that no sperm DNA was recovered from the mixture using organic differential extraction. This is consistent with studies that reported more than 95% loss of sperm sample using this method. It is important to note here that the samples were incubated for only 2 hours. In a crime lab, this incubation step is often increased to overnight to ensure adequate

recoveries. With longer incubation times the recovery may have been better than what we observed, but this defeats the purpose of reducing the analysis time. With a commercially available kit that is based on solid-phase extraction and magnetic bead separation, the yields were 5% more than with organic differential extraction but the overall recoveries were still poor ($5\% \pm 4$). On the other hand, alkaline lysis and pressure cycling resulted in ten times more sperm DNA recovery ($54\% \pm 9$) with the total lysis time lasting less than 15 minutes. (Figure 32)



Figure 31. STR profiles of sperm and epithelial fractions extracted from a post-coital swab. One swab was processed using Broward sheriff's office (BSO) crime lab method and another swab from the same volunteer was processed using alkaline lysis and pressure cycling technology. A) Sperm fraction recovered using BSO protocol B) Sperm fraction recovered using alkaline lysis and pressure cycling technology C) Epithelial fraction recovered using BSO protocol D) Epithelial fraction recovered using alkaline lysis and pressure cycling technology. The profiles indicate identical genotypes were obtained using both the protocols.

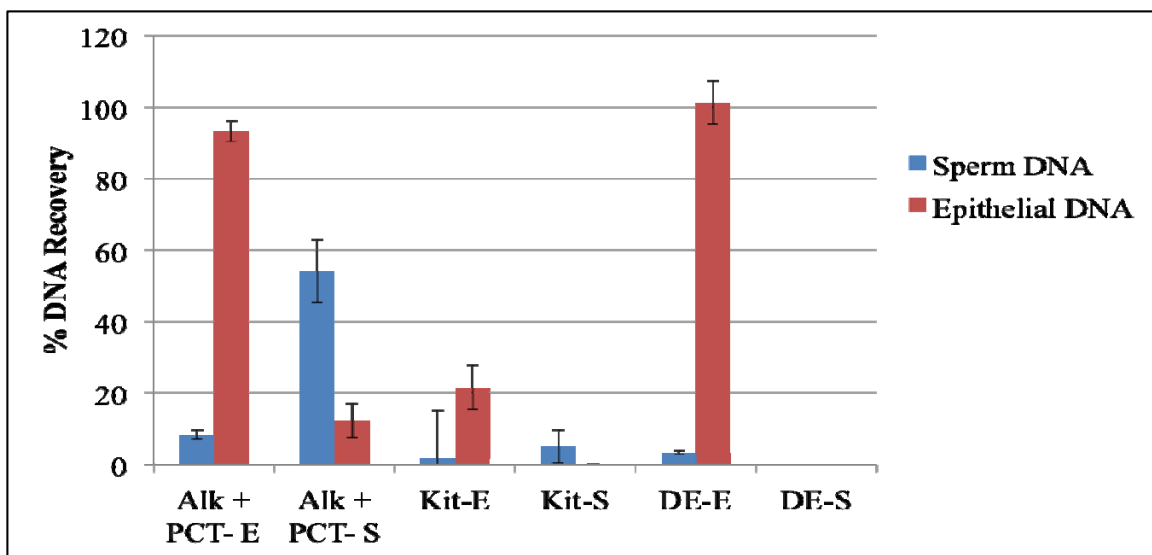


Figure 32. Comparison of PCT extraction with commercially available kit and organic differential extraction indicates better male DNA yields with alkaline lysis and PCT. (n=3 ± standard error) (Alk+PCT-E: Epithelial fraction obtained with alkaline lysis and PCT; Alk+PCT-S: Sperm fraction obtained with alkaline lysis and PCT; Kit-E: Epithelial fraction recovered with commercial kit; Kit-S: Sperm fraction recovered with commercial kit; DE-E: Epithelial fraction obtained with organic differential extraction; DE-S: Sperm fraction obtained with organic differential extraction)

D. Concluding remarks

Cotton swabs have been known to be a difficult matrix to work with considering the challenges associated with successfully recovering sample from tightly wound fibers. Alkaline lysis and pressure-based extraction uses inexpensive buffers, has a very short extraction time, and more importantly, can recover most of the DNA from the matrix, which is a significant improvement to the methods in existence. With a total of time of approximately 20 minutes to remove both sperm and epithelial cells from spiked swabs, this new process is quick and efficient. Current methods for removal of cellular debris from swabs require an incubation time of 2-4 hours, which does not include the differential DNA extraction. By using sodium hydroxide solution for lysis and phenol-chloroform-isoamyl alcohol to purify the extracted sample, it has been shown that with

the aid of simple solvents available in every lab, sperm and epithelial fractions could be successfully separated in a rapid and efficient manner. This gives leeway to substitute different methods for downstream purification to further streamline the process that is suited to individual labs.

CHAPTER X. DEVELOPMENTAL VALIDATION

A. Introduction

Different variables such as environmental conditions, inhibitors, and sample substrate affect the final outcome of analysis. This may be a departure from how the samples respond to certain treatments in a controlled environment. It is important to develop methods that can overcome obstacles that interfere with downstream genetic analysis. Since all the studies so far were performed using clean laboratory conditions, validation studies were done to determine if good quality genomic DNA would be obtained in high yields even when the sample is exposed to different kinds of trauma.

Following the SWGDAM guidelines, the efficiency of the new lysis method was evaluated in the presence of PCR inhibitors, after exposure of the samples to environmental insults, and when the sample was present on different substrates. The sensitivity of the method was evaluated to determine the effect of variable mixture ratios and quantity of sample on the ability to generate a conclusive autosomal STR profile. A reproducibility study to gauge the consistency of this method and a correlation study to evaluate how this method compares to existing protocols were also done.

B. Materials and methods

B.1. Samples and reagents

Sperm samples were collected from two male donors, and vaginal epithelial swabs were collected from four female donors according to the protocols approved by the Institutional Review Board (IRB) of Florida International University. Indigo was

obtained from TCI (Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan), hematin and humic acid were purchased from ICN Biomedicals Inc. (Aurora, Ohio) and Alfa Aesar (Ward hill, MA) respectively.

B.2. Extraction and purification

Samples were extracted using the new lysis method comprising of pressure cycling technology and alkaline lysis. The sample suspended in 0.4 N NaOH was exposed to 20,000 psi pressure for 10 cycles followed by separation of the lysate, and incubation of the swab in a water bath at 95° C for 5 minutes. Post-extraction purification was performed using phenol chloroform isoamyl alcohol (25:24:1 v/v) and ethanol precipitation. The purified DNA pellet was suspended in 50 µL of 1X Tris-EDTA (TE) buffer.

B.3. Quantification

The Plexor® HY system (Promega Corp., Madison, WI) was used to quantify autosomal DNA and male-specific Y-chromosomal DNA. All the samples were analyzed in triplicate and Plexor® HY analysis was performed following the manufacturer's instructions (Plexor® HY system, Technical Manual# TM299, Promega Corp., Madison, WI).

B.4. STR Analysis

DNA extracts obtained from the biological samples were amplified with the PowerPlex® 16 HS system (Promega Corp., Madison, WI) according to manufacturer's

protocols (PowerPlex[®] 16 HS system, Technical Manual# TMD022, Promega Corp., Madison, WI). Quantification data obtained using real-time PCR was used to normalize the input DNA for STR analysis. 1 ng of sample was amplified using GeneAmp[®] 9700 thermal cyclers and 1 μ L of the amplified products were separated using ABI Prism[™] 310 genetic analyzer (Applied Biosystems, Foster City, CA). Data analysis was performed using GeneMapper[®] ID v3.2 (Applied Biosystems, Foster City, CA).

B.5. Stability studies

Two sets of samples (S1E1, S2E1) were prepared using two different sperm donors (S1, S2) and one female epithelial cell donor (E1). For environment exposure, two sets of samples (S1E1, S1E3) were created using one sperm donor (S1) and two female donors (E2, E3). DNA was extracted in triplicate from samples exposed to three different conditions:

- 1) Inhibitor mix: An inhibitor mix was prepared consisting of 12.5 mM indigo, 0.5 mM hematin and 2.5 mg/mL humic acid. Semen samples were diluted to 1:50 and 50 μ L of the diluted semen was added to a vaginal swab. The swab containing the mixture was spotted with 5 μ L of inhibitor mix. A small cutting of the air-dried swab was extracted.
- 2) Stains on denim were created by adding 50 μ L each of sperm and epithelial cell samples. A 1 x 1-cm cutting of this stain was extracted
- 3) Mixture on cotton swab exposed to outdoor environment for 7 days. Semen samples were diluted to fifty times and 50 μ L of diluted semen was added to

vaginal swab. The swab containing the mixture was left outside where it was exposed to environmental elements for 1 week.

B.6. Reproducibility studies

Reproducibility studies were performed over a course of three consecutive days. Six sets of samples (S1E1, S1E2, S1E3, S2E1, S2E2, S2E3) were prepared by mixing sperm cells from two male donors (S1, S2) and epithelial cells from three female donors (E1, E2, E3). Semen samples were diluted to fifty times and 50 μ L of diluted semen was added to vaginal swab. The swabs were left to air-dry at room temperature. A small cutting from the swab was used for each extraction. All six sets of the samples were extracted in triplicates on three different days to check for reproducibility in the results.

B.7. Sensitivity studies

Two kinds of studies were performed to determine the sensitivity of this extraction method. In the first study, variable ratios of mixture of sperm cells and epithelial cells were extracted to evaluate how this method performs in enriching sperm fraction and generating a conclusive male DNA profile when the sample is overwhelmed with vaginal epithelial cells. Semen sample from one male donor and epithelial swab from one female donor were collected. The cells were eluted into 1X PBS buffer and diluted to 1-1.5 million cells/mL using a hemocytometer for cell count. Swabs were prepared by adding a defined volume of each sample type to achieve the target ratio and allowing them to air dry at room temperature (Table 6).

Table 6. Sensitivity studies. Varying ratios of sperm cells and epithelial cells for sensitivity study

Ratio of male: female	Number of sperm cells	Number of vaginal epithelial cells
1:1	96000	96000
1:2	48000	96000
1:5	19200	96000
1:10	9600	96000
1:50	3000	150000

For the second sensitivity study, the total number of cells was reduced while maintaining a mixture ratio of 1:5 sperm to epithelial cells. This was done to determine the effect of alkaline lysis and PCT on recovering sufficient DNA yields to generate an autosomal STR profile from low samples levels. (Table 7)

Table 7. Sensitivity studies. Low sample levels in the ratio of 1:5 male to female cells

Total number of cells	Number of sperm cells	Number of vaginal epithelial cells
48000	8000	40000
24000	4000	20000
12000	2000	10000
6000	1000	5000
3000	500	2500

B.8. Case type samples

DNA was extracted from stains present on five different substrates. This study was performed to mimic the casework samples from sexual crimes and determine the effect of various substrates on DNA recovery using the new extraction protocol.

Stains were prepared on bedspread, cotton panties, socks, colored cotton T-shirt and denim. Semen sample and epithelial cells were diluted to 1:50 with 1X PBS buffer and 50 μ L of each sample type was added to the substrate. A 1x1 cm cutting was then excised from the substrate to perform extractions. All the extractions were performed in triplicate.

B.9. Correlation studies

Post-coital samples received in 2011 were extracted using alkaline lysis coupled with pressure cycling technology, organic differential extraction, and a selective digestion method used by the Broward sheriff's office (BSO) crime lab (Table 8). This was done to compare the DNA yields and STR profiles recovered using the new protocol with established practices. A small portion of the swab was used for extraction and all the extractions were performed in triplicate

Table 8. Correlation studies: Post-coital samples

Sample ID	Time since intercourse (hours)	Vasectomized partner	Race	Age range (years)	Time since menstruation (days)
PC161	7-12	No	Caucasian	31-40	8-14

PC162	7-12	No	Caucasian	21-30	8-14
PC176	13-18	No	Caucasian	31-40	8-14
PC177	13-18	No	Caucasian	21-30	8-14
PC178	13-18	No	Caucasian	21-30	8-14
PC179	13-18	No	Caucasian	31-40	15-21
PC180	13-18	No	Caucasian	31-40	15-21
PC181	13-18	No	Caucasian	31-40	15-21
PC164	31-36	No	Caucasian	31-40	8-14
PC163	37-42	No	Caucasian	31-40	8-14

C. Results and discussion

C.1. Stability studies

Stability studies were performed to determine if alkaline lysis and PCT method could extract high quality genomic DNA from samples exposed to environmental and chemical insults. Forensic samples are seldom found in a pristine condition in a crime scene. They are exposed to environment and come in contact with other agents that can degrade DNA. For example, a stain found on denim may be challenging to recover without co-extracting the indigo dye that can interfere with DNA and inhibit the PCR reaction. Humic acid found in soil binds to the DNA template and inhibits the processivity of polymerase enzyme. Hematin is found in red blood cells and is encountered in samples containing bloodstains. It interferes with the amplification of DNA molecule by binding to the Taq polymerase enzyme. These types of inhibitors are

common at crime scenes and it is therefore imperative to develop methods that can generate a full DNA profile despite these challenges.

In the presence of environmental and chemical insults, the concentration of Y-chromosomal DNA recovered from sperm donor 1 (S1) ranged from 24 ng/μL ± 1 to 43 ng/ μL ± 7 compared to 16 ng/μL ±4 extracted from the control. The concentration of Y-chromosomal DNA recovered from sperm donor 2 (S2) ranged from 5 ng/ μL to 9 ng/ μL compared to 2 ng/μL ±0.8 recovered from the control (Table 9). The results indicate that alkaline lysis and PCT successfully recovered DNA from samples exposed to different insults and produced higher yields compared to standard organic extraction. Reproducible DNA yields and complete male autosomal profiles were obtained from all samples. (Figure 33-36)

Table 9. Stability studies: Average concentration of Y-chromosomal DNA extracted from the samples.

Treatment	S1E1	S2E1	S1E3
Inhibitor mix	32 ± 5	9 ± 1	**
Denim	43 ± 7	5 ± 3	**
Outdoor environment	31 ± 4	**	24 ± 1

** *Note:* Stability study was not performed on these mixtures

DNA yield from control# 1 (sperm donor 1)- 24 ng/μL ± 1

DNA yield from control# 2 (sperm donor 2)- 16 ng/μL ± 4

The samples were extracted and quantified in triplicate ± standard error (SE)

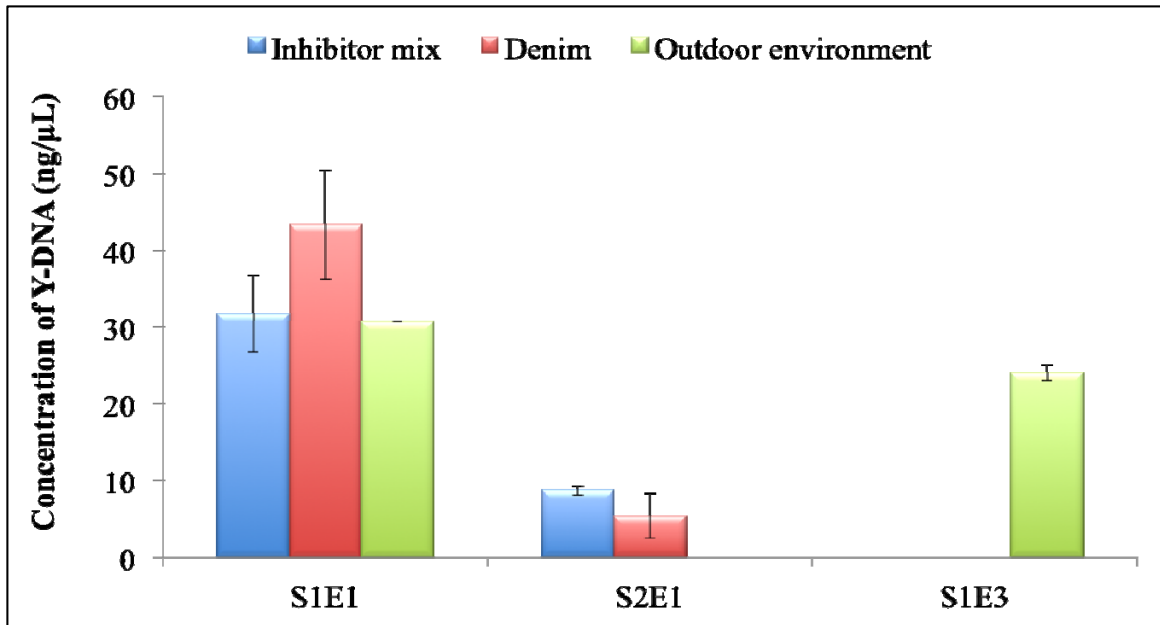


Figure 33. Stability studies. Concentration of Y-chromosomal DNA recovered from samples exposed to environmental insults. Alkaline lysis and PCT successfully extracted genomic DNA from all samples. (n=3 ± standard error)

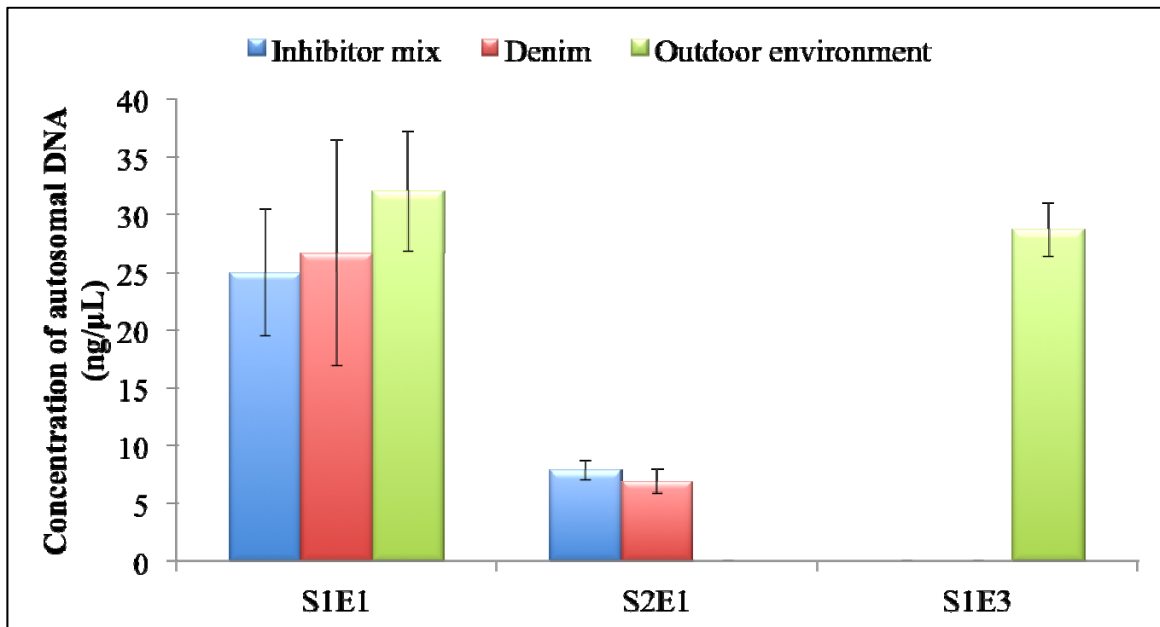


Figure 34. Stability studies. Concentration of autosomal DNA recovered from samples exposed to environmental insults. Alkaline lysis and PCT successfully extracted genomic DNA from all samples (n=3 ± standard error)

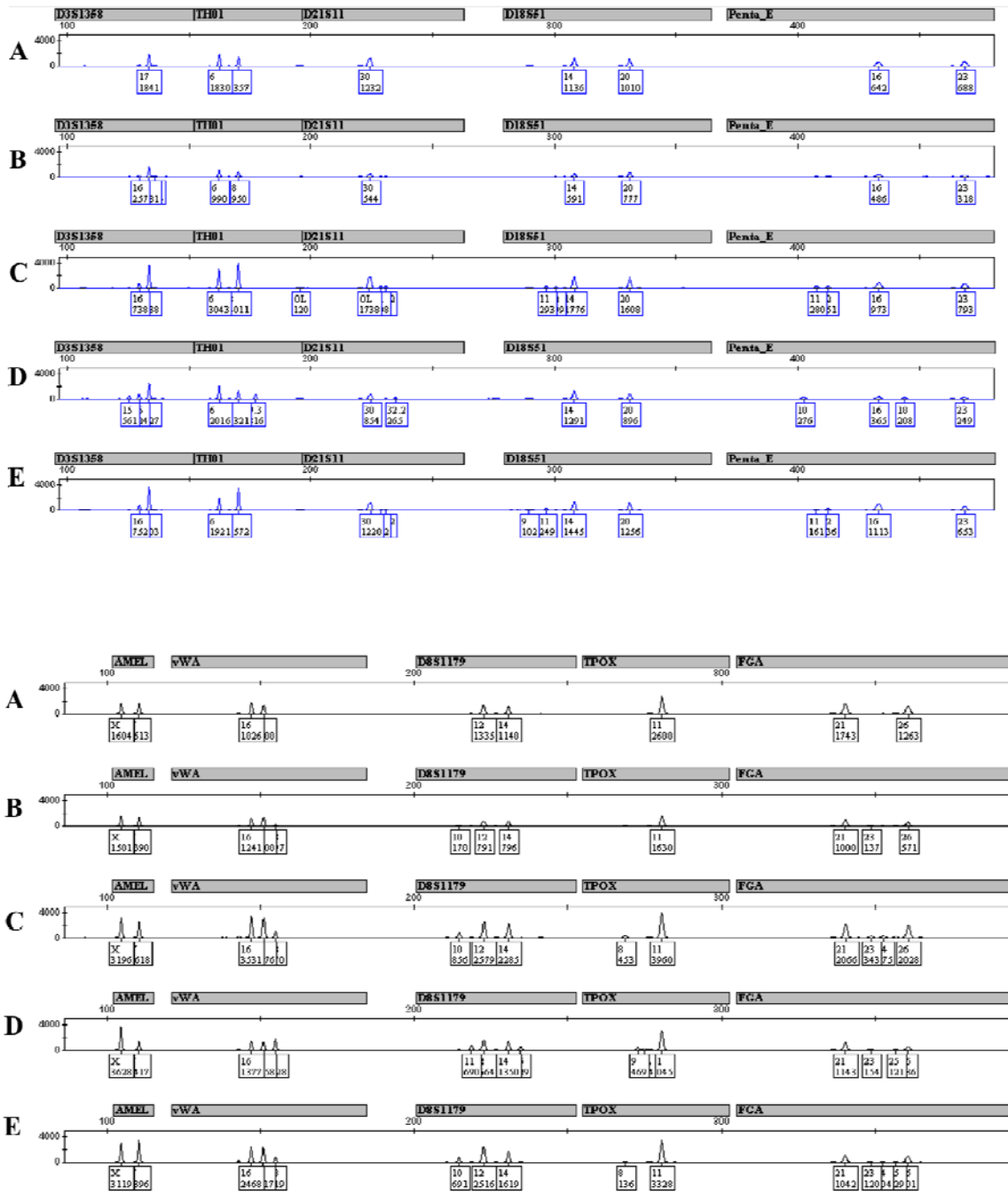


Figure 35. Electropherograms of DNA extracts recovered from samples exposed to environmental and chemical insults. A) Sperm control B) S1E1- Inhibitor mix C) S1E1-Denim D) S1E1- Outside environment E) S1E3- Outside environment. Full male DNA profiles were generated under all conditions. Blue panel represents fluorescein (FL)-labeled loci and black panel represents 6-carboxy-4', 5'-dichloro-2', 7'-dimethoxy-fluorescein (JOE)-labeled loci

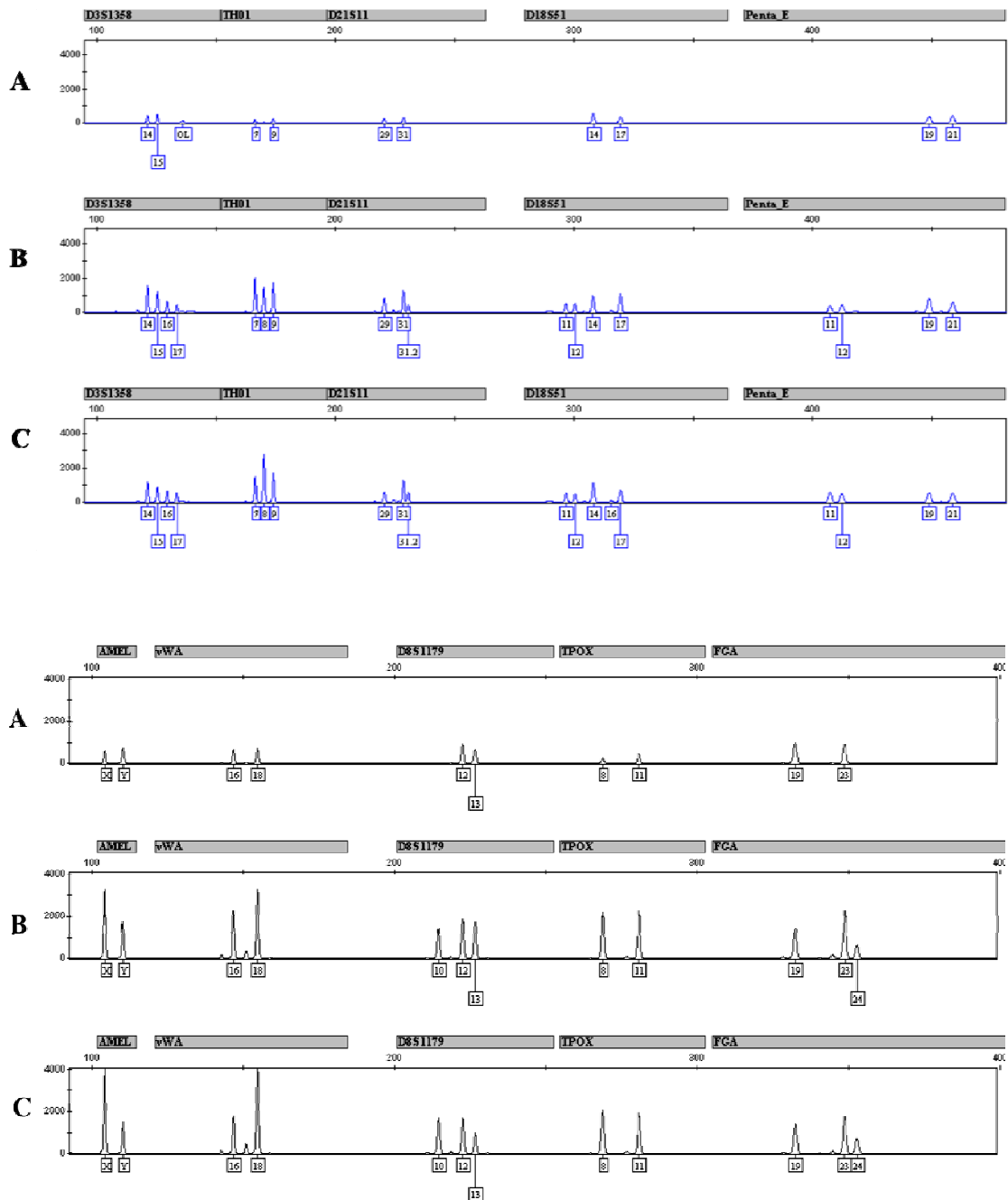


Figure 36. Electropherograms of DNA extracts recovered from samples exposed to environmental and chemical insults. A) Sperm control B) S2E1- Inhibitor mix C) S2E1-Denim. Full male DNA profiles were generated under all conditions but female DNA carryover is observed in the last two samples. Blue panel represents fluorescein (FL)-labeled loci and black panel represents 6-carboxy-4', 5'-dichloro-2', 7'-dimethoxy-fluorescein (JOE)-labeled loci

C.2. Reproducibility studies

To assess the reproducibility of the extraction efficiency using alkaline lysis and pressure cycling technology, six sets of samples were extracted in triplicate on three different days.

The average Y-chromosomal DNA concentration for sperm donor 1 (S1) ranged from 21 ng/ μ L to 23 ng/ μ L and for sperm donor 2 (S2) ranged from 4 ng/ μ L- 6 ng/ μ L. The values were reproducible in the presence of epithelial cells from different donors and when performed on three different days. The average concentration of male DNA recovered on three different days is given in Table 10. The control samples for sperm donor 1 and sperm donor 2 produced 16 ng/ μ L \pm 4 and 2 ng/ μ L \pm 0.8 Y-chromosomal DNA respectively. The results indicate that alkaline lysis and PCT gave reproducible and higher male DNA yields compared to control samples extracted with standard proteinase K organic extraction.

Table 10. Reproducibility studies: Average concentration of Y-chromosomal DNA

Sample	Concentration of Y-DNA (ng/ μ L)			SE
	Min	Max	Mean	
S1E1	17	25	21	2
S1E2	14	30	23	5
S1E3	16	29	22	4
S2E1	3	5	4	1
S2E2	4	8	6	1
S2E3	3	6	5	1

Note: The minimum, maximum and mean values were obtained from samples extracted over the course of 3 days.

SE, standard error

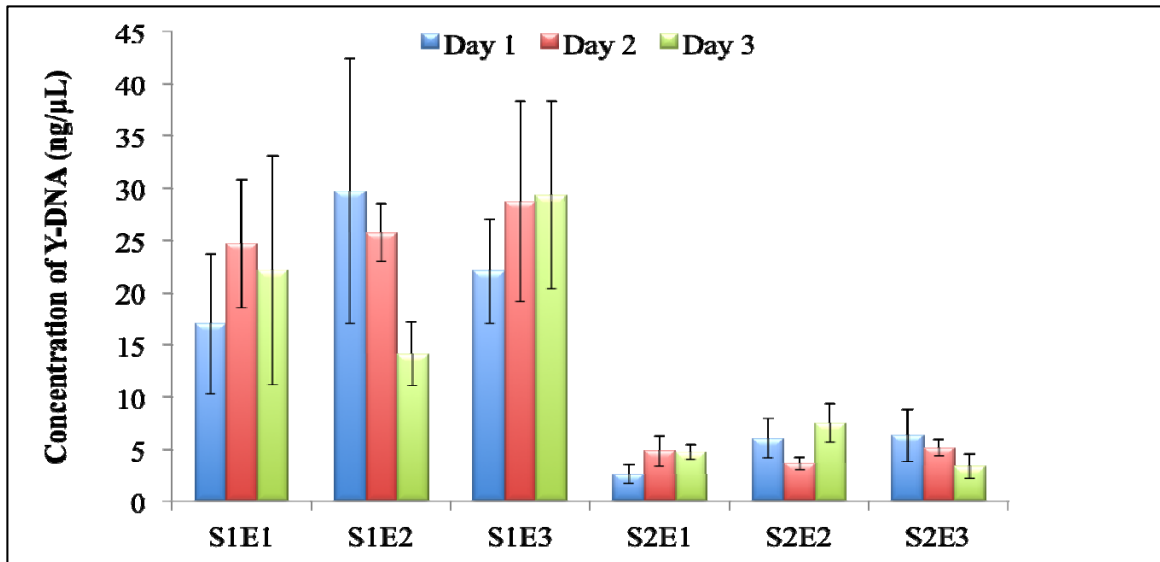


Figure 37. Reproducibility studies. Concentration of Y-chromosomal DNA extracted from samples on three different days indicates reproducibility of the extraction procedure remains unaffected. (n=3 ± standard error)

C.3. Sensitivity studies

Sexual assault samples obtained from real crime scenes are often overwhelmed with female epithelial cells resulting in a DNA profile that favors the female victim more than that of the perpetrator. In order to determine if the current method can lead to sperm fraction enrichment in the presence of overwhelming amount of vaginal epithelial cells, the method was applied to samples containing increasing amount of epithelial cells with a maximum of 150000 epithelial cells for a mixture containing fifty times more female cells compared to male cells.

Male autosomal STR profiles were obtained with samples containing up to ten times more female epithelial cells. The peak height ratio (PHR) of the Amelogenin marker indicates whether the profile is predominantly male or female. When the value of the relative fluorescence unit (rfu) of Y allele divided by the relative fluorescence unit (rfu) of the X allele is closer to 1.0, it indicates a predominantly male profile. The lower

the value is below 1.0, the greater is the amount of female DNA in the mixture. The results indicate a clean male profile in the presence of equal ratio of sperm cells to epithelial cells. Although a mixed profile was obtained with samples containing more than 1:2 ratio of male to female cells, a complete autosomal male STR profile could still be recovered from samples containing up to ten times more female cells (Table 11)

Table 11. Sensitivity studies: Effect of alkaline lysis and PCT on samples with mixture imbalance

Ratio of male: female	# Sperm cells	#Epithelial cells	Sperm fraction	Sperm fraction	Profile type/ Major contributor
			PHR (Amelogenin -Y/X)	#Male/ 16	
1:1	60000	60000	0.87	16	Single source
1:2	48000	96000	0.78	16	Mixed / Male
1:5	19,200	96000	0.44	16	Mixed/ Female
1:10	9600	96000	0.16	16	Mixed/ Female
1:50	3000	150000	0.06	15	Mixed/Female

Allelic peak heights from male and female contributors were divided by the total peak height at the respective locus. A total of seven loci with no shared alleles between the male and female DNA profiles were selected to calculate the percent contribution of male and female DNA in the sperm fraction.

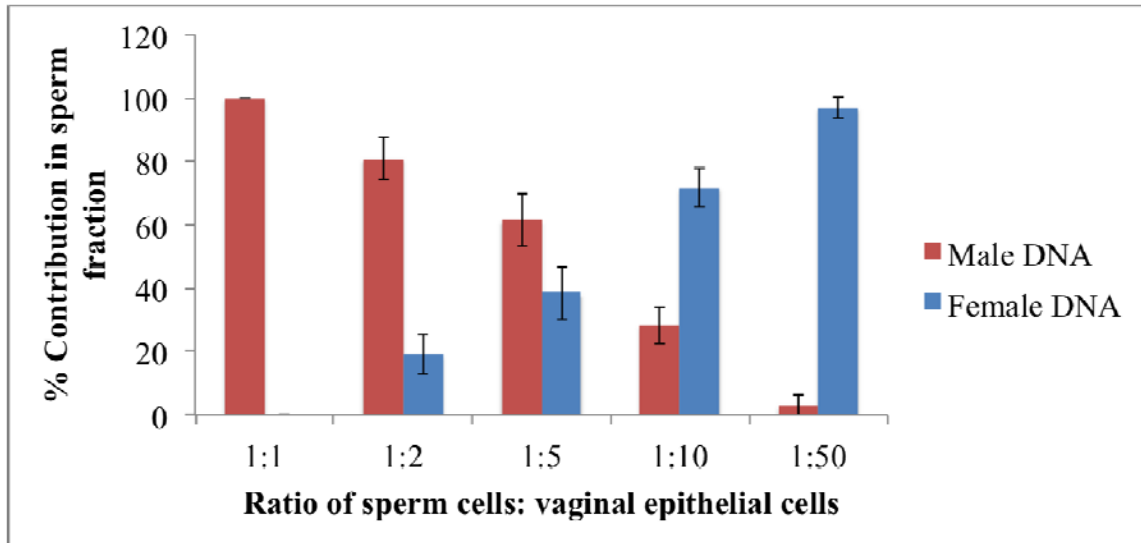
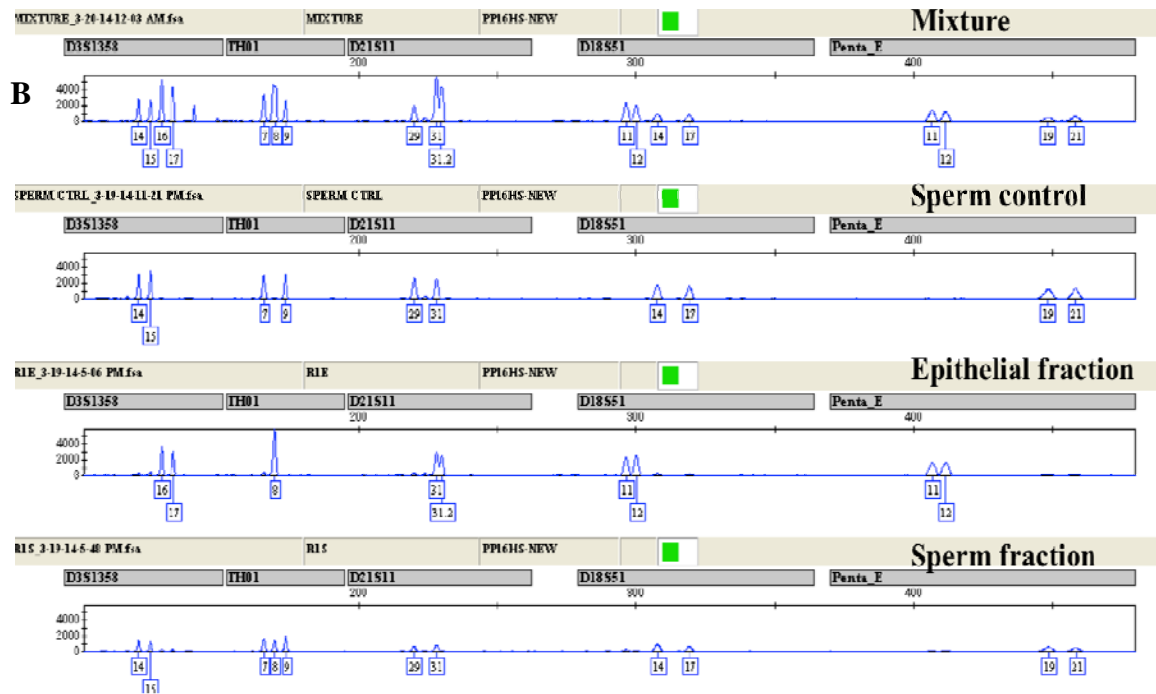
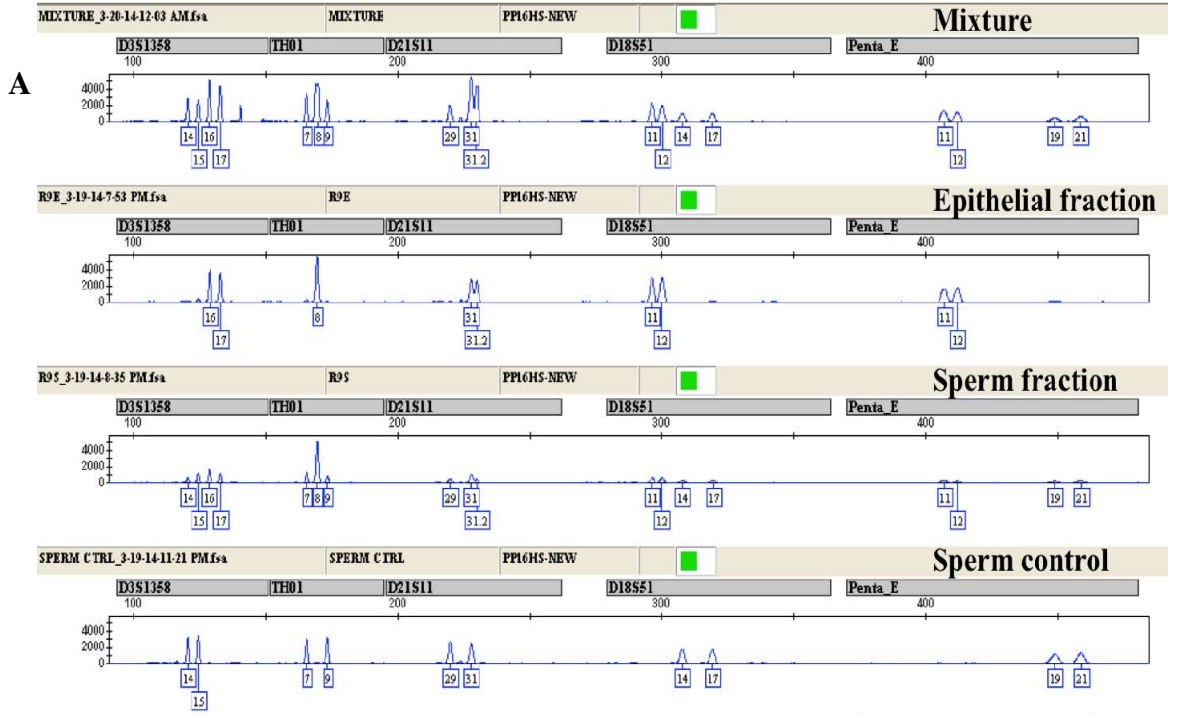


Figure 38. Sensitivity studies. Sperm fraction enrichment is observed in samples overwhelmed by almost ten times more female cells. More male DNA is recovered from samples containing up to five times more female cells.

The results from sensitivity studies indicate that a clean male autosomal STR profile can be obtained with samples containing a 1:1 mixture. Above this level, increasingly larger amounts of female DNA are present (Figure 38). The major and minor contributor in the final fraction was determined by dividing the sum of peak heights of male or female alleles by the sum of peak heights of all alleles at the respective locus. STR loci that are heterozygous for both male and female profile were chosen to calculate the percent contribution to the final DNA profile generated following extraction by alkaline lysis and pressure cycling technology. Results in figure 39 indicate that a complete male autosomal STR profile was obtained from samples with ten times more female cells. Loss of male alleles were observed when samples had female component in excess of fifty times to that of male cells.



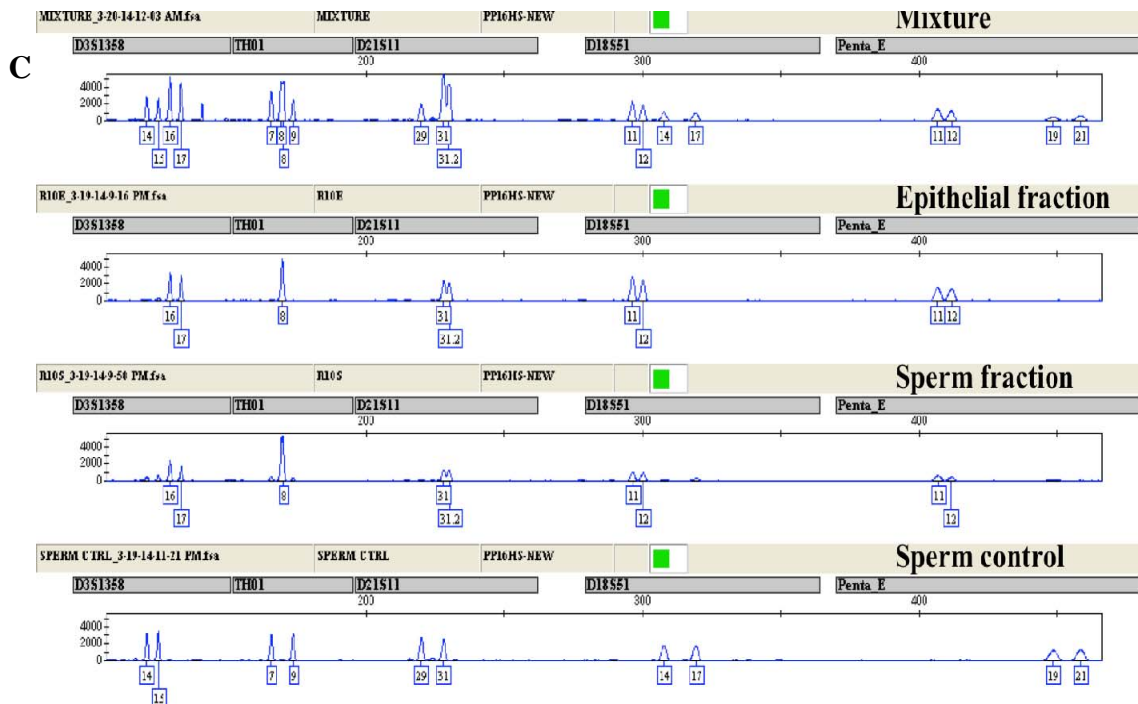


Figure 39. Electropherograms of DNA extracts recovered from different ratios of sperm cells and epithelial cells A) A clean separation of male profile is observed with 1:2 ratio of male to female cells B) A full male autosomal profile is seen with 1:10 ratio of male to female cells but female DNA carryover is also observed C) A complete loss of male alleles is observed in samples overwhelmed by female component (1:50 male to female)

In the presence of a 1:1 ratio of male sperm cells to female epithelial cells, a clean male profile was obtained but a mixed profile was seen with increasing amount of female cells. In the presence of double the amount of female cells, the final sperm fraction obtained with alkaline lysis and pressure cycling technology contained four times more male DNA compared to female DNA resulting in a predominantly male DNA profile. Male allelic dropout was observed when the sample was overwhelmed with 150,000 female cells compared to 3000 male cells (Figure 39).

To determine the sensitivity of the method, mixtures were created in the ratio of 1:5 sperm cells to vaginal epithelial cells and overall cell count was dropped from 48000

to 3000 cells. Sperm fraction enrichment was observed and male DNA was recovered from even a small sample volume of 500 sperm cells. (Figure 40)

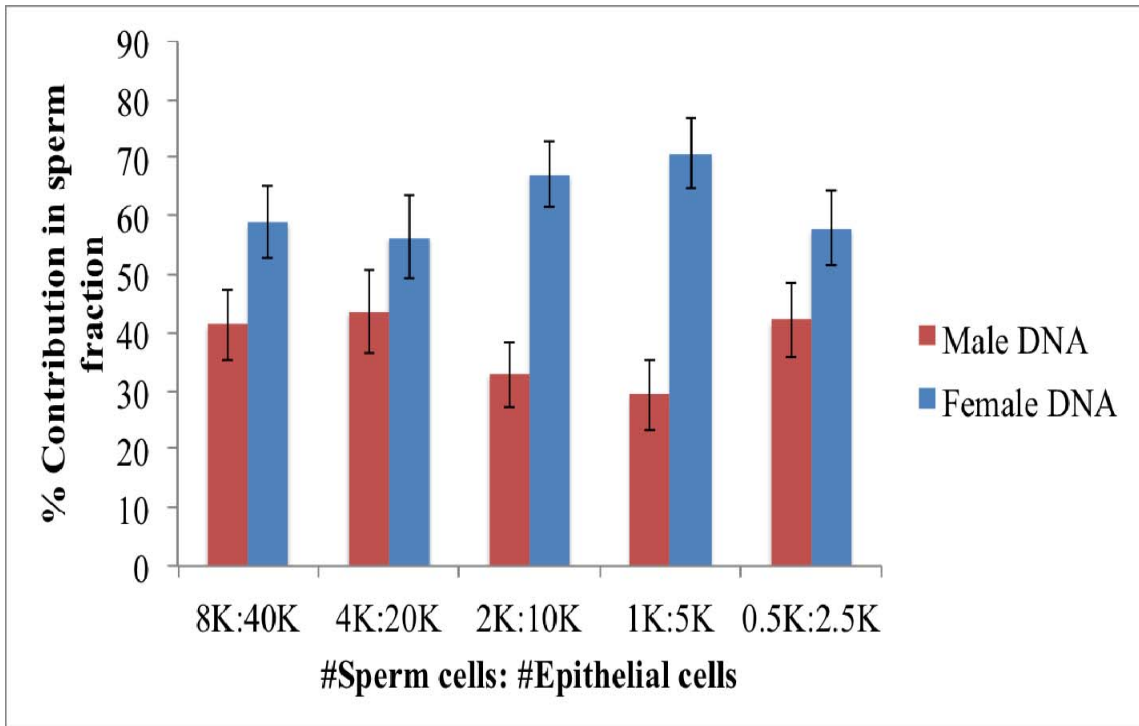


Figure 40. Sensitivity studies. The effect of alkaline lysis and pressure cycling technology on low sample levels. Male DNA was recovered from as little as 500 sperm cells. (n=3 ± standard error)

Evaluation of the peak height ratios at amelogenin marker indicates the presence of a predominantly female profile. But there was no loss of male alleles and all the 16 loci were detected in the male genotype. (Table 12)

Although a mixed profile was obtained with major contribution from female epithelial cells, a complete male autosomal STR profile was obtained with as little as 500 sperm cells (Figure 41). This indicates the effectiveness of pressure-based alkaline extraction on recovering sample from difficult substrates.

Table 12. Sensitivity studies. Effect of alkaline lysis and PCT on low sample volumes

Total # cells	# Sperm cells	#Epithelial cells	Sperm fraction		Profile type/ Major contributor
			PHR (Amelogenin -Y/X)	#Male/16	
48000	8000	40000	0.24	16	Mixed/ Female
24000	4000	20000	0.31	16	Mixed / Female
12000	2000	10000	0.25	16	Mixed/ Female
6000	1000	5000	0.13	16	Mixed/ Female
3000	500	2500	0.18	16	Mixed/ Female

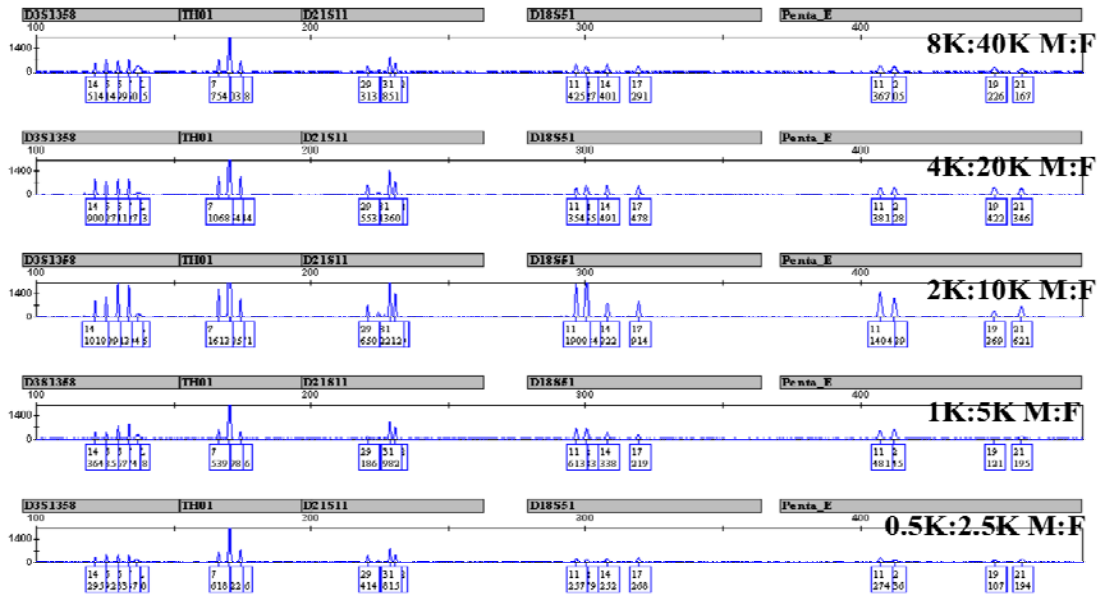


Figure 41. Sensitivity studies. Electropherograms of DNA extracts recovered from low sample levels. A complete male autosomal profile was obtained from as little as 500 sperm cells in the presence of five times more female cells.

C.4. Case-type samples

Clothing is one of the most common places to look for evidence in sexual assault cases. Sample deposited on different items of clothing was extracted with alkaline lysis and PCT to determine how the DNA yields and the genomic profile are affected.

Since the same volume of sample was applied to all substrates, similar DNA yields were expected. The amount of DNA extracted from the sperm control was $16 \text{ ng}/\mu\text{L} \pm 4$. The yields from case-type samples varied between $0.2 \text{ ng}/\mu\text{L} \pm 0.1$ and $7 \text{ ng}/\mu\text{L} \pm 1$ depending on the substrate and the sampling area. For example, yields dropped significantly when sperm DNA was extracted from a cotton panty ($0.2 \text{ ng}/\mu\text{L} \pm 0.1$) (Figure 42). It was observed during sampling that the fabric of the cotton panty had spandex in it that rendered the fabric more elasticity. The fabric got stretched when the substrate was excised and we hypothesize that as a result of this fewer cells were subjected to the extraction procedure compared to other more rigid and compact materials. The materials with less open structure and elasticity gave higher yields. For example, denim exhibited the best recovery among all the substrates with $7 \text{ ng}/\mu\text{L} \pm 1$ male DNA recovery. The recovery dropped with increasing elasticity and more open weave like a pair a socks ($2 \text{ ng}/\mu\text{L} \pm 1$) that caused the sample to seep out before it air-dried. Nevertheless, complete male autosomal STR profiles were still obtained from all the substrates. The major contribution came from the male DNA even in samples that showed a mixed profile. (Figure 43)

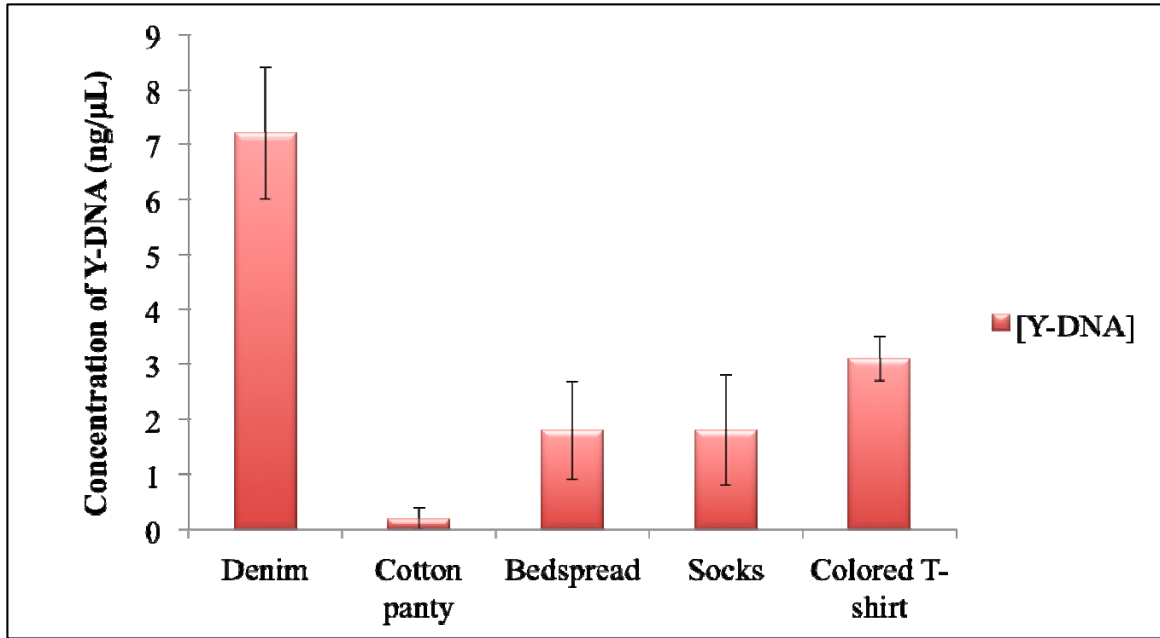
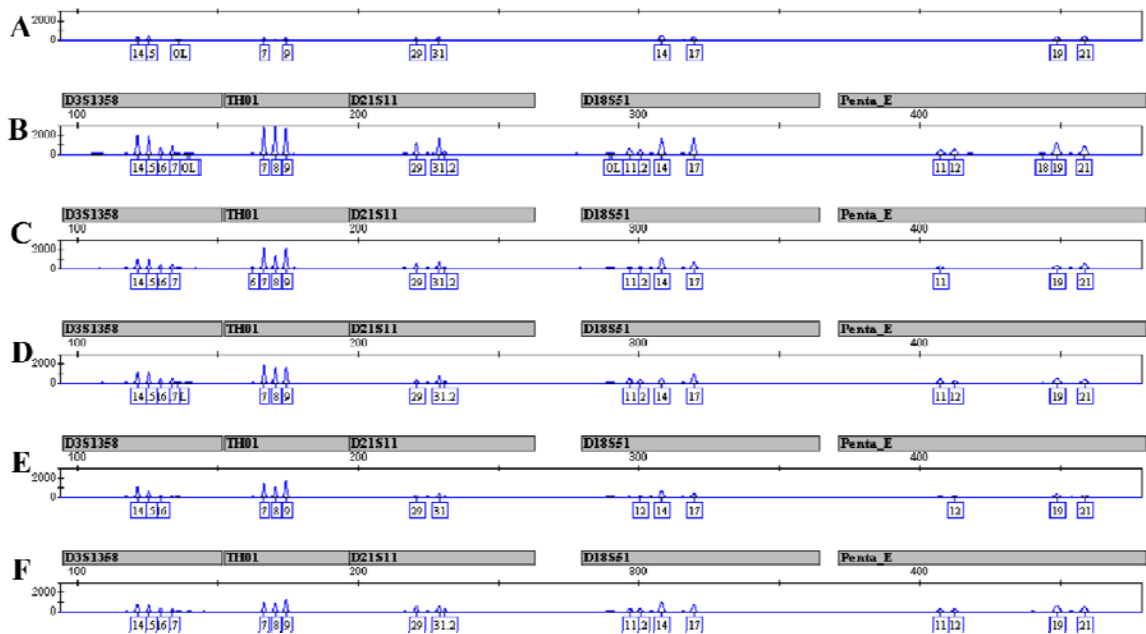


Figure 42. Case-type samples. The effect of alkaline lysis and pressure cycling technology on mixtures deposited on various substrates. DNA yields varied depending on the substrate but a complete male profile was obtained from all samples. (n=3 ± standard error)



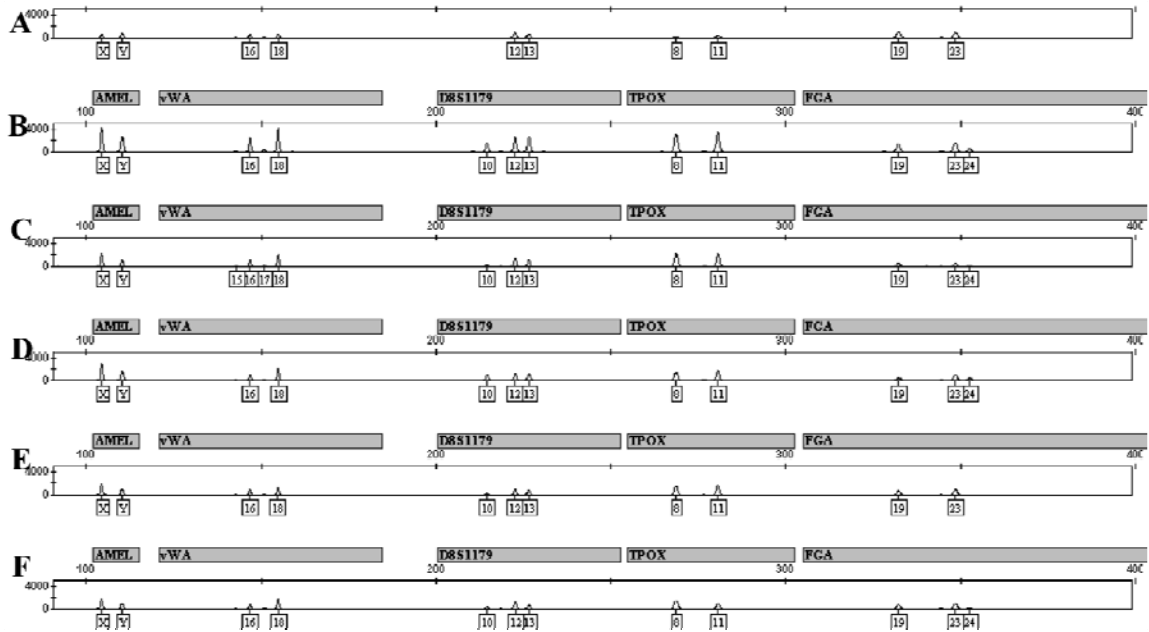


Figure 43. Electropherograms of DNA extracts recovered from samples deposited on various substrates A) Sperm control B) Denim C) Cotton panty D) Bedspread E) Socks F) Colored T-shirt. Full male DNA profiles were generated from all samples. Blue panel represents fluorescein (FL)-labeled loci and black panel represents 6-carboxy-4', 5'-dichloro-2', 7'-dimethoxy-fluorescein (JOE)-labeled loci

C.5. Correlation studies

Pressure-based alkaline lysis was compared with the method used by Broward sheriff's office crime lab and organic differential extraction. Most of the practicing labs use some modification of the organic extraction protocol. This comparison study was therefore done to evaluate the performance of the new alkaline method by comparing the DNA yields in the final sperm fraction. The concentration of Y-chromosomal DNA is used to determine the amount of male DNA recovered which is the target of interest. Autosomal DNA concentration was also evaluated to determine the amount of female DNA carryover and its effect on the STR profile.

Post-coital samples collected from ten volunteers were extracted in triplicate using each method for a total of 60 samples. The concentrations of autosomal and male DNA recovered is summarized below (Table 13). The data indicates that alkaline-based pressure cycling lysis recovered more male DNA from all post-coital samples compared to organic differential extraction. These yields were either comparable or better than the samples extracted using Broward sheriff's office (BSO) method. (Figure 44) This is a significant observation because alkaline lysis requires a total of less than 15 minutes incubation in lysis conditions whereas the two other protocols require couple of hours of incubation in the lysis buffer.

Table 13. Correlation studies. Comparison of DNA yields obtained using different extraction protocols reveals alkaline lysis and PCT to be comparable or better than BSO method and much more efficient than organic differential extraction.

Sample ID	BSO		Differential Extraction (DE)		Alkaline lysis + PCT	
	[Auto]	[Y]	[Auto]	[Y]	[Auto]	[Y]
PC161	2 ± 0.5	2 ± 0.5	4 ± 3	1 ± 0.6	25 ± 9	4 ± 2
PC162	0.4 ± 0.1	0.2 ± 0.03	0.2 ± 0.06	0.04 ± 0.01	0.7 ± 0.4	0.2 ± 0.4
PC176	3 ± 2	3 ± 1	3 ± 1	2 ± 1	11 ± 6	2 ± 1
PC177	0.03 ± 0.02	0.02 ± 0.02	0.004 ± 0.002	0.001 ± 0.00	0.08 ± 0.03	0.06 ± 0.06
PC178	0.2 ± 0.1	0.1 ± 0.08	0.05 ± 0.01	0.02 ± 0.01	1 ± 0.4	0.04 ± 0.01

PC179	0.5 ± 0.3	0.4 ± 0.3	0.09 ± 0.08	0.03 ± 2	11 ± 7	2 ± 1
PC180	4 ± 1	4 ± 1	2 ± 0.7	0.6 ± 0.2	33 ± 6	4 ± 2
PC 181	2 ± 1	2 ± 1	2 ± 0.7	0.6 ± 0.3	64 ± 9	5 ± 0.8
PC164	0.3 ± 0.08	0.3 ± 0.2	0.7 ± 0.1	0.03 ± 0.01	13 ± 4	0.5 ± 0.3
PC163	0.7 ± 0.3	0.2 ± 0.08	2 ± 1	0.02 ± 0.01	65 ± 13	0.3 ± 0.05

Note: BSO- Method validated and used by Broward sheriff's office (BSO) crime lab

Alkaline lysis + PCT- The novel differential extraction protocol developed using pressure cycling technology (PCT) under alkaline conditions

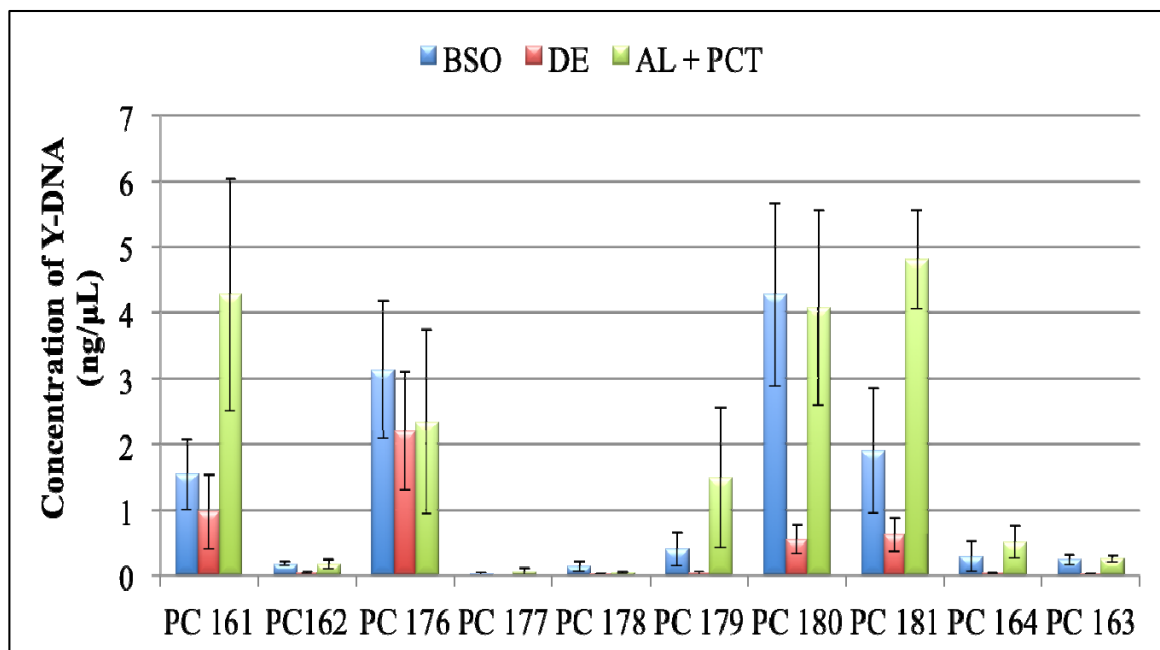


Figure 44. Correlation studies. Comparison of male-specific Y-chromosomal DNA yields revealed better recoveries with alkaline lysis and pressure cycling technology compared to existing differential extraction protocols.(BSO- Differential digestion method used by Broward sheriff's office crime lab; DE- Organic differential extraction; AL +PCT- Alkaline lysis with pressure cycling technology) (n=3 ± standard error)

All three methods failed to recover DNA from samples PC 177 and PC 178 that may be attributed to insufficient sample. Alkaline lysis with pressure cycling technology and BSO extraction protocol produced consistently better yields compared to organic differential extraction. Alkaline lysis with pressure cycling technology produced the highest yields from three samples (PC 161, PC 179 and PC 181) compared to the other two protocols (Table 13). The male DNA yields using alkaline lysis and PCT for these three samples ranged from 2 ng/ μ L \pm 1 to 5 ng/ μ L \pm 0.8 which is sufficient to obtain a complete male autosomal STR profile. But this was not the case when all the STR profiles were analyzed. Even with samples that gave higher male DNA yields using alkaline extraction, the male contribution to the STR profile was lower than with other extraction methods (Figure 45). This may be due to inefficient separation in the presence of large amount of female tissue. Studies done by other groups reported that male DNA can go undetected when it is overwhelmed more than ten times by female amplification products [85]. Since the input DNA for PCR amplification is normalized using autosomal DNA yields, samples containing at least twenty times more male DNA compared to autosomal DNA will result in loss of male profile.

The results from correlation studies therefore indicate that alkaline lysis coupled with pressure-based extraction gives the best DNA recoveries under 2 hours which includes 15-minute extraction procedure followed by phenol chloroform purification compared to other extraction protocols that include an incubation time ranging between 2 hours and overnight to lyse the cells [13, 23, 34, 48]. But female DNA carryover and loss of male profile is observed when the sample is overwhelmed by fifty times female tissue.

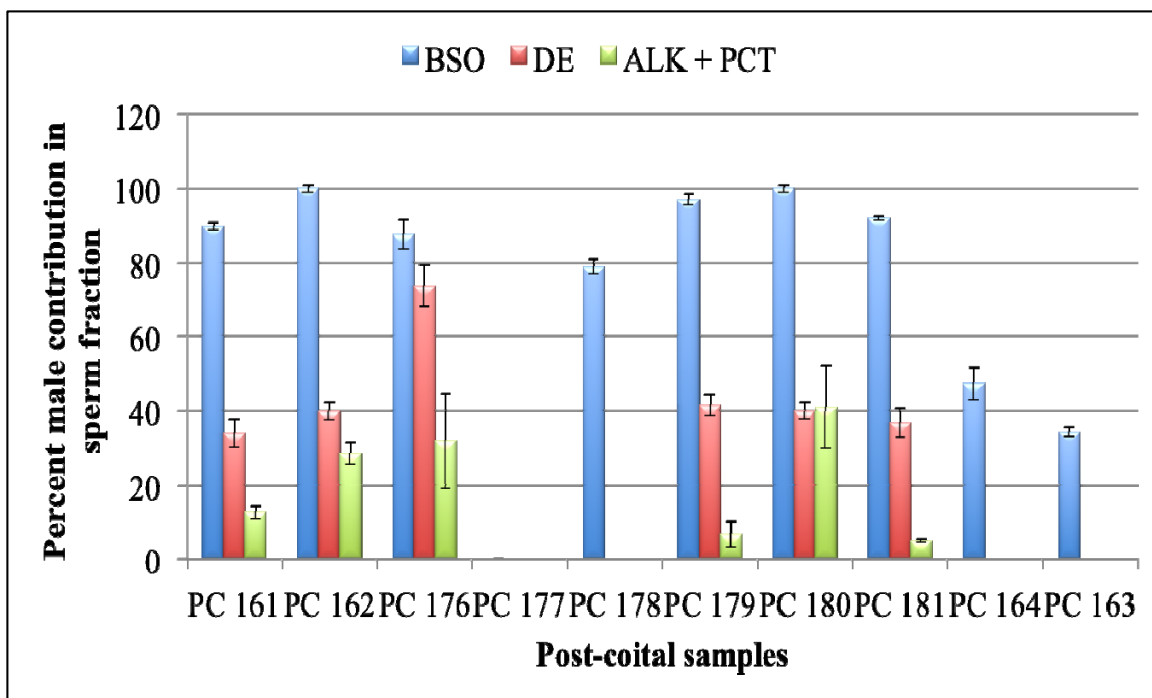


Figure 45. Correlation studies. Percent male contribution in sperm fraction indicates loss of male profile in samples containing more autosomal DNA compared to male-specific DNA indicating insufficient sample separation with alkaline lysis compared to other methods.

D. Concluding remarks

Using this method a clean male DNA profile was generated from mixtures containing a comparable amount of sperm cells and vaginal epithelial cells. Mixed profile was obtained in instances where an imbalance of mixtures favoring female tissue is observed. Loss of male alleles was observed when the sample was overwhelmed with fifty times more female cells but a complete male profile was obtained with mixtures containing low levels of sperm cells indicating that in contrast to previous reports that observed almost 90% sperm cell loss from cotton swabs due to inefficient recoveries this method proved to be more effective in recovering tissue from substrates. Therefore methods involving alkaline lysis combined with pressure cycling technology may provide

a new direction to address issues such as inefficient sample recoveries despite the long incubation times employed by current extraction protocols that create a bottleneck in forensic DNA testing.

XI. CONCLUSIONS

Sexual assault cases are plagued by challenges in sample analysis due to the presence of mixtures of male and female cells. The extracted sample is often overwhelmed by female epithelial DNA that results in the male autosomal STR profile masked by the female STR profile thus leading to difficulties in data interpretation. The goal of this study was to develop a method combining the use of pressure cycling technology and alkaline lysis to disrupt sperm cells and recover their DNA.

The extraction procedure is performed utilizing the Barocycler[®] NEP 2320, a commercially available instrument from Pressure Biosciences Inc. (South Easton, MA), equipped with a hydrostatic pressure chamber that generates alternating cycles of ambient and high pressures with a range of 5- 45 kpsi. Another goal of this study was to enhance sample recovery from cotton swabs. Cotton swabs are often used to collect evidence in a crime scene but the inefficient sample recovery from this substrate is a common problem. In order to enhance DNA recovery and hence improve downstream genetic analysis, the effect of alkaline lysis on sample recovery from cotton swabs was studied.

The current study involves the application of pressure cycling technology in the selective digestion of sperm cells from evidence mixtures with an emphasis on the role of buffer composition on sperm DNA yields and increase in selectivity of extraction. The cells were extracted into 1X PBS buffer (pH 7.4) with varying buffer compositions and subjected to 45000 psi pressure. To improve sample recoveries, cotton swabs containing sample were incubated in different concentrations of Sodium hydroxide under varying temperature and incubation times. Following extraction, all the samples were purified

using phenol chloroform isoamyl alcohol purification and quantified with Promega Plexor[®] HY system followed by an STR analysis using Promega PowerPlex[®] 16 HS system.

These results indicate that the application of pressure cycling technology, in the presence of appropriate buffers, can result in 50-60% recovery of male DNA from mixtures. These observations were reproduced with mixtures on cotton swabs where six times more male DNA was recovered compared to female epithelial DNA. Furthermore, alkaline lysis studies were performed to determine the effect of DNA recovery from cotton swabs, which have always posed a challenge in sample processing. Almost seven-fold increase in DNA recovery from swabs was observed under specific alkaline lysis conditions. Combining pressure cycling technology and alkaline lysis resulted in 5-6-fold enhancement in the relative ratio of sperm DNA in these mixtures and also improved DNA yields with an incubation time of less than 10 minutes in lysis buffer.

Validation studies were done to test the robustness and the reliability of this method in generating a male DNA profile from mixtures. Even in the presence of most commonly encountered inhibitors in forensic casework such as hematin (found in blood), indigo dye (denim) and humic acid (soil), full male DNA profiles were obtained. Exposure to environmental insults for one week did not affect male DNA yields. Reproducibility studies indicated that the yields are unaffected in the presence of different female contributors and when samples are extracted on three different days. The DNA recovery from sperm donor 1 ranged from a minimum of 21 ng/ μ L \pm 2 to 22 ng/ μ L \pm 4 and the DNA recovery from sperm donor 2 ranged from a minimum of 4 ng/ μ L \pm 0.7 to 6 ng/ μ L \pm 1. Sensitivity studies demonstrated that a predominantly male

DNA profile is obtained from samples overwhelmed by five times more female cells. Male allelic dropout was observed when the samples contained in excess of fifty times more female cells. This method was also able to generate a full male STR profile from as little as 500 sperm cells. Sperm DNA was successfully extracted from a variety of substrates including bedspread, cotton panty, t-shirt, socks and denim which proves the potential application of this method in forensic casework. Finally correlation studies were done to compare this method with established protocols. The observations from this study indicate that equal or better male DNA yields were obtained with alkaline lysis coupled with pressure cycling technology but female DNA carryover was observed in samples overwhelmed by female tissue which resulted in predominantly female DNA profile.

Short extraction times including less than 20 minutes incubation in a lysis buffer, high yields, inexpensive reagents and semi-automated platform further make alkaline lysis-based pressure cycling technology a potential candidate for extracting DNA from forensic evidentiary materials.

XII. FUTURE WORK

This study introduced a new technique to effectively extract biological tissue from difficult substrates and further demonstrated its potential in analyzing complicated mixtures but there are three points that need further exploration. One of the biggest limitations of this method is the ability to process only one sample at a time using PULSE tubes in a barocycler. The ability to expand this protocol for use with smaller MicroTubes that permit larger sample throughput will highly benefit this study. Second issue concerns the female DNA carryover observed in samples overwhelmed with more than fifty times female epithelial cells. The current method employs a short 5-minute incubation time to lyse female cells which is not sufficient when the sample has exceedingly large amount of female tissue. Increase in incubation time to ten minutes indicated a decrease in male DNA yields. Other approaches to further minimize this female DNA carryover need to be studied. Lastly, more validation studies including a large population will further strengthen the findings of this study.

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APPENDICES

Appendix 1

Stock Solutions

0.5M EDTA, 500 mL: Add 93.05 g Ethylenediaminetetraacetic acid di-sodium salt dehydrate to 400 mL ddH₂O. Adjust pH to 8.0 with 10N NaOH. Adjust final volume to 500 mL.

20% SDS, 500 mL: Dissolve 100 g sodium dodecyl sulfate in 400 mL ddH₂O and adjust final volume to 500 mL.

0.9% NaCl, 500 mL: Dissolve 4.5 g NaCl in 400 mL ddH₂O. Adjust final volume to 500 mL.

2M Tris-HCl, pH 8.0, 1000 mL: 242.2.10 g Tris base in 800 mL ddH₂O. Adjust pH to 8.0 with HCl. Adjust final volume to 1000 mL.

10 N NaOH, 50 mL. 20 g NaOH to 30 mL ddH₂O. Adjust final volume to 50 mL. Store in plastic bottle.

Buffers:

TE (10mMTris-HCl/ 0.5 mM EDTA, pH 8.0), 500 mL: Add 5 mL of 1 M Tris-HCl and 100 μ L of 0.5 M EDTA to 395 mL ddH₂O. Autoclave.

310 Buffer 10X (1M TAPS, 20 mM EDTA pH 8.0), 100 mL: Add 24.33 g TAPS and 4 mL 0.5M EDTA to 70 mL ddH₂O. Adjust pH to 8.0 with 10N NaOH. Adjust final volume to 100 mL.

Differential Lysis Buffer (DEB) (100 mM NaCl, 10 mM EDTA, 0.4% SDS), 500 mL: 2.92 g NaCl, 10 mL 0.5 M EDTA, 10 mL 20% SDS. Adjust final volume to 500 mL with ddH₂O.

Stain Extraction Buffer (SEB) (10 mM Tris, 100 mM NaCl, 10 mM EDTA, 2% SDS, 38 mM DTT), 500 mL: Dissolve 2.92 g NaCl in 250 mL ddH₂O. Add 5 mL 1M Tris, 10 mL 0.5 M EDTA, 50 mL SDS. Titrate to pH 8.0 with HCl. Adjust final volume to 500 mL with ddH₂O. Add 6 mg/mL DTT when ready to use. Keeps for 2 weeks in fridge after DTT is added.

Appendix 2

Protocol: Standard organic extraction

I. Purpose

To extract and purify human DNA from cotton swabs containing single-source samples.

II. Safety considerations

Observe all standard laboratory precautions

Warning: The following reagents are considered to be hazardous and require the use of appropriate personal protective equipment. These reagents should be handled in a fume hood.

Phenol/chloroform/isoamyl alcohol has toxic, corrosive and suspected carcinogenic properties that render it harmful or fatal if ingested, inhaled, or exposed to the eyes. Phenol is also a skin irritant and may cause burn if it comes in contact with skin. It is mandatory to perform experiments involving phenol in a fume hood and wear protective gloves.

DTT is an irritant and can be harmful if ingested, inhaled or if it comes in contact with skin.

Reagents

PCR ddH₂O
Stain Extraction Buffer with dithiothreitol (DTT)
Proteinase K (20 mg/mL)
Phenol/Chloroform/Isoamyl Alcohol (25:24:1) (PCIA)
ddH₂O

Procedure

- 1) Cut the swab with sterile scissors and place it in a 2 mL microcentrifuge tube
- 2) Preparation of stain extraction buffer (SEB).
 - a. Weigh out 6 mg of Dithiothreitol (DTT) per mL of SEB to be used.
 - b. Add DTT to filtered SEB in 15 mL tube, and mix. May be stored up to two weeks in the refrigerator.
- 3) Add 300µL of SEB and 2 µL of Proteinase K to each swab sample.
- 4) Incubate samples at 56°C for 2-4 hours.

- 5) Remove swab sample from tube and place in spin basket. Place spin basket back in tube, cap, and spin in microcentrifuge for 1 minute at 5000 rpm.
- 6) Remove spin basket from tube and throw away.
- 7) Add 300 μ L PCIA to each sample. Mix and spin at 13000 rpm for 10 minutes.
- 8) Remove aqueous (top layer) from sample tube with 100 μ L pipettor and tips. Transfer to another fresh tube. Make sure not to remove any of the organic layer.
- 9) Add 2.5 volumes of absolute ethanol and 1/10 the volume of 3M Sodium acetate solution to the aqueous layer. Place the samples in the freezer for overnight incubation.
- 10) Centrifuge the samples at 13000 rpm for 10 minutes
- 11) Remove the supernatant and do not disturb the DNA pellet
- 12) Wash the pellet with 70% alcohol
- 13) Re-suspend the pellet in 60 μ L water
- 14) Incubate in the water bath at 56° C for 15 minutes.

Appendix 3

Protocol: Differential DNA extraction using alkaline lysis and pressure cycling technology (PCT)

I. Purpose

To extract and purify DNA from cotton swabs containing a mixture of male sperm cells and female vaginal epithelial cells using pressure-based extraction procedure

II. Safety considerations

Refer to the safety instructions in buccal swab extraction

Reagents

1X Phosphate buffered saline (PBS)
PCR ddH₂O
Phenol/Chloroform/Isoamyl Alcohol (25:24:1) (PCIA)
0.4 N Sodium hydroxide (NaOH) solution
2M Tris HCl pH 7.5
ddH₂O

Instrumentation

a. Barocycler set up

- i. Turn the compressor tank on by flipping the switch to “auto” or “on”.
- ii. Turn and barocycler on and ensure that the waste container is empty and the supply bottle is filled with ddH₂O up to the fill line.
- iii. Clear tube goes into the supply bottle and the red and blue tubes go into the waste bottle.
- iv. On the keypad, click “edit” and give a name for the protocol, using the touch pad, to save it for future runs.
- v. Click “enter” which leads to the next selection where the user can define the target high pressure. Choose a pressure value between 5000 psi and 45000 psi and click “enter”.
- vi. The next screen displays “Time1” which is the number of seconds that the sample will be exposed to high pressure. Choose a value between 1 and 99 seconds and hit “enter”.

- vii. The next screen displays “Time2” which is the number of seconds that sample is exposed to ambient pressure. Choose a value between 1 and 99 seconds and hit enter.
- viii. Next, choose the number of pressure cycles by selecting a value between 1 and 99 and hit enter.
- ix. The user is given an option to save this protocol, which can be done by hitting “yes” or “no” on the touchpad.

b. Barocycler operation

- i. Chamber closure seals the pressure chamber and lock pin holds the chamber closure to create a tight seal.
- ii. The vent button on top of the chamber closure is used to purge air from the chamber before the beginning of a run.
- iii. Slide the door open and pull the lock pin out while holding down the vent button. This allows the user to remove the chamber closure.
- iv. Place the tube containing the sample in the pressure chamber.
- v. Seal the chamber with chamber closure and locking pin.
- vi. Push the “precharge” key on the touch pad which pushing on the vent button to purge any air from the chamber.
- vii. Select a saved protocol or create a new one following the procedure described in the barocycler set up section.
- x. Hit “run” to begin.

c. PULSE tube loading

- i. Use the provided PULSE tube tool to assemble the tube.
- ii. Place the ram into the ram end of the tube and push it in using the PULSE tube tool until it hits resistance.
- iii. Place the sample in the sample chamber and add the reagents specific to the protocol. The PULSE tube can hold between 0.2 mL and 1.5 mL of liquid sample and the optimum sample input if working with solid or semi-solid samples is between 50 and 300 mg per tube.

- iv. Screw the blue screw cap in the sample end of the tube with the help of the PULSE tube tool to seal the tube.
- v. Use a forceps to retrieve the PULSE tube from the pressure chamber following pressure treatment.

Procedure

- 1) Cut the swab with a sterile scissors and place it in the PULSE tube.
- 2) Add 800 μ L of 0.4 N NaOH solution to the sample chamber and seal the tube with the blue screw cap.
- 3) Select the appropriate protocol, “precharge” the chamber by pushing down the vent button and hit “run” to begin. The optimized pressure parameters are,
Pressure- 20,000 psi
Time1- 15 seconds
Time2- 20 seconds
Number of cycles- 10
- 4) After pressure treatment, remove the PULSE tube from the chamber and transfer the solution and the swab cutting to 2.0 mL microcentrifuge tube.
- 5) Add 57.6 μ L of 2M Tris-HCl solution.
- 6) Mix and spin at 5000 rpm for 2 minutes.
- 7) Remove swab sample from tube and place in spin basket. Place spin basket back in tube, cap, and spin in microcentrifuge for 1 minute at 5000 rpm.
- 8) Transfer the swab cutting to 1.5 mL microcentrifuge tube to proceed with alkaline lysis at high temperature and purify the remaining solution by adding equal volume of PCIA. Refer to the standard organic extraction for PCIA purification and ethanol precipitation. This fraction contains female epithelial DNA.
- 9) To the swab cutting in 1.5 ml microcentrifuge tube, add 400 μ L of NaOH solution.
- 10) Mix and spin at 5000 rpm for 2 minutes.
- 11) Incubate the sample at 95°C for 5 minutes.

- 12) Remove the sample from the water bath and spin at 5000 rpm for 2 minutes.
- 13) Add 28.8 μ L of 2M Tris-HCl solution.
- 14) Mix and spin at 5000 rpm for 2 minutes
- 15) Remove swab sample from tube and place in spin basket. Place spin basket back in tube, cap, and spin in microcentrifuge for 1 minute at 5000 rpm.
- 16) Remove spin basket from tube and throw away. Proceed with PCIA purification.
- 17) Add 1 volume of PCIA to each sample. Mix and spin at 13000 rpm for 10 minutes.
- 18) Remove aqueous (top layer) from sample tube with 100 μ L pipettor and tips. Transfer to another fresh tube. Make sure not to remove any of the organic layer.
- 19) Add 2.5 volumes of absolute ethanol and 1/10 the volume of 3M Sodium acetate solution to the aqueous layer. Place the samples in the freezer for overnight incubation.
- 20) Centrifuge the samples at 13000 rpm for 10 minutes.
- 21) Remove the supernatant and do not disturb the DNA pellet.
- 22) Wash the pellet with 70% alcohol.
- 23) Re-suspend the pellet in 60 μ L water.
- 24) Incubate in the water bath at 56° C for 15 minutes. This fraction contains sperm DNA.

Appendix 4

Protocol: Organic differential extraction of DNA from semen stains

I. Purpose

To extract and purify DNA from cotton swabs containing a mixture of male sperm cells and female vaginal epithelial cells using organic differential isolation procedure.

II. Safety considerations

Refer to the safety instructions in standard organic extraction

Reagents

PCR ddH₂O
Stain Extraction Buffer
Proteinase K (20 mg/mL)
390 mM dithiothreitol (DTT)
Phenol/Chloroform/Isoamyl Alcohol (25:24:1) (PCIA)
ddH₂O

Procedure

- 1) Cut the swab with sterile scissors and place it in 2.0 mL microcentrifuge tube.
- 2) Add 500 μ L SEB and 5 μ L Proteinase K to the sample.
- 3) Incubate samples at 37°C for 2-4 hours.
- 4) Remove swab sample from tube and place in spin basket. Place spin basket back in tube, cap, and spin in microcentrifuge for 5 minutes at 13000 rpm.
- 5) Discard the spin basket and the swab.
- 6) Transfer the liquid portion above the sperm cell pellet into a new microcentrifuge tube (D1 fraction) and retain the pellet for further processing (D2 fraction)
- 7) Add 350 μ L SEB, 40 μ L 390 mM DTT and 10 μ L Proteinase K to the sperm cell pellet (D2 fraction)
- 8) Incubate at 37°C for two hours.
- 9) Remove the sample from the water bath and spin at 5000 rpm for 2 minutes.
- 10) Perform PCIA purification on D1 and D2 fractions

- 11) Add 1 volume of PCIA to each sample. Mix and spin at 13000 rpm for 10 minutes.
- 12) Remove aqueous (top layer) from sample tube with 100 μ L pipettor and tips. Transfer to another fresh tube. Make sure not to remove any of the organic layer.
- 13) Add 2.5 volumes of absolute ethanol and 1/10 the volume of 3M Sodium acetate solution to the aqueous layer. Place the samples in the freezer for overnight incubation.
- 14) Centrifuge the samples at 13000 rpm for 10 minutes
- 15) Remove the supernatant and do not disturb the DNA pellet
- 16) Wash the pellet with 70% alcohol
- 17) Re-suspend the pellet in 60 μ L water
- 18) Incubate in the water bath at 56° C for 15 minutes.

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