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IMPROVING DNA RECOVERY FROM FIRED CARTRIDGE CASINGS (FCCs) USING CHELATION FILTRATION AND STRMIX

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DEDICATION

I dedicate this dissertation to my family for their love, encouragement, and support.

Kaylee Crenshaw

Matthew Crenshaw

Ann Newman

John Graham

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ABSTRACT OF THE DISSERTATION

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by

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Professor Bruce McCord, Major Professor

Gun-related crimes are the cause of many homicides and injuries every year across the United States. According to the Federal Bureau of Investigation (FBI) statistics, firearms were used in 68% of murders, 41% of robberies, and 21% of aggravated assaults in 2011 alone(Reduction of Crime n.d.). Cartridges and casings left on the scene often link shootings to a firearm and many times are the only evidence. Traditional swabbing of fired cartridge casings (FCCs) has yielded usable profiles only approximately 1-2% of the time, causing many laboratories to refrain from processing FCCs.

A method for collection of the cellular material from the FCCs for downstream DNA analysis was developed and evaluated. Chelation filtration is a simple, easy-to-perform, two-step filtration method using Chelex resin in the initial incubation step and only requires a vacuum device along with two filter devices. The process does not create any need for alterations to downstream

DNA analysis. Comparisons of the Chelation filtration method (N=20) to traditional swabbing (N=20) were conducted and the quantity of DNA recovered (ng) as well as the percentage of expected alleles observed was evaluated using A-NOVA analysis, resulting in 40% ($P<0.001$) more alleles detected with the Chelation filtration method.

An anecdotal study of 122 cases was performed to evaluate the effectiveness of the Chelation filtration method on real casework evidence, where 21 interpretable DNA profiles were obtained yielding 5 CODIS matches. With the assistance of STRmix analysis, over 50% of those profiles were eligible for statistical calculations and STRmix was also used to deduce profiles for CODIS entry. The Chelation Filtration method can be an invaluable tool to help solve gun crimes where there is little to no other evidence besides FCCs.

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ABBREVIATIONS AND ACRYONYMS

°C	Degree Celsius
A	Adenine
ABI	Applied Biosystem by Life Technologies
Bp	Base Pair
BSA	Bovine Serum Albumin
C	Cytosine
CE	Capillary Electrophoresis
CODIS	Combined DNA Index System
CPE	Combined Probability of Exclusion
CPI	Combined Probability of Inclusion
Ct	Critical Threshold
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
dsDNA	Double Stranded DNA
EDTA	Ethylenediaminetetraacetic Acid
FBI	Federal Bureau of Investigation
FCC	Fired Cartridge Casing
G	Guanine
ILS	Internal Lane Standard
LR	Likelihood Ratio
MCMC	Markov Chain Monte Carlo
NRC	National Research Council

PCIA	Phenol-Chloroform Isoamyl
PCR	Polymerase Chain Reaction
PHR	Peak Height Ratio
POP	Performance Optimized Polymer
PVP	Polyvinyl Pyrrolidinone
QC	Quality Check
qPCR	Quantitative PCR
RFLP	Restriction fragment length polymorphism
RMP	Random Match Probability
SDS	Sodium dodecylsulfate
STR	Short Tandem Repeat
ssDNA	Single Stranded DNA
SWGDAM	Scientific Working Group for DNA Analysis Methods
T	Thymine
TAPS	3-{[1,3-Dihydroxy-2-(hydroxymethyl)-2-propanyl]amino}-1-propane-1-sulfonic Acid
Taq	Thermus Aquaticus Polymerase
tDNA	Trace DNA
UV	Ultraviolet Radiation
VNTR	Variable Number Tandem Repeats
Y-STRs	Y-Chromosome STRs

INTRODUCTION

A. STRUCTURE OF DNA AND ITS USE IN FORENSIC SCIENCE

DNA, known as the genetic blueprint of organisms, is contained inside the nucleus of all human cells and contains a specific code that allows for the cell to produce the specific proteins needed for life along with providing the phenotype characteristics such as our eye color, skin color, hair color and many other traits (Fig. A.1). This large molecule is a chain of repeating units called nucleotides. Each nucleotide consists of a 5-carbon sugar known as 2'deoxyribose, a phosphoric acid molecule and a nitrogenous base (Adenine, Guanine, Cytosine or Thymine which are abbreviated A, G, C and T, respectively). The nitrogenous bases are divided into two groups: Purines which have a double ring structure (A and G) and Pyrimidines which have a single ring structure (C and T) as shown in Figure A.2.

The DNA in each chromosome is separated into genes that are comprised of coding regions (exons) and non-coding regions (introns). The exons contain the sequence of nitrogenous bases that code for a particular protein of a gene, and the introns, or "junk DNA" as they have been called due to their lack of apparent function, which interrupt the gene sequence, potentially assisting with minimizing the deleterious effect of mutations (Alberts 2017). Researchers determined these non-coding regions play a role in gene activity and expression[1].

[1] (Barrett, Fletcher, and Wilton 2012)

Every gene is made up of certain number of nucleotide bases in a row which then provide the code to produce messenger RNA and thereby coding for the polypeptide chain with a particular function in the organism (Pearson 2006). A gene may exist in different forms (alleles) which vary in some way through the process of recombination or mutation, causing a change in the expression or protein made. Within any given population, variations in alleles may be few or many, depending on the gene under consideration.

The DNA molecule contains two complementary polynucleotide chains that form a double stranded helix as shown in Figure A.1, with the nucleotide bases attached to the sugar-phosphate backbone. These two complementary chains are joined to each other by base pairing involving the hydrogen bonding between the nitrogenous base of one chain pairing with its complementary base (A-T and C-G) on the other chain. The hydrogen bonding between the two chains involves a hydrogen atom being shared between two other atoms with two hydrogen bonds per A/T pair and three hydrogen bonds per G/C pair. In this way, the strands are held together with the weak hydrogen bonding and twist around each other due to the specific shapes of the purines and pyrimidines to form the stable double helix structure of DNA (Hartl 2009).

Once the DNA double helix supercoils around proteins known as histones to form what is called chromatin (Fig. A.3). During cell division, chromatin further condenses into the structures called chromosomes. Each species has a specific number of chromosomes, each of a certain size, with humans having 46

chromosomes in total (23 pairs of chromosomes), one set inherited from the maternal parent and one from the paternal parent. Two chromosomes (the X and Y chromosomes) determine the sex of the individual with females having two X chromosomes (XX genotype) and males having one X chromosome and one Y chromosome (XY genotype).

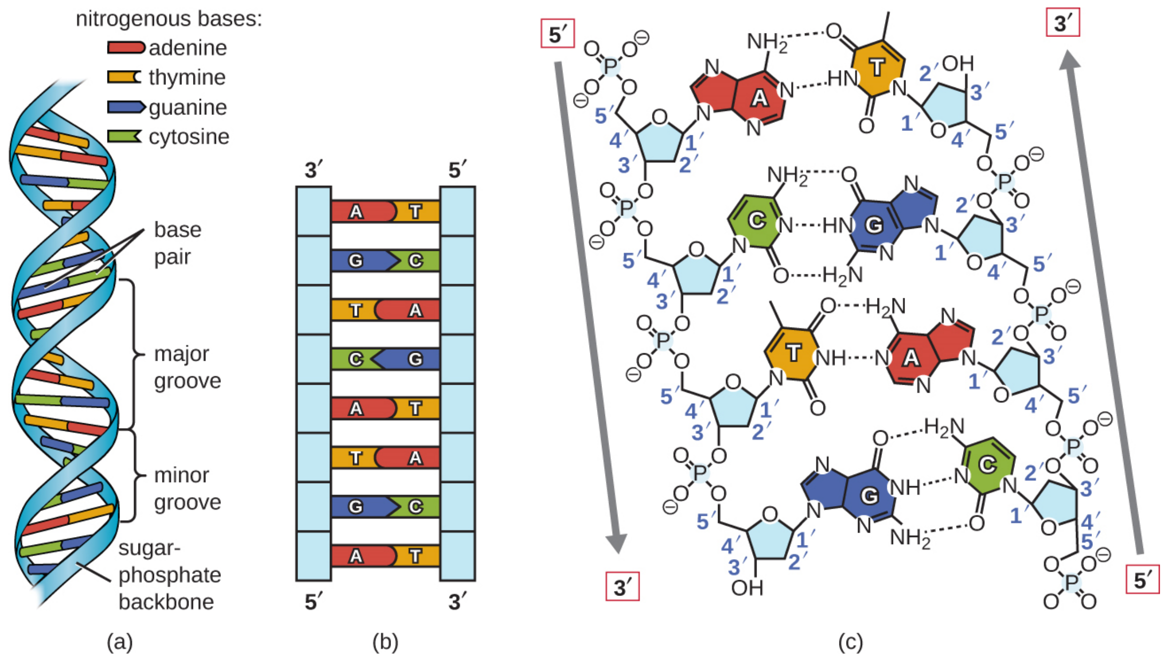
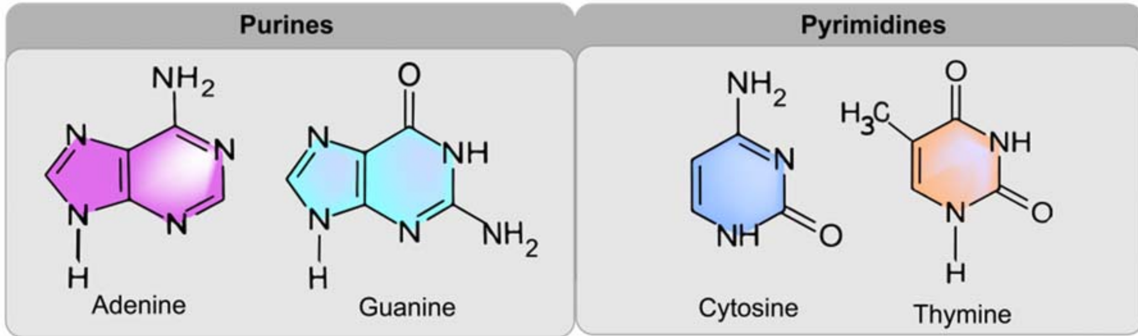


Figure A.1: The DNA double helix structure, made up of antiparallel strands. Retrieved from [Structure and Function of DNA · Microbiology (philschatz.com)] on July 15, 2023.



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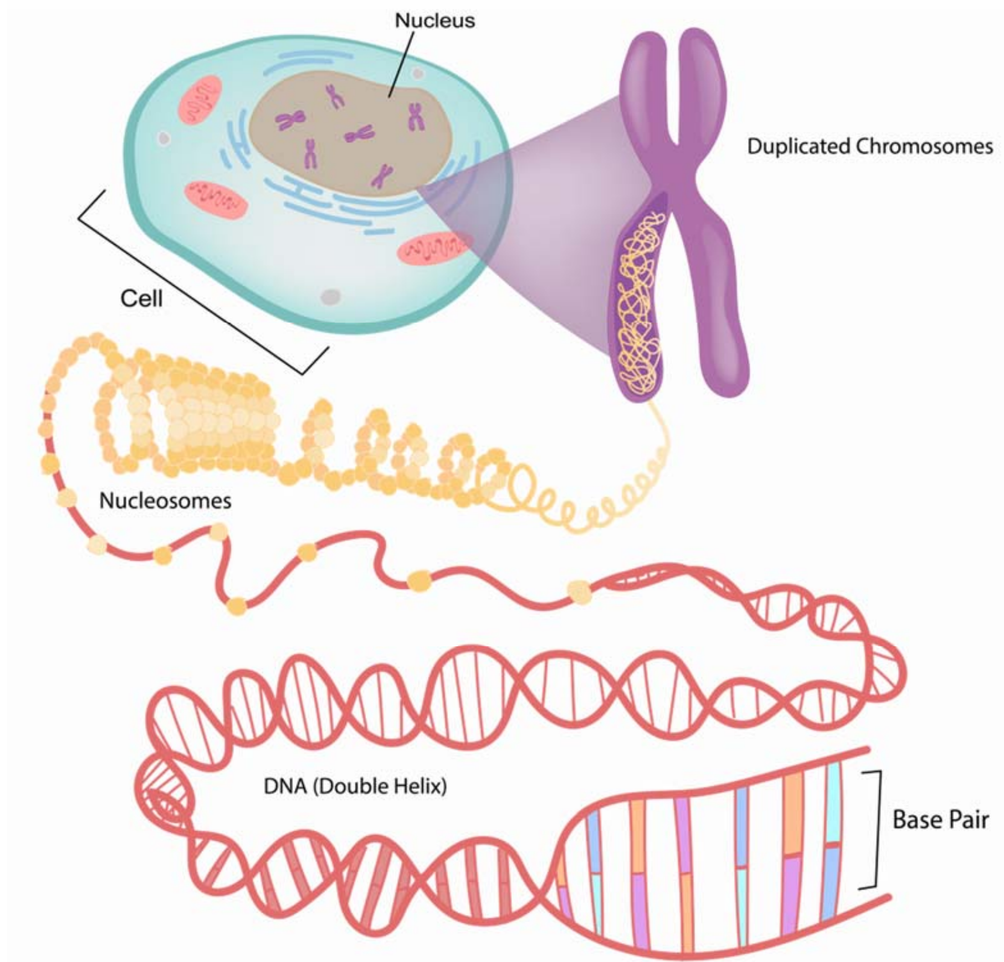

Figure A.3: Structure of Chromosome. Retrieved from [5.2 The Genetic Basis of Gene Expression – The Evolution and Biology of Sex (umn.edu)] on July 15, 2023.

There are approximately 3 billion base pairs that comprise the 23 pairs of chromosomes found in the human genome. Each chromosome can contain several hundred to even several thousand genes in some cases. Of the 3 billion base pairs, only about 1.3% contain coding sequences, while the remaining 98.7% are non-coding sequences. Some of these non-coding sequences exhibit many observed allele types within the populations and are therefore considered to be highly polymorphic which means there is a higher-than-average variability in their bp sequence and/or bp sequence repeats which makes them forensically valuable for identification purposes. The variances of these areas are due to random mutations, which form a variant allele that is then passed on to future offspring. The field of forensic science uses DNA sequences that have a specific known chromosome location [2]. A DNA marker refers to a location on a chromosome and is also known as a locus, the plural form being loci (referring to multiple locations). When it comes to STR loci, the locus name gives information about both the individual sequences and the chromosome it is found on. For example, an STR locus having the following designation D16S539, includes a D for DNA, the 16 refers to the chromosome number, the S stands for a single copy sequence, and the number at the end, in this case 539, showing how many other loci were discovered on that chromosome beforehand. Therefore, this location was the 539th marker described on chromosome 16. These locus names are often abbreviated to include the first letter and number for ease of use. For

[2] “Genetics: A Conceptual Approach, 7th Edition | Macmillan Learning, US.”

example, the locus previously mentioned, D16S539, is referred to simply as D16 by forensic scientists. There are some loci that have unique nomenclature, generally when it falls inside the coding region of a gene, using an abbreviation. For example, TPOX is a locus that is found within the coding region of the gene for thyroid peroxidase.

B. HISTORY OF FORENSIC BIOLOGY

Forensic science can be described as the application of scientific investigations to legal questions. To solve crimes, detectives typically rely upon the evidence found at the crime scene. Edmund Locard was the laboratory director of the first forensic crime lab early in the 20th century, located in France. Locard developed a doctrine surrounding the belief that each time there is contact between two different items, either biological or non-biological, there will be a transfer of some type of microscopic material. This tenet can be simplified to "every contact leaves a trace" and is also known as "Locard's Exchange Principle". There are many examples throughout history of instances where scientific principles were used to help determine how a crime occurred. A notable example is Julius Caesar's murder, where it was determined that despite being stabbed 23 times, only one of the stab wounds was fatal (The Lives of the Twelve Caesars, by C. Suetonius Tranquillus; n.d.). Another famous example of application of science to law is "His Duan Yu" a Chinese book translated as the "Washing away of Wrongs", written by Song Chi in 1248 AD. The book explains how to differentiate between a death by drowning compared to strangulation (Bell

2008). These examples demonstrate that the application of scientific reasoning to legal questions was established many years ago.

More advances appeared around the 1800s when Mathieu Orfila, also known as the “father of toxicology”, developed a method for determining the presence of blood. Then in the late 1800s Sir Francis Galton and Sir Edward Henry described a method for classifying and identifying individuals by their fingerprints. Following that, in the 1900s Leone Lattes was able to determine blood typing from dried bloodstains. This was the first real step on the road towards “forensic biology” as a separate science. However, it was not until the end of the 1900s that the concept of DNA profiling came about.

In the late 1940s, Erwin Chargaff, an Austrian biochemist discovered that organisms contain specific base pairing rules based on the percentages of each of the four nitrogenous bases that make up their DNA. He found that there was approximately ~30% each of Adenine (A) and Thymine (T) and ~20% each of Cytosine (C) and Guanine (G), leading to the conclusion that A must connect to T and G must connect to C, which later became known as “Chargaff’s Rule” (Chargaff, Zamenhof, and Green 1950). Chargaff’s work, along with the efforts of Rosalind Franklin, James Watson, and Francis Crick, eventually led to the discovery of DNA’s double helix structure of DNA in 1953, winning a Nobel Prize in 1962. They discovered that the A/T and C/G interactions prevented the two strands of the helix from dissociating and providing the necessary stability for the structure (Watson and Crick 1953). The structure of DNA was the first step,

although it took a little over 30 years for any further significant progress within the field.

In 1984, Sir Alec Jeffreys made a discovery that kicked off the field of DNA typing. Jeffreys discovered that there are certain regions within the human genome that contain repeat sequences whose lengths differ between individuals. These regions, or areas, are called Variable Number of Tandem Repeats (VNTRs) and at these regions the differences in the number of variations of the region's length can help discriminate between individuals, a process initially termed DNA fingerprinting (Jeffreys, Wilson, and Thein 1985). A method for analyzing these repetitive sequences was developed in 1980 by Arlene Wyman and Ray White, known as restriction fragment length polymorphism (RFLP) (Wyman and White 1980). This process, originally designed to detect single base variations in the human genome (Botstein et al. 1980); however, the researchers were able to use special enzymes called endonucleases, which they harvested from bacteria, to cut the DNA into segments at specific locations. Then, electrophoresis causes the fragments to be separated by their size, based on the mobility pattern of DNA as it moves through the agarose material, with the smaller fragments will travel faster than the larger fragments.

Southern blotting uses a process where the fragments are transferred onto a nylon membrane which is then hybridizes using radioactive probes with the specific sequences that are complementary to the ones on the gel. The radioactive probes are then exposed to radiographic film, resulting in a banded

pattern image that can be analyzed compared to an allelic ladder to permit individualization of human genotypes (Fundamentals of Forensic DNA Typing - 1st Edition n.d.).

In the very beginning, Dr. Jeffreys used multi-locus probes to digest the DNA, which yielded several fragments and made the interpretation very complex and confusing, especially if there were multiple contributors. Due to this issue, Dr. Jeffreys started using single locus probes and termed his method “DNA fingerprinting” [3]. The first known case using Jeffreys’ method was in 1985 involving a paternity case in England (Jeffreys et al. 1993), and then in 1986 it was applied in a famous homicide case (The Blooding by Joseph Wambaugh n.d.). The case involved the rape and murder of two teenage girls in a small English village, where the suspect they had developed was only confessing to one of those rape/murder. His denial of the other murder was troublesome and there were those who questioned his mental state. Since there was DNA from the victims’ bodies, the police decided to attempt Jeffreys’ DNA fingerprinting method. They compared the evidence found at the crime scene to the known DNA samples from everyone in that village and did not find any matches. The police found out one day that a man was boasting that he had submitted a DNA sample in place of his friend’s DNA. When the police were informed, they found the man’s friend, obtained his DNA, resulting in a match to the DNA from the crime scene leading to his arrest and later conviction for both rapes and murders

[3] (Jeffreys et al. 1993; Jeffreys, Wilson, and Thein 1985)

of the two teenage girls (The Blooding by Joseph Wambaugh n.d.). However, although it was accurate and reproducible, it was also a time-consuming process, labor-intensive, required the use of harmful reagents and a large amount of starting DNA (Fundamentals of Forensic DNA Typing - 1st Edition n.d.).

Shortly after Dr. Jeffreys started implementing his DNA fingerprinting method, Kary Mullis, an American biochemist, theorized about a method to make more copies of the DNA, which could make it easier to analyze and interpret these DNA fingerprints (Mullis 1990). His idea was to use a set of two primers for each of the areas (loci) of the DNA molecule that he wanted to copy. His theory, later termed the Polymerase Chain Reaction (PCR) involved essentially replicating the process used by cells to create copies, adding the correct polymerases, nucleotides, and the specific heating and cooling cycles (Mullis 1990). The Polymerase chain reaction (PCR) has since been proven to be a sound method capable of making millions of copies of the same DNA sequence, leading to exponential increases in the amount of DNA that biologists started with. Now it would be possible to start with as little as 0.5ng or less, instead of the 0.5μg or more that is required for the VNTR/RFLP method (Fundamentals of Forensic DNA Typing - 1st Edition n.d.). The PCR method, when applied to the forensic field, was able to produce DNA profiles from crime scene samples that previously would have yielded no results with the VNTR/RFLP methodology because of the small starting amount or if it were degraded.

One problem encountered that made the process time-consuming was that fresh polymerase needed to be added after each denaturation step, due to the heat of the denaturation step, the polymerase would then become useless(Mullis 1990). Then, in the 1990s, the new heat stable polymerases were discovered (ex. Thermus aquaticus, a.k.a Taq. Bacteria found in geothermal springs) that rendered this step unnecessary (PCR Applications - 1st Edition n.d.). Additional developments with these heat-stable polymerases eventually led to the creation of what is now known as “hot start” polymerases, which essentially have a bound repressor molecule that separates and activates the polymerase once it reaches a certain temperature. These polymerases were only activated after the primers annealed, which greatly improved both the efficiency as well as the specificity of the reaction (Fundamentals of Forensic DNA Typing - 1st Edition n.d.; PCR Applications - 1st Edition n.d.).

The first PCR assay developed was the leukocyte antigen (HLA) locus, specifically the DQα1 marker found on chromosome 6. In addition, another method, known as the “dot blot”, was being used where the DNA would be bound to a substrate material and following that, the complementary probe would hybridize to its corresponding target region, thereby causing a color change to occur (Sajantila et al. 1991). This dot blot method had a low power of discrimination power was low and it became difficult to interpret when there was a mixed sample; therefore, alternative assays were sought. Then a new assay was developed which included five additional loci, known as the AmpliType Polymarker kit.

Another PCR-based assay was developed to help further improve discrimination power which amplified specific regions of repeated base pairs, between 8-100bp repeated a certain number of times, that vary in length between individuals, also called variable length tandem repeats (VNTRs) or amplified fragment length polymorphisms (AmpFLPs). The resulting PCR products from these assays were loaded in a well of an agarose gel sitting in a buffer solution, followed by the application of an electrical current. The DNA would migrate through the gel separating the fragments by size as the smaller fragments would travel faster than the longer ones. The gel would then be stained for visualization using the intercalating agent ethidium bromide followed by using UV light. The alleles would show up as a band and those bands were sized by direct comparison to the bands of an allelic ladder containing all the known alleles for the gene which would be run in parallel with the samples.

Then the discovery of Short Tandem Repeats (STRs) created a big change for the field of forensics, since the repeat units were now only 2-7 base pairs. There were many such regions located throughout the human DNA that were contained mostly in the non-coding regions (Fundamentals of Forensic DNA Typing - 1st Edition n.d.; Lins et al. 1998). Several factors were involved when choosing the STR markers to put into the forensic assays, since privacy concerns had to be considered. It was important to choose non-coding regions to avoid any genetic information being obtained, and it is preferable that the STRs have short sequence as well as many different possible alleles to improve the power of discrimination. The greater the number of loci, the higher the power of

discrimination, yet there are limitations as each locus must have a fluorescent label and there cannot be overlap in the lengths for different loci, so the limiting factor is also the number of different fluorescent dyes that can be separated on the instrument.

Most commercial STR kits now contain up to 24 loci, with enhanced sensitivity and power of discrimination of >1 in octillion. The FBI established the Quality Assurance Standards for Forensic Testing Laboratories which is periodically updated, and they also initiated a technical working group (TWGDAM) which is now called SWGDAM that produce sets of guidelines to help standardize the field of forensic biology. There are also two accrediting bodies, ANSI Accreditation Board (ANAB) and A2LA, that will audit laboratories to ensure compliance with the standards set forth for forensic testing laboratories. The National Academy established the National Research Council (NRC) who consisted of worldwide experts in the field of forensic science with the goal to publish guidelines for interpretation of DNA profiles and how to perform statistical calculations such as random match probability (RMP) and likelihood ratio (LR) (The Evaluation of Forensic DNA Evidence 1996). The FBI also created a software database of 13 core loci called the Combined DNA Index System (CODIS) to share DNA profile information between the labs within a state and between states to assist with investigative leads. The CODIS database has three levels to its architecture with the National DNA Index System (NDIS) at the FBI, the State DNA Index System (SDIS) for each state, and the Local DNA Index System (LDIS) for each regional lab within the state. There are several

categories for the DNA profiles entered into the database ranging from crime scene samples (forensic unknowns) to convicted offender samples, and even missing persons data, which allows public forensic laboratories to search its crime scene samples against other labs' samples or against other known individuals in the database (convicted offenders or missing persons). There were 13 original core loci to include: CSF1PO, FGA, TH01, TPOX, VWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11 and a sex marker, Amelogenin (Figure B.1). These 13 loci generally have a high power of discrimination of about one in a trillion or more depending on the rarity of the alleles in the sample, except for identical twins [4], and they were chosen and validated extensively by scientists around the world specifically for the purpose of creating this nationwide database (B. Budowle et al. 1999). The inclusion of a sex marker, Amelogenin, with only two alleles (X and Y) allows scientists to determine the gender of the person (Sullivan et al. 1993). More recently, CODIS increased its core set to include: D1S1656, D2S441, D2S1338, D3S1358, D5S818, D7S820, D8S1179, D10S1248, D12S391, D13S317, D16S539, D18S51, D19S433, D21S11, D22S1045, CSF1PO, FGA, Penta D, Penta E, SE33, TH01, TPOX and vWA (Hares 2011). The additional loci produce a higher power of discrimination and help to differentiate between close relatives.

[4] (Hill 2012)

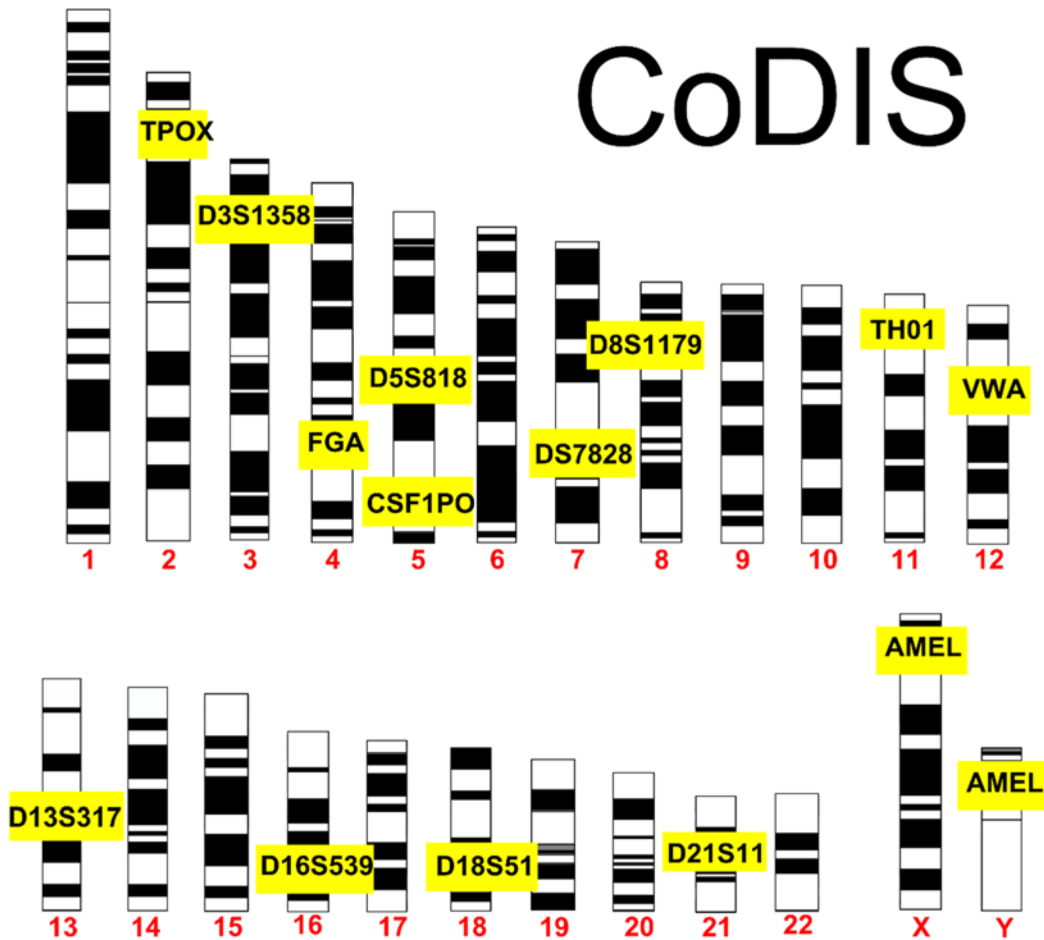

Fig.B.1: Original CODIS 13 Core Loci with chromosome positions, retrieved from [8.3: Variable Number Tandem Repeats - Biology LibreTexts](#) on July 15, 2023. Credit: Jeremy Seto (CC-BY-NC-SA)

C. EVALUATION OF TRACE DNA AND COLLECTION METHODS

The physical evidence found at a crime scene and the method used to collect that evidence can play a crucial role for convicting the guilty as well as freeing the innocent [5]. This physical evidence, if not correctly collected, stored, and

[5] (Lee and Ladd 2001)

transported, could become compromised and hinder its usefulness. Collection is the first step and if not done properly, then it could result in unusable data. Biological evidence such as blood, saliva, semen, or even epithelial cells, if handled improperly could either become contaminated with exogenous DNA or possibly degraded due to mold or fungal growth. Therefore, protocols must be established and followed for the proper collection, storage, and transportation of biological evidence, including maintaining an accurate chain of custody. These protocols and how the evidence should be treated depend on the type of biological material being collected and on the substrate material. Sometimes it is important to collect a substrate blank, depending on the scenario, which is taken from a section of the substrate untouched by any biological evidence. In addition, reference samples (usually a buccal swab) from the victim, consensual partners, family members and any possible suspects should also be collected for comparison to the crime scene evidence (Tilstone, Savage, and Clark 2006).

Skin cell evidence is generally left behind on items of evidence that are touched or handled by an individual and can be collected in a variety of ways. The DNA left behind from contact/touching of items (including FCCs) can be classified as “trace DNA”, which has previously been described as DNA coming from a multitude of potential sources including epithelial cells from the individual touching the item, secondary transfer from other individuals, sweat droplets or even saliva from speaking. This trace DNA can be valuable in attempting to identify potential investigative leads in many cases that otherwise have no leads, such as drive-by shootings. There are many factors that can potentially affect the

recovery of DNA left behind when someone touches an object. One of the primary factors is the limited amount of DNA initially deposited on the item. The other factors include the degradation of the DNA, inhibition from the metals or other inhibitors such as hematin, calcium from bones, and indigo from jeans, or exposure to chemicals or heat. In addition, if the surface where skin cells were deposited is outside, it may be exposed to elements, such as the sun, the rain, or bacteria from soil, all of which can further degrade the trace DNA.

The study of DNA transfer, prevalence, persistence, and recovery (TPPR) involves many different variables or factors at each step. DNA transfer can be from any number of potential sources and studies have shown that the amount of DNA on the hands of an individual can vary significantly depending on the time of day, how often the individual touches their face, specifically any mucosal membrane areas such as the eyes, nasal secretions, or saliva from the mouth (Burrill, Daniel, and Frascione 2019). The persistence of the DNA on the hands of the individual varies with activity including hand washing. This variability makes it difficult to conduct scientific studies of DNA TPPR using mock scenarios as many of the studies only focus on the recovery and it is not possible to accurately measure how much DNA is transferred initially. Designing experiments to study DNA transfer, prevalence, persistence, and recovery poses significant challenges. Factors such as the variability of donor DNA, the complexity of mixtures, and the limitations of controlled environments can affect the accuracy of results and usefulness for making inferences. Additionally, the practical constraints of conducting experiments that mimic real-world scenarios

can hinder the ability to capture the full spectrum of DNA transfer events. The development of standardized protocols, representative sample collections, and innovative experimental designs can help mitigate these challenges and enhance the validity of research outcomes.

Environmental conditions can have a considerable influence on DNA transfer, prevalence, persistence, and recovery. Factors such as temperature, humidity, UV exposure, and substrate composition can significantly impact DNA degradation and survival rates (Burrill, Daniel, and Frascione 2019). However, reproducing realistic environmental conditions in controlled laboratory settings can be challenging. Simulating dynamic and diverse environmental scenarios and utilizing advanced technologies such as environmental chambers and microclimate monitors can aid researchers in better understanding the effects of environmental factors on DNA dynamics [6].

The properties of different substrates present complexities in DNA transfer and recovery. Porous materials, touched surfaces, and items exposed to environmental elements may impede DNA recovery due to substrate interactions and interference. Substrate properties such as texture, porosity, and chemical composition can influence DNA adsorption, desorption, and degradation rates. Addressing these challenges requires the development of specialized collection

[6] (Burrill, Daniel, and Frascione 2019)

methods, improved extraction techniques, and the integration of material science principles into forensic research.

In the past, scientists have assumed that the DNA collected from items touched by a suspect originated from the outer layer of skin cells, a belief that continues to this date. However, the data from multiple studies conducted on the composition of tDNA deposited from contact with an item has provided greater insight into the potential sources of DNA. Burrill et al. (2019) proposes that this tDNA could arise from one of several sources to include: naturally shed keratinocytes, endogenous nucleated epithelial cells (potentially transferred from the individual's own mucous membrane surfaces such as saliva, nasal fluid, or eyes), or fractionated cell-free DNA (Burrill, Daniel, and Frascione 2019).

During the evaluation of crime scene evidence and with the advances in DNA technology with the advent of PCR, investigators will often want to know the identity of the person who touched or handled an item. Examples include the gun left at a crime scene, the knife handle used to stab a victim, the door handle of the point of exit, and the pant pockets of the victim that were turned inside out. This type of DNA commonly encountered on crime scene evidence is described as many things such as "touch DNA", "contact DNA", "trace DNA" or even "low template DNA", and herein will be referred to as trace DNA (tDNA). Although in principle this sample will often contain the DNA from the person who contacted the item, there are times when the DNA from other individuals who may not have had contact with the item will also be present.

When an individual touches an item, there is the possibility of transfer both to and from the individual. The nature of the material left behind on the item can be classified into several categories as shown in Fig. C.1: cell-free DNA, fragment-associated residual DNA, transferred exogenous DNA from other people or surfaces touches, endogenous nucleated cells from sweat glands or from touch other mucous membrane areas such as eyes, mouth, or nasal secretions; and anucleate corneocytes.(Burrill, Daniel, and Frascione 2019)

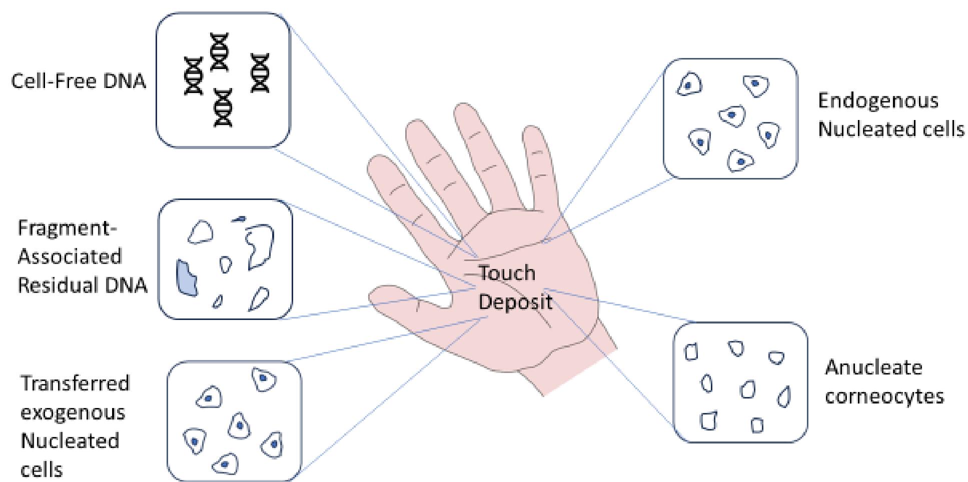

Fig.C.1: Explanation of the various types of cellular material contained on the hands that is transferred when someone has contact with an item. Image created by author.

When the DNA sources that are on the surface of the hand are transferred to an item upon contact, the amount of DNA sources that are left behind is dependent on many different variable to include: the length of time of the contact event, the pressure applied during the contact, how long ago the individual washed their hands, how much the person was sweating, if there was any

rubbing or just single contact, the surface material touched (such as cloth vs. countertop vs. rough grip of a gun), among other variables. These multiple variables have led to significantly large studies that researchers have been conducting to scientifically determine the likelihood of a transfer event occurring(van Oorschot et al. 2019).

In addition to the DNA sources discussed above that can be transferred from a contact event, there are other avenues that can result in DNA being found on an item of evidence. One of those being from miniature saliva droplets that come out of our mouth when we speak, cough, or sneeze. This became something more people realized during the COVID pandemic resulting in the masks worn to prevent the spread of these airborne droplets. This has an impact on crime scene personnel since these people are collecting evidence and are sometimes doing so without wearing facemasks. Also, the detectives who may walk around a crime scene talking to each other during their investigation may also contribute their DNA onto evidence, and with the sensitivity of our methods, these DNA profiles may show up on the evidence. Ideally, though, the goal is to determine who may have touched the item.

Dried biological samples are most often collected for DNA testing by employing a double swab technique. This method involves first using a moistened cotton swab to collect the material, followed up with a dry cotton swab to remove any DNA left behind on the item. Wet stains are normally collected with a dry cotton swab, or two. Any samples and fabric should be dried prior to

packaging and all biological material is normally packaged in paper or card containers to promote complete drying, preventing further bacterial or fungal growth which may cause DNA degradation. Once collected and dried, samples can be stored in a refrigerator at 4ºC or even at room temperature. They can also be stored in a freezer at 20ºC for long-term storage [7]. It is also important to check for trace evidence such as hairs, fibers, or even swabs of areas that may have had contact with the perpetrator. To preserve the biological evidence for other forensic, it is important that the collection of cellular material from evidence items be performed carefully, with as little disruption to the item as possible, while still preserving enough biological material for DNA testing. Therefore, the sampling method and reagents used in the collection and preservation should not affect any downstream applications. Care should be taken in avoiding any method that may cause inhibition of the PCR step to ensure the most robust DNA profile is obtained.

When it comes to tDNA there are several different methods that have been developed over the years to include different swabbing techniques [8], the swab materials [9], tape-lifting methods [10], vacuum tools[11], different buffers[12],

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[7] (Fundamentals of Forensic DNA Typing - 1st Edition n.d.)

[8] (Pang and Cheung 2007)

[9] (Phetpeng, Kitpipit, and Thanakiatkrai 2015)

[10] (Bhoelai, Beemster, and Sijen 2013)

[11] (Vickar et al. 2018)

[12] (Aloraer et al. 2017)

electrostatic equipment[13] and even skipping the collection and going straight to direct amplification from the evidence item itself (Cavanaugh and Bathrick 2018).

D. DNA PROCESSING METHODS

There are four basic steps to the processing of samples collected for DNA analysis. The first step is called extraction, which is where the cells are lysed releasing the DNA that is contained inside the nucleus. After lysis, the extraction process also requires a purification step where the remaining cellular components are removed from the sample, leaving purified DNA behind. This is the step where certain inhibitors are normally removed as well. The second step in the process is quantitation, where an estimate of how much DNA is in the sample is made. This is important because the third step in the process, amplification, has an optimal DNA template input amount window, so knowing the amount in the solution will enable the analyst to add more or even dilute the sample to ensure that the amplification reaction provides interpretable results. The third step is the PCR amplification where the primers are added for the target sequences or loci and the heating/cooling cycles allow for the copying of those areas resulting in exponential amounts of each starting template. The fourth step is separation of the alleles at each locus by their size (bp length) and allele calls using the ladder and internal lane standards run with the samples. Each step of the DNA analysis process has gone through many advancements

[13] (Plaza et al. 2015)

over time which will be discussed further in more detail, all with the goal of improving the quality and power of discrimination of the DNA profiles obtained at the end.

The first step in the DNA analysis process is extraction. During extraction the cells must be first be separated from the substrate, then the cells are lysed to releasing the DNA from the nucleus along with the cellular components, and finally the DNA is purified by removing all cellular components such as enzymes, other proteins, and potential inhibitors that could negatively affect amplification reaction [14]. During the extraction procedure, a negative reagent control is processed simultaneously, with the same reagents and handled alongside the evidence samples. This negative control is generally an empty tube containing no biological material and serves to ensure that the reagents and consumables used in the process are free from contamination. If a negative control sample shows allelic peaks after amplification, then investigation should be conducted to determine the possible source of contamination, if possible, and the samples may require re-extraction.(Giglio, Monis, and Saint 2003).

When the field of forensic biology first began, the primary extraction method was Phenol-Chloroform Isoamyl Alcohol (PCIA) Phenol-Chloroform Isoamyl Alcohol (PCIA) extraction, also known as organic extraction, being called the “gold standard” due to its ability to yield high levels of DNA. Even today it is

[14] (Demeke and Jenkins 2010)

used for many special sample types such as bones. The reason the field moved away from the method is due primarily to its use of a toxic chemical, phenol, requiring careful handling in a fume hood with proper ventilation. The procedure also has many steps, introducing more possibilities for contamination from tube handling, and is not amenable to automation, making it a labor-intensive method. The basic steps involved in the PCIA extraction are shown in Fig.D.1 where a detergent is added (SDS) along with an enzyme (Proteinase K) and Dithiothreitol (DTT) and incubated to help with cell lysis and the degradation of proteins. The phenol, chloroform and isoamyl alcohol are then added which separates out the hydrolyzed proteins from the DNA molecules, allowing for the transfer of the upper aqueous phase containing the purified DNA into a clean tube. The sample is then cleaned up to remove potential PCR inhibitors with ethanol precipitation. This step usually involves a high concentration of ethanol (100% for first step, followed by two to three washes using 70%).

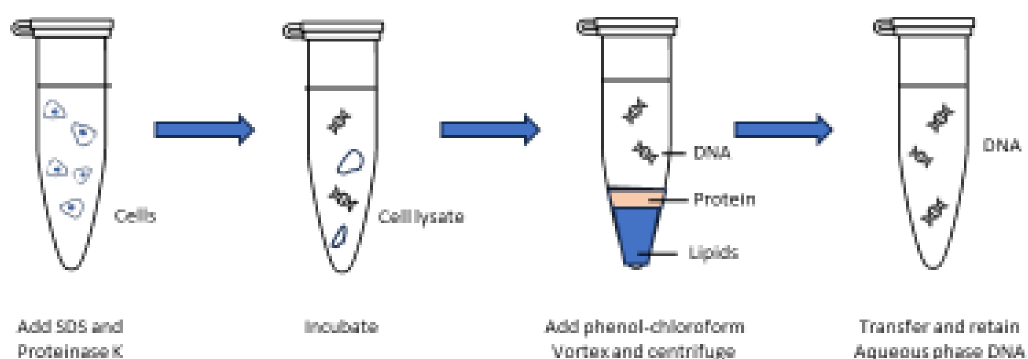

Fig.D.1: Diagram of PCIA (organic) DNA extraction method. Image created by author.

Another extraction method called Chelex Method uses a resin (Chelex 100) that binds to metal ions like Magnesium reducing the impact of DNA nucleases or PCR inhibitors [15]. The method involves boiling the sample with Chelex resin in water for several minutes, which lyses the membrane releasing the cellular components followed by a washing step to remove any resin that could have carried over [16]. The Chelex extraction method does have an advantage over PCIA in that it minimizes tube transfer steps making it not only faster, but also will have less chances for contamination. The only drawback is that it creates single stranded DNA due to the boiling, which is less stable than the double stranded form of DNA. requires less pipetting and tube transfer steps than the PCIA method [17].

Solid phase extraction (SPE) methods are a type of extraction method that can vary depending on the kit or manufacturer. In general, these SPE methods use a solid substrate of some kind which varies (typically it is either silica particles or possible magnetic beads) which will selectively bind to DNA. The methods involve an initial cell lysis step releasing the DNA along with cellular debris, proteins, and other artifacts. The DNA binds to the solid substrate while multiple washes are carried out to remove the remaining components, resulting in purified DNA. The final elution step adds a low ionic strength buffer (TE^{-4}) to

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[15] (Walsh, Metzger, and Higushi 2013)

[16] (Willard, Lee, and Holland 1998)

[17] (Walsh, Metzger, and Higushi 2013)

release the DNA from the solid substrate back into solution. Figure D.2 shows the summary of the general process [18].

Several commercial kits that use the SPE method, also known as bead chemistry, have been developed in recent years such as the QIAamp® Kit (QIAGEN, Valencia, CA) [19] , the DNA Investigator Kit (QIAGEN, Valencia, CA), the Prepfiler Kit (Applied Biosystems), and the DNA IQ™ system (Promega Corporation, Madison, WI) [20]. Each kit is slightly different from the other, using a different bead composition or size and different buffers. These kits have also been adapted by the manufacturers to be automated onto a robotic platform such as the BioRobot EZ1 workstation (Qiagen, Inc., Valencia, CA), QIAcube system (Qiagen, Inc., Valencia, CA), Maxwell Robot (Promega Corporation, Madison, WI) and the Automate Robot (Applied Biosystems) allow for automation of the extraction process [21].

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[18] (Nagy et al. 2005)

[19] (Greenspoon et al. 1998)

[20] (Frégeau and De Moors 2012)

[21] (Bruce Budowle, Bieber, and Eisenberg 2005)

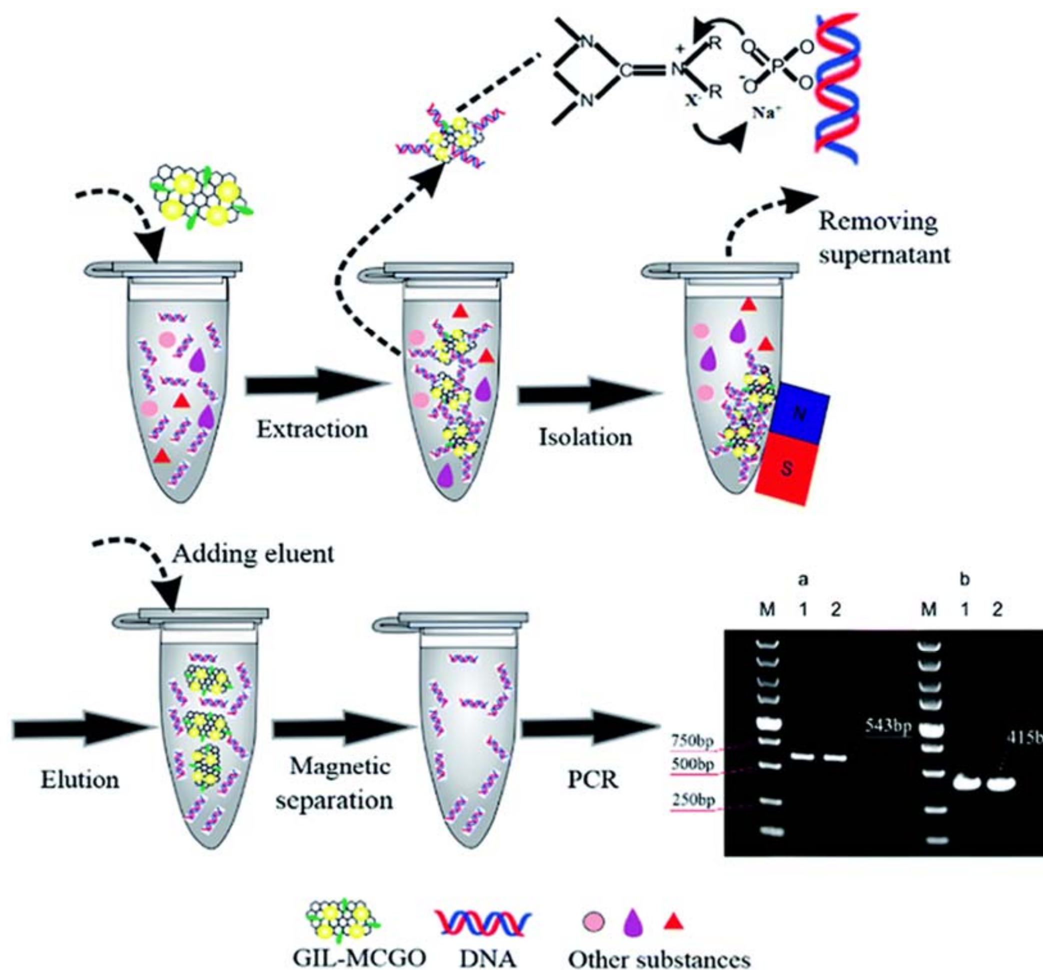

Fig.D.2: Principle and procedure of the DNA extraction method using guanidinium ionic liquid modified magnetic chitosan/graphene oxide (GIL-MCGO) nanocomposite particles via magnetic sold-phase technology. Retrieved from Extraction of DNA from complex biological sample matrices using guanidinium ionic liquid modified magnetic nanocomposites - RSC Advances (RSC Publishing) DOI:10.1039/C9RA01505A on July 15, 2023. (CC BY-NC 3.0)

The elution buffer will also contain proprietary additives designed to enhance DNA stability and improve overall yield which are. At this point the DNA is in a clear liquid and is considered “purified”. This will now need to go through some form of quantitation to see how much, if any, DNA is in the liquid extract.

The quantitation of DNA in forensic science has undergone significant advancements over the years, driven by technological developments and the increasing demands of forensic DNA analysis. In the early days of forensic DNA analysis, quantitation methods were relatively crude and labor-intensive. One of the initial approaches involved visual estimation of DNA concentration based on the intensity of DNA bands on agarose gels, where the DNA bands were transferred onto nitrocellulose membranes that were exposed to hybridization with probes that would bind to the DNA and visualized with a Charged Coupled Device (CCD) camera. The intensity of the resulting band for the sample was then compared to known standards. However, this method lacked accuracy and reproducibility.

The introduction of spectrophotometric (optical) methods revolutionized DNA quantitation in forensic science. Spectrophotometry measures the absorbance of light by DNA samples at specific wavelengths, allowing the estimation of DNA concentration. There are two types of optical technologies, UV-Vis which is a photometric measurement of the DNA based solely on the absorptive properties of the nucleic acids. Initially, ultraviolet (UV) spectrophotometry at 260 nm was used, but it suffered from inaccuracies due to the presence of contaminants that also absorb light at this wavelength. Later, dual-wavelength spectrophotometry using 260 nm and 280 nm became more common, as it provided better assessment of DNA purity by measuring protein contamination(Nielsen et al. 2008) . The concentration can be calculated using the absorbance values at 260nm and applying the Beer-Lambert's equation (*A*=

e*LC*), where A is the absorbance reading, e is the molar absorptivity, *L* is the path length in cm, and *C* is the concentration.

A second optical technology used in forensic science today is known as Qubit, manufactured by ThermoFisher Scientific. This system uses a fluorescent dye that binds selectively to the DNA or RNA molecule and once bound they emit a fluorescent signal that is then measured by the internal fluorometer [22]. To calculate the concentration with this method, it requires a standard curve which involves testing at least two samples with varying known concentrations to create a regression model standard curve (see Fig.D.3).

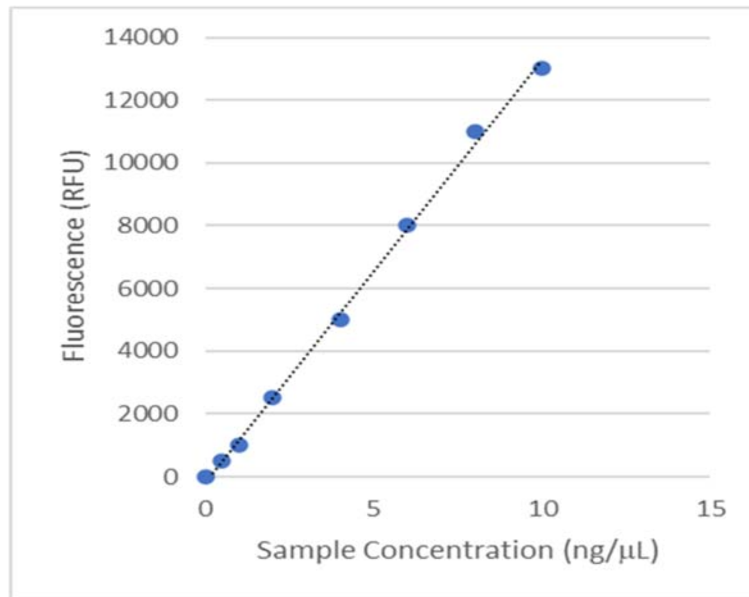

Fig.D.3: The regression line standard curve for the Qubit fluorometer. This line is used to estimate the concentration of DNA in the sample. Image created by author.

[22] (RNA/DNA Quantification - US n.d.)

The advent of real-time PCR techniques brought about significant improvements in DNA quantitation for forensic purposes. Real-time PCR allows the quantification of DNA by monitoring the amplification of specific DNA targets using fluorescent probes or intercalating dyes. This method enables accurate and sensitive quantitation of DNA, even from limited or degraded samples. Quantitative (real-time) PCR, also known as “qPCR” has become the standard approach in forensic laboratories, offering high-throughput capabilities and improved accuracy compared to earlier methods. One of the advantages of qPCR methods is that it can also provide additional information about a sample such as the possible presence of inhibitors or the amount of degradation. These factors can provide needed information for decision-making during the amplification reaction. The qPCR method is essentially a PCR reaction which is monitored in real-time with the amount of fluorescent dye detected in the sample. The system will either detect the amount of dye that is accumulating, or the amount of dye being quenched. Most forensic laboratories use what is known as TaqMan assay qPCR technology, see Fig.D.4 below, which involved a fluorescent dye attached to a quencher molecule located on a complementary target sequence to the DNA region of interest. As the PCR process cycles, the polymerase removes the dye from the quencher allowing the dye to fluoresce which is then monitored by the instrument detector. The targets used in the Quantifiler Trio kit (Applied Biosystems) include the Small Autosomal target, the Large Autosomal target, the Male target, and the Internal Positive Control (IPC), each with their own specific reporter dye (Holt et al. 2016). The software

calculates a cycle threshold (CT) value which indicates the point at which the fluorescent signal crossed the pre-established threshold and compares it to a standard curve which is also run on the same plate.

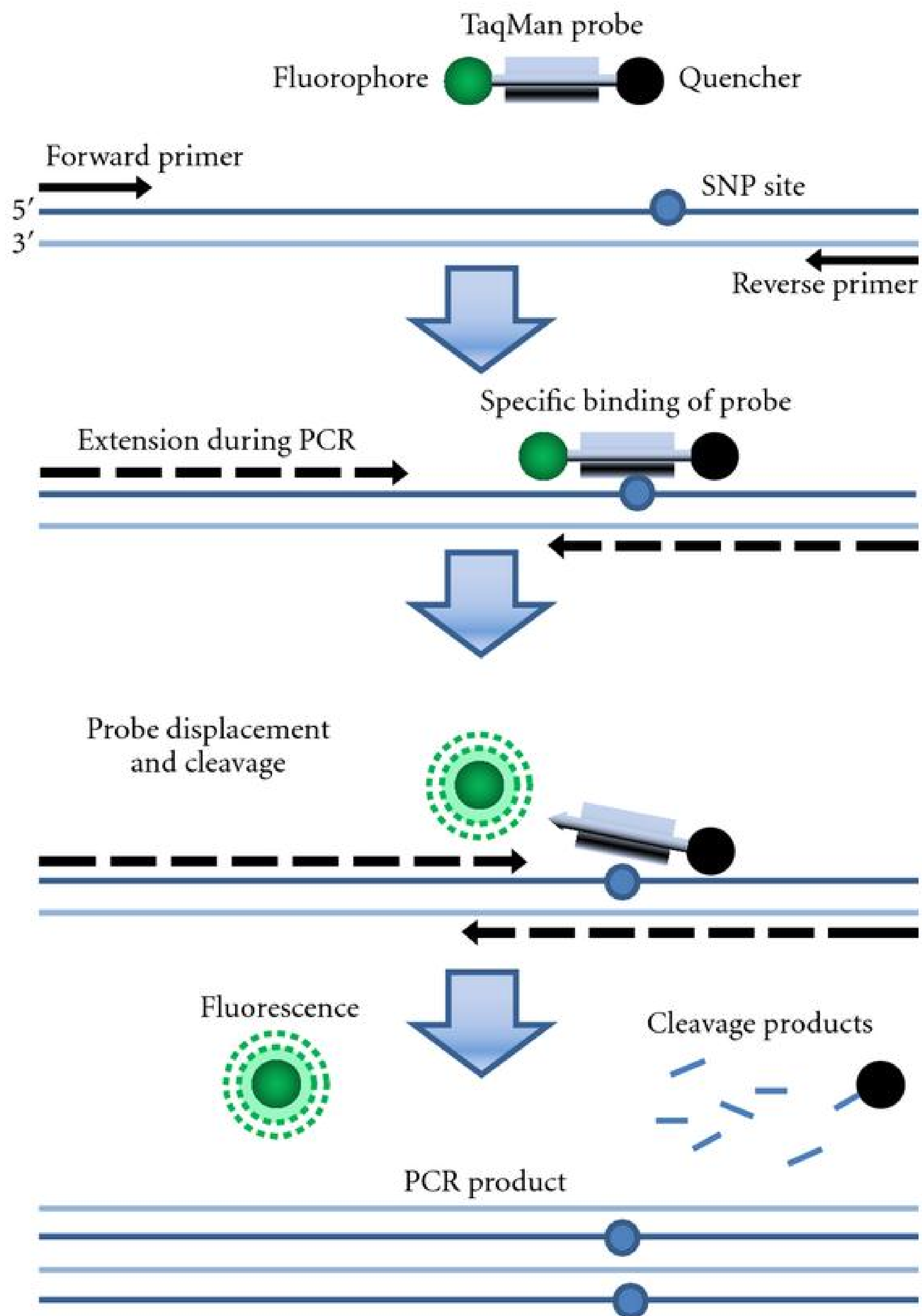

Fig.D.4: TaqMan probe chemistry mechanism. Retrieved from Figure 1 | The Vector Population Monitoring Tool (VPMT): High-Throughput DNA-Based Diagnostics for the Monitoring of Mosquito Vector Populations (hindawi.com) on July 15, 2023. (CC BY-NA 3.0)

Recent advancements in microfluidic devices and digital PCR have further refined DNA quantitation in forensic science. Microfluidic platforms offer enhanced sensitivity, reduced sample volume requirements, and increased automation. They allow precise quantitation of DNA by performing PCR and quantitation steps in miniaturized, high-throughput systems. Digital PCR (dPCR) is a technique that partitions DNA samples into thousands of individual reactions, enabling absolute quantitation of DNA copies. dPCR provides increased precision and sensitivity for DNA quantitation.

Following quantitation, the samples are then amplified using the Polymerase Chain Reaction (PCR) for a defined set of loci or target DNA sequences. The process is exponential in nature with one molecule producing two and then those two produces four, which in turn produces 8 copies, doubling with every cycle, with most PCR kits requiring about 28-30 cycles for optimal results (Butler 2014). The PCR process involves the creation of a reaction where the template DNA molecules are added along with a set of primers, which are short DNA fragments (single-stranded) that are complementary to the target region, DNA polymerase, buffer, and dNTPs. The fluorescently labeled primers used to make the copies of each region or marker are later excited with a laser which can then be detected to capture the intensity of the signal of the different fragments.

The PCR process takes place in three essential steps: denaturation, annealing and elongation, all occurring at different temperatures. There are optional steps such as an initialization step, which involves the heating of the sample to activate the hot-start enzymes and is commonly used. The denaturation step is when the double stranded DNA molecule is separated into two single strands, which requires a high temperature to cause the hydrogen bonds holding the nitrogenous bases together to break apart. After the denaturation is complete, the temperature is lowered allowing the primers to anneal to the complementary target sequence on the template DNA strand. The precise temperature where this will happen is dependent on the melting temperatures of the primers used. In PCR kits that have multiple loci that each have their own set of primers, a median temperature will be selected and optimized if needed during testing of the kit. The primers chosen need to be long enough to bind specifically to the selected marker or locus. The PCR reaction requires both a forward and reverse primer to isolate the region of interest. Lastly, the sample is heated to around 72°C as part of the elongation step allowing the polymerase to elongate the complementary strands, as it adds complementary nucleotides to each strand[23,24]. The PCR process steps and temperature ranges can be seen in Fig.D.6. Most forensic PCR reactions amplify STRs regions that are somewhere between ~60-500 base pairs (bp) in length.

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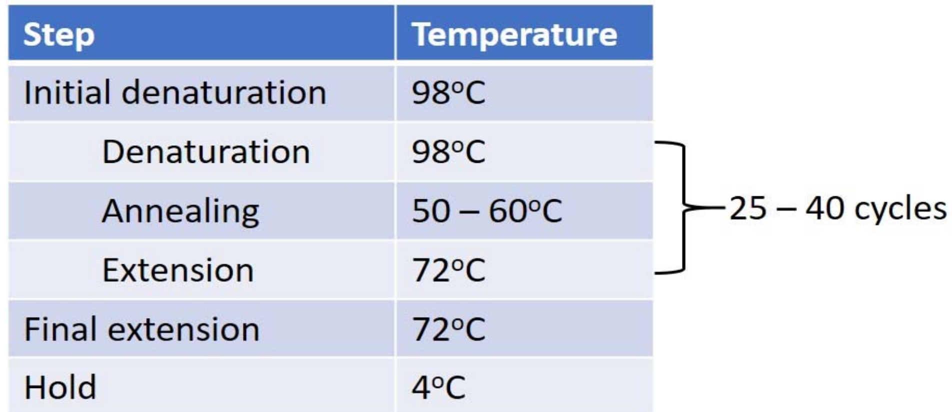
[23] (Delidow et al. 1993)

[24] (Demeke and Jenkins 2010)

The PCR reaction has limiting factors which control the final amount of product and there are feedback inhibitory effects produced by the reaction products (Schochetman, Ou, and Jones 1988).



Step	Temperature
Initial denaturation	98°C
Denaturation	98°C
Annealing	50 – 60°C
Extension	72°C
Final extension	72°C
Hold	4°C

Fig.D.5: Summary of the three main steps of PCR process. Image created by author.

Magnesium Chloride ($MgCl_2$) is a chemical required for the PCR reaction to work and needs to be in a specific concentration. It acts as a co-factor that binds to the DNA and helps to activate the polymerase and maintain its stability. If there is not enough $MgCl_2$ then the polymerase will not activate and the reaction will fail, and if there is too much it may create non-specific amplification of other regions. Primers are short pieces of DNA that are manufactured to be complementary to a specific target site on the DNA template. The two primers required are called the forward and reverse primers. Both are needed to amplify a specific region with a defined beginning and ending point.

The Deoxynucleotide Triphosphates (dNTPs) are the nitrogenous bases or building blocks of DNA. These are used to create each new complementary strand as the polymerase moves along the template, adding one at a time. The PCR reaction will contain all four dNTPs (A, T, G, and C) in equal proportions.

The buffer solution is critical to the success of the PCR reaction as it keeps a stable environment for polymerase to function properly, including optimal pH and salt concentrations. This buffer may contain other PCR enhancers to include bovine serum albumin (BSA), Dimethyl sulfoxide (DMSO) as well as the $MgCl_2$ previously discussed. These additives are utilized to increase the reaction efficiency and help to reduce any potential effects of inhibitors[25]. Bovine serum albumin (BSA) is typically used to prevent the inhibitor from binding to the polymerase by binding to the inhibitor itself [26].

The template DNA molecule is also added to the PCR reaction and serves as the original strand that is separated and copied. This process is semi-conservative where one strand of the double stranded product consists of a template. The amount of DNA template added to the PCR reaction should be within a certain window, with most kits requiring between 2pg and 1ng of DNA. Therefore, once the quantity is obtained from the qPCR step, a sample may need

[25] (Mamedov et al. 2008)

[26] (Satoh et al. 1998)

to be diluted or concentrated accordingly to fall within the target input amounts (Fundamentals of Forensic DNA Typing - 1st Edition n.d.).

The steps involved in the PCR process are shown in Figure D.6 and are as follows:

1. Initialization step (not shown): Sample is heated to 94–98 °C for 1-15 minutes to activate polymerase and denature the DNA.

2. Denaturation: Step 1 of the cycle involves heating to 94–98°C for ~30s, to separate the two strands of the DNA.

3. Annealing step: Step 2 of the cycle lowers the temperature to 50–70°C (dependent on melting temperature of primer) for ~30s so that the primers anneal to the target sequences.

4. Extension/elongation/Replication step: Step 3 of the cycle is where the polymerase creates the new complementary strand. Normally a temperature of 70-72°C is used for about 3 minutes.

5. Final elongation or incubation (not shown): Once the 28-30 cycles are complete, this temperature is kept around 65°C for approximately 10 minutes to complete the adenylation to its full extensions.

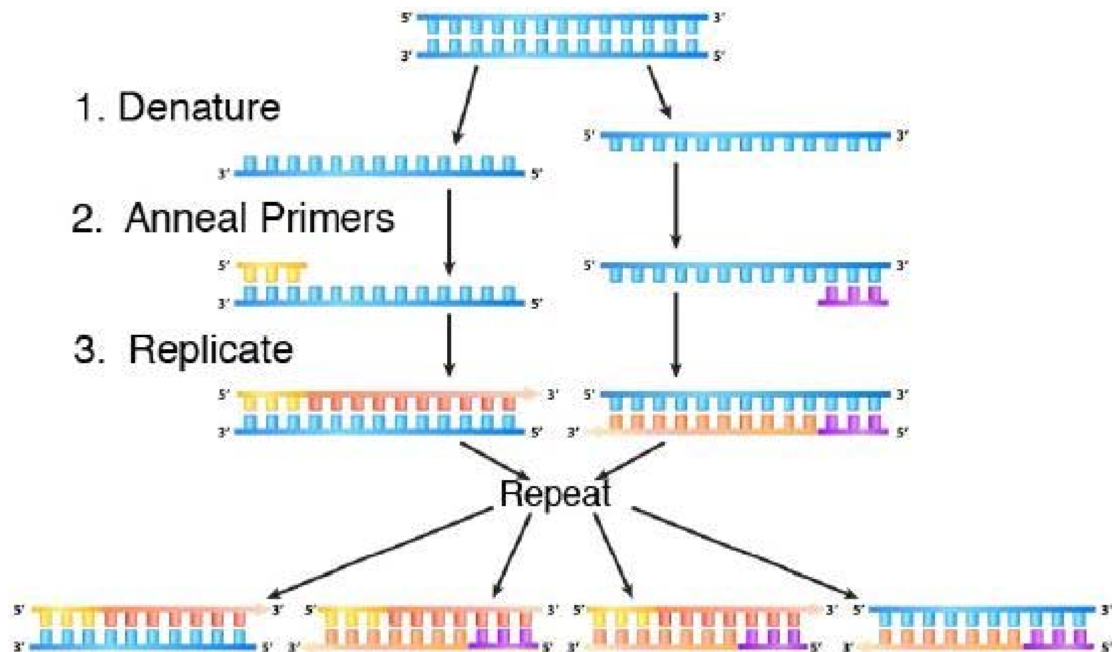


Fig.D.6 Schematic of the PCR thermocycling steps. Denaturation at ~98°C, Annealing ~50-60°C and Elongation ~72°C. Image by Aleia Kim, retrieved from 8.7: Polymerase Chain Reaction (PCR) - Biology LibreTexts on July 15, 2023. (CC-BY-NC-SA)

The PCR process of heating and cooling is performed in an instrument called a thermal cycler, which rapidly heats and cools a metal block according to the PCR parameters that were programmed. The current thermal cyclers are much more advanced compared to the first thermal cyclers which had issues with evaporation and/or condensation, requiring the addition of mineral oil to the top of each sample. Newer thermal cyclers now have a heated lid to eliminate the need for mineral oil. The thermal cyclers allow users to adjust the cycle conditions by programming into the control panel of the instrument. Parameters that can be adjusted include the ramp rate, temperature at each step of the cycle, time for

each step of the PCR, the number of cycles of denaturing, annealing and elongation, and the addition of any pauses such as the infinity hold at 4°C.

PCR inhibition is a condition where a specific chemical molecule that is present in the DNA sample will inhibit the PCR reaction in some way. Some inhibitors make it more difficult for cell lysis to occur while other inhibitors will bind to the polymerase or even to the DNA template strand leading to no amplification or poor amplification[27]. Other inhibitors include hematin, calcium, humic acid (from soil) and indigo (dye used in denim). Hematin in blood and calcium from bone can both inhibit the PCR reaction to varying degrees by binding to the polymerase, thereby competing with the magnesium, resulting in little to no amplification product. In certain cases, it is possible for an inhibitor to be introduced into the sample during collection or preamplification steps [28]. One method for removing the inhibitory effects is to simply dilute the sample which reduces the concentration of the inhibitor in the sample.

It is also possible to add higher concentrations of the DNA polymerase to counteract the lack of polymerase that may have been bound to inhibitors and therefore rendered non-functional. This additional amount would allow the polymerase to be able to complete the PCR steps (Butler 2014). The ideal situation is the complete removal of possible inhibitors from the DNA extract

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[27] (Demeke and Jenkins 2010)

[28] (Butler 2014)

when possible. It is critical for the analyst to determine if a sample is degraded or just inhibited. This is why it is important to have the information from the quantitation step regarding inhibition, to treat the sample according to the specific issue. There is nothing that can be done for degraded DNA, besides amplifying as much template DNA as possible; however, the data from the quantitation step, specifically the internal positive control (IPC) can indicate the presence of potential inhibitors in the DNA samples.

After amplification, the PCR product must then be separated using a capillary electrophoresis (CE). CE is a technique originally performed on an AB 310 genetic analyzer, which was then replaced with an AB 3100 or 3130 genetic analyzer and today CE is commonly performed on the AB 3500 genetic analyzer. CE is a process used to separate and analyze DNA fragments based on their size and charge. The process involves using a sample that has been amplified by PCR and target regions were labeled with a fluorescent dye specific to the nucleotide bases (e.g., dyes such as 6-FAM, VIC, NED, etc.).

Once prepared, the fluorescently labeled PCR product is loaded onto a plate with a denaturing chemical (formamide) and an internal lane standard in each sample. The capillary array within the AB 3500 genetic analyzer consists of multiple fused silica capillaries with a thin inner wall coated with a polymer matrix to reduce electroosmotic flow. This capillary array is precisely aligned within the instrument. The next step involves applying an electric field across the capillary array. High-voltage power supplies within the AB 3500 genetic analyzer generate

the necessary electrical potential. The positive electrode, known as the anode, is placed at the start of the capillary array, while the negative electrode, called the cathode, is positioned at the opposite end.

As the electric field is applied, the negatively charged DNA molecules migrate through the capillaries toward the positive electrode. The migration rate of DNA fragments is determined by their size, with smaller fragments migrating faster than larger ones. This separation is based on the principle of sieving, as the DNA molecules encounter resistance from the polymer matrix within the capillary. During the migration process, a laser within the AB 3500 genetic analyzer excites the fluorescent dye molecules attached to the DNA fragments. This excitation causes the dye to emit light at specific wavelengths, which is detected by a sensitive detector located at the end of the capillary array. The detector captures the emitted light and converts it into an electrical signal (Hill 2012).

The electrical signal generated by the detector is then amplified, digitized, and processed by the AB 3500 genetic analyzer's software. The software analyzes the signal and converts it into an electropherogram, which represents the intensity of the detected fluorescence over time. The electropherogram provides a visual representation of the separated DNA fragments, with peaks corresponding to different fragment sizes. By comparing the position and intensity of the peaks in the electropherogram to known size standards, the AB 3500 genetic analyzer software determines the sizes of the DNA fragments in the

analyzed sample. The software sizes the fragments in the evidence sample by applying an algorithm to the retention time data for the sample using the retention time of the fragments in the Internal Lane Standard that is added to each sample containing fragments of known specific base pair sizes ranging from about 60 base pairs to around 1,000 or so, depending on the kit being used (Fundamentals of Forensic DNA Typing - 1st Edition n.d.). During a CE run, the plate will also have a well containing Allelic Ladder, which contains fragments that encompass all possible allele variations that are normally observed in the population for each DNA locus (Fig.D.7). The software performs the allele calling by comparing the sizes of the alleles in the allelic ladder to the base pair sizes of the unknown evidence sample.

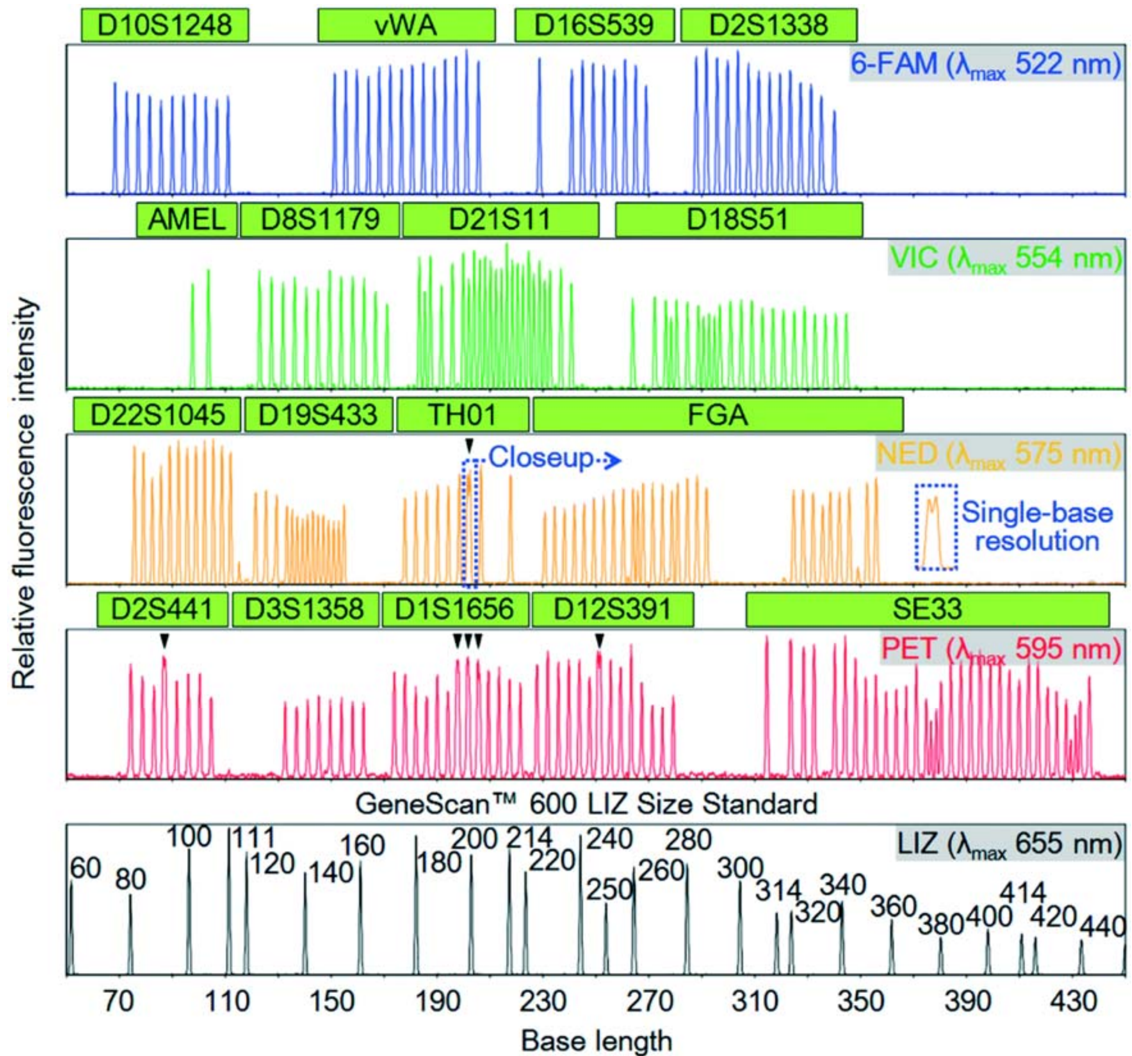

Fig.D.7: Example of allelic ladder and internal lane standard, used to size the fragments and assign allele calls in a PCR kit (Anazawa et al. 2017) (CC-BY-NA)

E. DNA ANALYSIS AND INTERPRETATION

The Genemapper ID-X software is a sophisticated tool created by Applied Biosystems and used in genetic analysis to perform allele calls. It follows a well-defined algorithmic process, which can be summarized in several steps. Initially, the software receives raw electrophoresis data, typically obtained from capillary

electrophoresis or other similar technologies. This raw data contains information about the size and intensity of DNA fragments that have been separated based on their size and charge.

The software starts by performing a baseline correction step to remove any background noise or artifacts. This ensures accurate measurements of fragment sizes and intensities. Next, the data is normalized to a standard size ladder, which serves as a reference for fragment sizing. Once the data is preprocessed, the software applies peak detection algorithms to identify individual peaks corresponding to DNA fragments. These peaks represent all possible alleles for each locus tested.

Assignment of the allele calls to the detected peaks in the sample is performed by the software utilizing a combination of size and intensity information. It compares the observed sizes of the peaks with a predefined set of expected sizes, which are based on known genetic markers and their associated alleles found in the Allelic Ladder. The software calculates a similarity score between the observed sizes and the expected sizes to determine the best match.

In addition to size information, the software considers peak intensities to ensure accurate allele calls. It analyzes the relative intensities of the peaks and compares them with expected patterns for each allele. This helps in distinguishing between true alleles and potential artifacts or noise. To enhance accuracy, the software incorporates statistical models such as Gaussian mixture modeling and quality control measures. It uses statistical methods such as

Gaussian mixture modeling or likelihood ratio tests to estimate the probability of each observed peak belonging to a specific allele.

The Genemapper ID-X software also allows for user-defined parameters and customization. It provides options to adjust thresholds, define peak height or area requirements, and apply allele-calling rules specific to the analysis being performed. The analyst must look carefully at the data called and check for artifacts. There are many kinds of artifacts, the most common one is called stutter. This artifact is produced when there is strand slippage during the amplification causing a PCR product peak that is one repeat sequence shorter or longer due to the insertion or deletion of a repeat unit. Stutter artifacts can complicate the interpretation of mixed DNA samples, especially when the stutter peak intensity overlaps with the true allele peak.

Another artifact analysts must look for is drop-in and drop-out, which are two distinct artifacts that can affect the interpretation of low-template or degraded DNA samples. Drop-in occurs when extraneous DNA contaminates the reaction, leading to the amplification of alleles not present in the original sample. This can occur during sample handling or due to contamination in the laboratory. On the other hand, drop-out refers to the failure to amplify alleles that are genuinely present in the sample and is often caused by the limited amount of template DNA, inhibition, or degradation.

Allelic imbalance can also occur, when both alleles are amplified; however, one allele amplifies preferentially over the other in heterozygous loci,

resulting in an imbalance in peak heights. This artifact can arise from factors such as differences in primer binding efficiencies, secondary structures within the template DNA, or preferential amplification of shorter amplicons. Allelic imbalance can affect the accurate determination of DNA profiles and may impact the interpretation of mixed DNA samples.

Contamination is always something that needs to be monitored carefully in forensic DNA analysis. In general, it refers to the unintended introduction of foreign DNA into the sample or reagents, which can lead to erroneous results. Contamination can occur at any stage of the analysis, including sample collection, handling, or amplification. Should it occur during sample handling, it may be undetectable, for instance if the crime scene technician touches one item at the crime scene, then forgets to change gloves and handles a different item, contaminating with the cells from the first item. When it happens inside the laboratory during analysis it is easier to detect due to the controls and monitoring by checking evidence samples for matches to a staff DNA database. Strict laboratory protocols and appropriate controls are essential to minimize the risk of contamination. Lab protocols always require the processing of what is known as a reagent blank, which is a quality control sample that is processed alongside the evidence samples and serves to monitor the reagents used and the tube handling by the analyst. If there are any DNA peaks that appear in a reagent blank, then further evaluation must be done to determine the possible source of the contamination and ensure that the evidence samples do not contain the

contaminating profile. If there is evidence of contamination, often the sample will need to be re-extracted from new cuttings.

It is crucial to be able to recognize and mitigate these PCR artifacts or contamination in forensic DNA analysis to accurately interpret the results and minimize their impact on casework. Laboratory practices, quality control measures such as reagent blanks, and validation studies are employed to address these artifacts and ensure the reliability of the sample interpretations.

F. DNA STATISTICAL CALCULATIONS

In forensic DNA analysis, statistical methods are crucial for assessing the significance of DNA evidence and providing a quantitative measure of the strength of the association between a suspect and a crime scene sample. Three commonly used statistical methods in forensic DNA analysis are random match probability, the combined probability of inclusion, and likelihood ratios.

Random match probability (RMP) is a statistical measure that determines the likelihood of a randomly selected individual from a population having the same DNA profile as the one observed in the crime scene sample. RMP is calculated based on the frequencies of the specific alleles in the observed evidence profile, commonly obtained from a specific reference database or population database. Frequencies of each allele in the population group are calculated by counting the number of observations of that allele within the population group divided by the total number of individuals in the reference

population group. Since the loci used in forensic testing are independently assorted, the frequencies of alleles for each locus can be multiplied together to obtain the estimated frequency of an entire DNA profile (multiple loci) within the population group. The RMP provides an estimate of the rarity of a particular DNA profile and thus the significance of the match[29].

The combined probability of exclusion (CPE) or its inverse, the combined probability of inclusion (CPI) is another statistical measure used in cases where a mixture of DNA from multiple contributors was obtained from the evidence (Butler 2014). The CPI is The CPI determines the probability that a randomly selected individual, not necessarily the suspect, would be included as a contributor to the observed mixture. It considers the frequencies of DNA profiles in the reference population, as well as the number of contributors and their relative contributions to the mixture. The CPI is typically used to quantify the strength of evidence when multiple individuals could have contributed to the DNA mixture. The limitation of CPI is that it requires that all alleles from all contributors are present in the DNA mixture, which is often not the case when analyzing samples containing low level contributors.

The forensic community has moved towards the use of Likelihood ratios (LRs) for expressing the weight of the evidence obtained. LRs are used to express the strength of DNA evidence in favor of two competing propositions: the

[29] (Butler 2014)

prosecution hypothesis (typically involving the suspect) and the defense hypothesis (typically involving an alternative explanation or another individual's involvement). The LR compares the probability of observing the DNA evidence under each hypothesis. It is calculated by dividing the probability of the DNA evidence given the prosecution hypothesis by the probability of the evidence given the defense hypothesis. The LR provides a measure of the weight of the evidence and allows for the evaluation of the likelihood of different scenarios based on the DNA profiles observed. The likelihood ratio is calculated using a ratio of two propositions. The proposition in the numerator of the ratio is known as the prosecutor's hypothesis and generally favors the inclusion of the person of interest (POI) and an unknown person or POI and victim for example[30]. The denominator of the ratio is known as the defense hypothesis and always will favor exclusion of the POI; therefore, in the example of a two-person mixture it would be that the evidence comes from two unknown individuals or possibly from the victim and an unknown individual. The formation of the proposition set is critical to the resulting likelihood ratio and both propositions (Hp and Hd) must be exclusive, which means that they both cannot be true simultaneously.

It is possible to calculate a LR manually, although it would be very time consuming, therefore laboratories will use a software program to assist with this calculation. There are two types of software systems available for calculating likelihood ratios, one is semi-continuous in that it will apply a single probability of

[30] Buckleton et al., "The Probabilistic Genotyping Software."

drop out to model the entire sample and does not consider the height of each peak when performing the calculation, examples include LRmix, Lab Retriever, and LikeLTD [31]. The other category is fully continuous, which utilizes the Markov chain Monte Carlo (MCMC) resampling method. Examples of the fully continuous software programs are STRmix, LikeLTD and EuroForMix [32]

G. INTERACTIONS BETWEEN METAL IONS AND DNA MOLECULES

In general, the interactions between metal ions and DNA molecules encompass a wide range of mechanisms that play crucial roles in biological processes, including gene regulation, DNA repair, DNA replication, and transcription. Metal ions, being positively charged, interact with the negatively charged phosphate groups on the backbone of DNA primarily through electrostatic attractions. The extent of their interaction is dependent on the position of the ion on the Period Table. There are three basic types of interactions that metal ions can have with a DNA molecule, which include groove association, intercalation, and irreversible covalent bonding (Bonsu, Higgins, and Austin 2020) (see Fig.G.1). Some common metal ions, such as magnesium (Mg^{2+}), sodium (Na^{+}), and potassium (K^{+}), interact with the negatively charged phosphate groups, leading to activation of processes, linkage between enzymes and nucleotides, and the condensation and compaction of DNA. This

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[31] (Buckleton et al. 2019)

[32] (Buckleton et al. 2019)

condensation plays a vital role in DNA packaging within cells and facilitates interactions with proteins.

The alkali metals, which are monovalent, do not typically bind strongly to DNA and will normally associate with the minor grooves (Hud and Polak 2001). The alkali earth metals tend to associate with the major grooves, with Mg^{2+} being a major component of cellular activity due to it forming Mg-dNTP-complexes during PCR which is why it is such a crucial component of the PCR reaction (Bonsu, Higgins, and Austin 2020). The presence of Mg^{2+} ions between DNA strands helps neutralize the negatively charged phosphate backbone, reducing electrostatic repulsion, and promoting DNA stability. Mg^{2+} ions therefore contribute to the maintenance of DNA structure, facilitating DNA-protein interactions, and influencing DNA replication and transcription.

These grooves provide distinct chemical and structural environments that metal ions can exploit. Major groove binding occurs when a metal ion coordinates with functional groups (such as nitrogen or oxygen) of nucleotide bases exposed in the major groove. This interaction can influence DNA structure and affect protein-DNA recognition. Similarly, metal ions such as Na^{+} can bind to the minor groove, although with less structural specificity (Bonsu, Higgins, and Austin 2020).

The transition metal ions can form coordination complexes with specific sites on the DNA molecule, primarily involving coordination bonds with nitrogen or oxygen atoms. Metal ions such as copper (Cu^{2+}), zinc (Zn^{2+}), iron (Fe^{2+}/Fe^{3+}),

and cobalt (Co^{2+}) can coordinate with DNA bases or the phosphate backbone or sometimes both. These interactions often lead to structural alterations and destabilization in DNA and can affect its flexibility, and conformation [33]. Certain transition metals like Cu^{2+} and Fe^{2+}/Fe^{3+} can catalyze the cleavage of DNA strands. This cleavage can occur through two main mechanisms: oxidative DNA cleavage and direct metal participation. Oxidative DNA cleavage involves the generation of reactive oxygen species (ROS) by metal ions, which can damage DNA by causing single- or double-strand breaks, most commonly with copper ions (Bonsu, Higgins, and Austin 2020). Direct metal binding occurs when the metal ion directly coordinates with the DNA backbone or bases, leading to strand breakage. In addition, the irreversible binding can cause problems with its electrophoretic mobility due to the alteration in conductance with the bound metals.

Yet, other metal ions can induce DNA unwinding and destabilization, which is often essential for DNA-related processes. For instance, during DNA replication and transcription, helicases unwind the DNA double helix to expose the template strand. Metal ions like zinc (Zn^{2+}) and nickel (Ni^{2+}) can interact with specific sites on DNA, facilitating local helix destabilization and promoting unwinding.

[33] (Bonsu, Higgins, and Austin 2020)

H. HISTORY OF FIREARMS AND GUN VIOLENCE

The term “firearm” can be defined as any device that can expel a projectile with the use of an explosion typically contained along with the projectile inside what is called “ammunition”. This explosion occurs inside the firearm and is caused by the ignition of a combination of chemicals that make up the gunpowder, which creates a pressure build-up propelling the projectile out through the barrel of the gun at an extremely high speed. Gunpowder was initially created using a combination of charcoal, potassium nitrate, and sulfur. The first uses of gunpowder were for firecrackers and propelling projectiles such as cannonballs or lead pellets and has since evolved to use of smokeless powder (nitrocellulose) which can produce more energy and burns cleaner. Figure H.1 shows the basic parts of a typical semi-automatic handgun which is involved in most gun crimes. The firing pin is what causes the spark as it strikes the primer of the cartridge, igniting the gunpowder which causes a pressure buildup and propels the bullet out of the firearm.

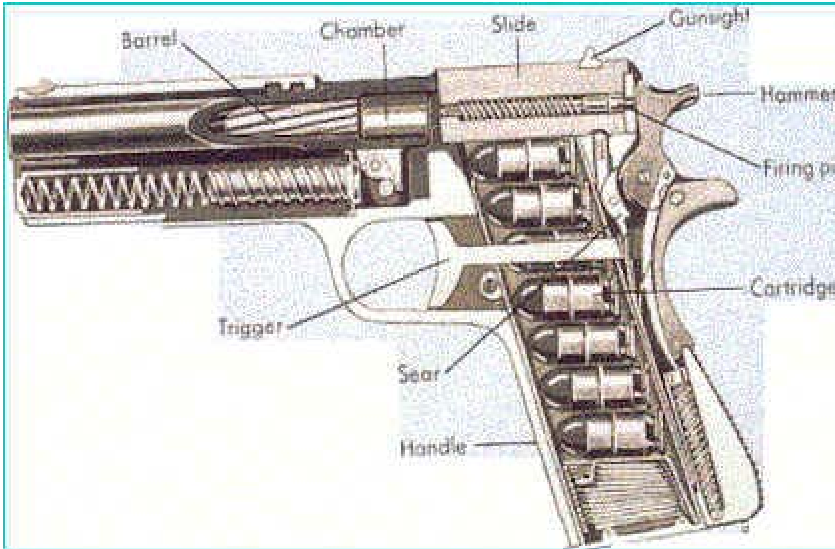

Fig.H.1: Anatomy of a semiautomatic pistol. Retrieved from Jayrod P. Garrett: The Weapons Cache: Guns and Gun Control (the1stog.blogspot.com) on July 15, 2023.

The modern bullet cartridge, as shown in Fig.H.2, shows how the primer, which causes the spark to occur, is in proximity to the propellant located inside the casing. The casing, composed of one of many possible metals, is ejected from the firearm after the bullet is ejected. The most common material used in the manufacture of cartridge casings is brass(Cartridge case | artillery | Britannica n.d.). Brass is an alloy composed primarily of copper (Cu) and zinc (Zn), along with some other minor elements in smaller quantities. It is widely used in various applications, including ammunition casings. Brass is typically chosen as the metal used in casings due to some of its properties, such as its mechanical strength, resistance to corrosion, and how easy it is to manufacture.

The composition of the brass can vary depending on the specific application and desired properties. Cartridge casings typically use 260 brass, which is also

known as cartridge brass, yellow brass, or 70/30 brass, and is an alloy composed of approximately 70% copper and 30% zinc by weight. This composition strikes a balance between strength, malleability, and corrosion resistance. Some other elements can be present in brass alloys in small quantities which help to improve specific traits. One of these other elements which is sometimes included is lead (Pb); however, due to the various environmental and health concerns surrounding lead, the use of lead in brass alloys has been reduced or eliminated in many applications. Tin (Sn) is also sometimes added to improve resistance to corrosion that can occur in brass exposed to certain environmental conditions. Aluminum (Al) can be added to increase the strength and hardness of the brass. Nickel (Ni) is another metal that can be included to improve the strength or toughness of the brass, making it more resistant to corrosion as well. Manganese is added to enhance the strength and hardness (Quality Assurance n.d.). The precise composition of the brass used in the manufacturing of Winchester 9mm ammunition casings can vary to some extent, depending on the specific product line. Additionally, ammunition manufacturers may have proprietary formulations or variations in their brass alloys to suit their specific requirements. Overall, brass provides an excellent material choice for ammunition casings due to its combination of strength, corrosion resistance, and ease of manufacturing.

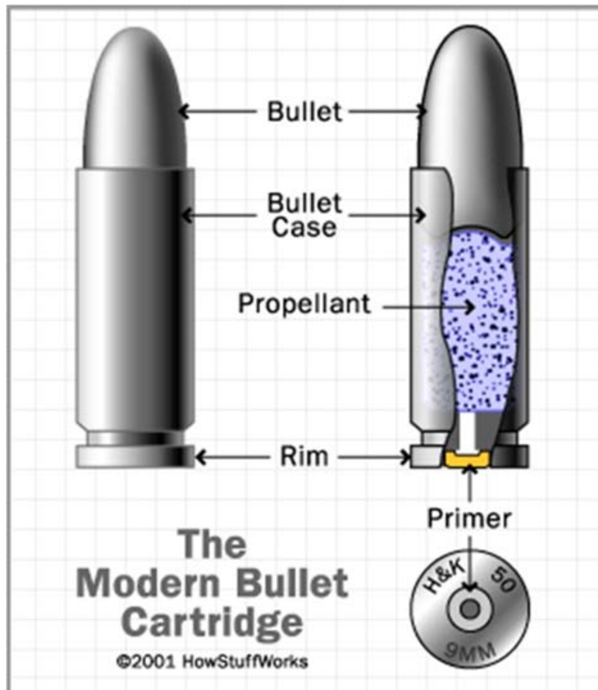

Fig.H.2: Anatomy of a cartridge showing the projectile, casing, propellant and primer. Retrieved from dc - What is the purpose of ballistics tests in The Dark Knight? - Science Fiction & Fantasy Stack Exchange on July 15, 2023.

Since the advent of gunpowder, firearms were slowly improved upon and made to be lightweight and more user friendly compared to their World War II counterparts. Eventually, semi-automatic firearms began to become increasingly popular with civilians, especially the more portable handguns, primarily for reasons of self-protection. This caused a peak in handgun ownership in the late 1980s and along with it came an increase in gun-related crimes.

Gun crimes have been on the rise since 2019, rising 26% in 2020 and continuing to remain at similar levels through 2022 (Gun Violence Archive n.d.). According to the Gun Violence Archives, there were 87,940 people in the United States that were either injured or died from gun violence in 2022 [1]. Data from the

FBI's Uniform Crime Report reveals that in 2019 alone, almost 74% of all homicides were committed with a firearm, compared to 60% of homicides in 2015 (Expanded Homicide Data Table 8 n.d.). In addition to homicides, there are also many nonfatal firearm-related victimization, with 439,200 reported to be the average from 2014 to 2018 according to the Bureau of Justice statistics (Kena 2022). Solving these types of crimes can be challenging since many times the only evidence left behind are fired cartridge casings (FCCs). When a cartridge is fired from a gun, the metal casing that previously held the gunpowder is ejected from the gun through a side chamber, often falling to the ground. This casing is typically very hot while it is being ejected due to the explosion of the gunpowder. It is estimated that the temperature inside the chamber reaches approximately 1800°C for between 0.5 and 5ms.

CHAPTER 1: MODIFICATIONS TO THE BARDOLE METHOD TO INCREASE DNA RECOVERY USING A 2-STEP FILTRATION

ABSTRACT:

Once the tDNA is left on the cartridge when it is loaded into the magazine or chamber of the firearm, the effects of metal ion interactions begin. This may cause any number of downstream effects such as inhibition or even degradation to the extent that DNA amplification yields no result. This study aimed to investigate the potential impact, if any, on the firing process on DNA recovery. Two sets of samples were prepared, one set using 10uL of saliva and the other set using 15uL of saliva. The results showed no significant difference between the fired and unfired casings. The Bardole Method was modified, followed by adding a pre-filter step beforehand and found to have a significant effect on the DNA recovery, $p<0.03$. All further studies will incorporate this 2-step filtration method using the pre-filter followed by the modified Bardole (using a funnel to concentrate the fluid onto the center of the capture filter).

INTRODUCTION

Normal methods for obtaining DNA from fired cartridge casings (FCCs) have primarily involved swabbing the casings collected to remove the cellular material, followed by processing for DNA extraction and typing. Unfortunately, these traditional methods have not been very successful in yielding DNA data that can be used for comparisons, or for entry into a national database to search for a perpetrator. As usable DNA profiles are only produced approximately one

percent of the time using these methods, many laboratories have chosen to refrain from processing FCCs. The Bardole Method (Bonsu et al. 2020) was developed by Francine Bardole with support from M-Vac Systems, Inc. and utilized a soaking of the FCCs in Butterfield's buffer followed by processing through the M-Vac concentration filter to collect cellular material. The M-Vac is a vacuum system that applies a buffer to the surface of an item followed by suctioning the liquid along with any cellular material and depositing it onto the filter of the concentration filter device. The process is not optimal since the filter size is too large to fit into one microcentrifuge tube, leaving behind potential cellular material during extraction.

MATERIALS AND METHODS

Sample selection and preparation

The overall goal of this study is to improve the current methods of obtaining DNA from fired cartridge casings (FCCs) commonly encountered in crime scenes involving shootings. Therefore, these studies were purposely designed to control as many variables as possible and used large amounts of DNA from saliva of volunteers, to more accurately determine which methodology resulted in the largest recovery and lowest variability in amount of cellular material deposited on each cartridge casing. This procedure was used instead of mock casework studies as such procedures significantly increase the variability in recovery rates due to innate differences in the transfer of cellular material when handling the cartridges while loading a magazine. Once a methodology

was identified as obtaining the largest % recovery using known high quantity cellular material, then it was reasonable to expect that this procedure would produce the largest DNA recovery regardless of the amount of cellular material present when used in the real casework samples.

The ammunition used in these studies was 9mm full metal jacket Winchester cartridges which are the most common type seen in casework. The cartridge casings are made of brass consisting of approximately 70% copper and 30% zinc. Casings were spotted on the headstamp groove area with liquid saliva diluted 1:1 to reduce viscosity and represented 200 cells based on cell counting performed with a hemocytometer. The cartridges were allowed to dry thoroughly and were loaded into a magazine using gloves. The cartridges were next fired using a Glock 9mm semi-automatic pistol through a cardboard box to catch the FCCs as they were ejected. The FCCs were placed into glassine envelopes and processed within two days using the specific method being tested.

Fig. 1.1: Method for collecting fired cartridge casings, using a cardboard box to catch the FCCs so they did not fall on the ground. Photo taken at Broward County Sheriff’s Office by author.

Cell Collection via Bardole Method with Modification

The BSO Crime Scene Unit has been using the M-VAC System for processing evidence since approximately 2014. This method has been used to process clothing and other types of evidence using a patented wand and buffer system which collects cellular material embedded into the fibers of fabrics or the crevices of surfaces such as rocks. The system utilizes a concentration container with a 0.45mm filter to capture cellular material for downstream DNA processing. A method utilizing the M-VAC filter device for processing FCCs was previously developed by Francine Bardole and termed the Bardole Method (Bonsu, Higgins, and Austin 2020). This method involves soaking the FCC in the special buffer

solution (called surface rinse solution or SRS,) also known as Butterfield's Buffer. The sample in the buffer is next aspirated onto the filter (Fig.1.2) using the vacuum pressure of the M-VAC.

Fig.1.2: Concentration Filter device, reproduced with permission from M-Vac Systems, Inc.

Fig.1.3: Modification to Bardole method with addition of a funnel to concentrate the cells into a smaller diameter portion of the concentration filter. This enables the entire sample to be extracted at one time. Photo taken at Broward Sheriff's Office by author.

The diameter of the filter membrane contained in the concentration filter device as shown in Fig.1.2 is 50mm. This filter is capturing the cellular material on its surface as the solution flows through the membrane. The overall size of the membrane makes it unable to be extracted in its entirety in a 1.5mL microcentrifuge tube, even with cutting into smaller pieces. Therefore, a modification to the procedure was devised whereby a plastic funnel is inserted into the upper portion of the device and held down to form a seal over the membrane while the filtrate is filtered through the device using the vacuum pressure. This concentrates the cellular material to a smaller portion of the filter,

approximately 20mm in diameter, and allows for downstream processing of the entire sample (Fig.1.3).

Cell Collection via 2-Step Filtration Method

Individual FCCs were added to a 50mL sterile conical tube followed by the addition of 25mL of Butterfield's Buffer (M-VAC Systems). After vortexing for about 30 seconds, the sample and buffer were pre-filtered to remove debris or large contaminants using a device consisting of a Durapore 40-micron polyvinylidene fluoride (PVDF) filter (Millipore Steriflip SKU#SCNY00040) which was connected to a tube that ultimately leads to an MVAC or other vacuum system (Fig.1.4). The 50mL conical containing the FCCs and debris was next inverted while applying a vacuum pressure to allow the solution to flow through the pre-filter, capturing larger debris and allowing cellular material to flow through. This filtrate collected in the lower 50mL conical contains the cellular material and any free DNA (Fig.1.5). The filtered solution was then poured into a sterile Nalgene Rapid-Flow Sterile Disposable Filter Unit containing a 0.45mm polyethersulfone (PES) membrane (Thermo-Fisher cat#124-0045) or cellulose nitrate (CN) membrane (Thermo-Fisher cat#121-0045). This filter unit has a large surface area; therefore, a sterile plastic funnel was placed inside the filter unit to concentrate the cellular material into a smaller subset of the filter surface. (Fig.1.6). Vacuum pressure was next applied to filter the sample liquid through the filter unit. Filter membranes were then allowed to dry. Next, the center-

stained circular area of the membrane containing the sample was cut out and placed into a small sterile petri dish for DNA processing.

Fig.1.4: Pre-Filter Device with tubing that leads to the vacuum, reproduced with permission from M-Vac Systems, Inc.

Fig.1.5: Pre-Filter device after attachment and inversion of conical containing casings. Bottom conical contains filtrate with cellular material for step 2. Photo taken at Broward County Sheriff's Office by author.

Fig.1.6: Concentration filter using modified funnel insertion for additional concentration of cellular material into smaller diameter area of the filter. Photo taken at Broward County Sheriff's Office by author.

DNA Processing

Samples were extracted using the Qiagen EZ1 DNA Investigator Kit and BioRobot EZ1 (EZ1). A modified version of Qiagen's protocol was utilized, incorporating a special in-house buffer. Samples were lysed using 690mL of the in-house buffer (TNE, 1%SDS, 1 drop of Anti-foam A), 10 mL proteinase K (20mg/mL) and 1mL carrier RNA. The samples were incubated for 30 minutes on an Eppendorf Thermomixer C at 1400rpm in 30 sec intervals. The large volume protocol was used on the EZ1, and samples were eluted in 40ml. Samples were quantified using the Quantifiler™ Trio DNA Quantification Kit on the ABI 7500 Real-Time PCR System. Each extract was amplified using the GlobalFiler™ PCR Amplification Kit and subsequently detected on an AB 3500 Genetic Analyzer. Samples were injected for 15s at 1.2kV and separated electrophorectically in performance optimized polymer (POP-4) using the HID36_POP4 module. The data was analyzed using GeneMapper IDX software v1.5. The analytical threshold was set at 150 relative fluorescence units (RFUs).

RESULTS

This study consisted of two (2) sets of ten (10) cartridges each, with 10 mL of saliva containing a total of ~1,000 buccal cells spotted onto each casing and allowed to dry. Each cartridge was fired, and the casings processed with set one (10 samples) prepared using only the Bardole method (adding the use of a plastic funnel for DNA processing ease) and set two (10 samples) processed

through the 2-step Filtration method. The data obtained showed a statistically significant increase in the average quantity of DNA recovered with the 2-Step Filtration method with a p-value of 0.03 (see Fig.1.6). Based on the data obtained, all future studies involved optimization of the 2-Step Filtration method.

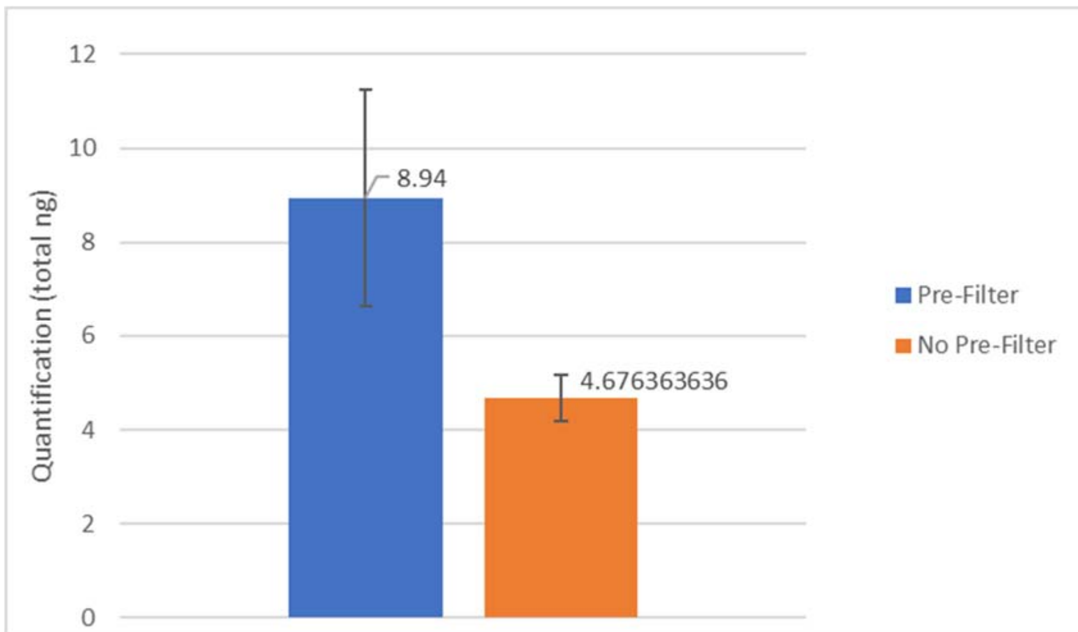

Fig.1.6: Quantification results for Modified Bardole Method alone (No Pre-Filter) compared to 2-Step Method (Pre-Filter use followed by Modified Bardole Method), n=10, p-value=0.03.

DISCUSSION

One of the observations during the preliminary testing of the methods involved the collection of the FCC from the crime scene. Normal protocols at that time involved the use of paper coin envelopes which would be used for one or more FCCs at a time. This process was linked to much lower DNA recovery, and it is believed to be potentially caused by the paper of the envelope rubbing against the FCC surface. The change to using glassine envelopes greatly

improved the recovery of DNA. Ideally, isolating the FCCs using a clean sterile collection container that prevents the casing from touching the sides of the container would be the best methodology for collection.

CHAPTER 2: CHELATION FILTRATION IMPROVES DNA RECOVERY COMPARED TO TRADITIONAL SWABBING

ABSTRACT:

Studies have provided increasing evidence that the metallic surface of the casings release metal ions that bind to the DNA and cause inhibition during the DNA amplification step, and potentially degrade the DNA (Bonsu, Higgins, and Austin 2020). More recent techniques for improving DNA recovery involve specialized methods for soaking casings during extraction[34], which can result in additional time and expense. Furthermore, since it has been shown that the presence of metal ions in a sample can result in gradually increasing DNA strand breakage (Bonsu, Higgins, and Austin 2020), there is a need to develop a rapid and simplified method to increase DNA recovery. This study describes the development and proof of concept for a quick two-step cellular collection method using Chelex to isolate metal ions followed by vacuum filtration. This method, being called “Chelation Filtration” is designed to be used after crime scene collection of the casings, and prior to submission to the DNA laboratory. It enhances the recovery of DNA from casings by up to 40% over the current swabbing techniques and is easily incorporated into crime scene processing utilized.

[34] (Bille et al. 2020)

INTRODUCTION

The original belief regarding the cause of the poor success rates was that degradation of the DNA occurred due to the extreme heat generated when the bullet was fired in the chamber[35]. This theory has been tested over the years, and many studies have since provided increasing evidence that the metallic surface of the casings that causes PCR inhibition during the DNA amplification step due to DNA binding and intercalation, as well as degradation[36]. Therefore, overcoming this metal inhibition would theoretically help to recover more DNA, and result in a higher number of cases with DNA data that is usable.

In 2010, Dieltjes et al. published a paper outlining a method for soaking the FCCs in a lysis buffer followed by swabbing to help capture all the DNA in the sample [37]. This method was expanded upon by Montpetit et al. in 2015[38], who made modifications to the lysis buffer. Both groups claimed to achieve about 25% or more success. These soaking methods were attempted at Broward Sheriff's Office (BSO) in September of 2019 however, the results could not be replicated (data not shown), therefore other methodologies were investigated. Importantly, these soaking methods do not specifically address the removal of PCR inhibitors. These inhibitors can include metals ions that are released from

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[35] (Bille et al. 2020; Burrill, Daniel, and Frascione 2019; Horsman-Hall et al. 2009)

[36] (Dieltjes et al. 2011)

[37] (Dieltjes et al. 2011)

[38] (Montpetit and O'Donnell 2015)

the metallic surfaces of the casings which cannot be removed using standard filtration methods such as the Bardole method (Bonsu, Higgins, and Austin 2020).

MATERIALS AND METHODS

Several experiments were performed to optimize sample recovery. In general, these experiments involved soaking the FCCs in sterile 50mL conical tubes containing 25mL of Butterfield's Buffer (M-VAC Systems) and 2-3 grams of Chelex resin (100-200 mesh, molecular biology grade) prior to incubation/shaking. The sample conical tube was vortexed for approximately 30 seconds using a level 3 on the VWR Mini vortexer. The sample tube containing the casings/Chelex Resin/Buffer was attached to the Steriflip Pre-filter and inverted with vacuum pressure applied to filter out the casings and Chelex along with any other debris or contaminants. The remaining liquid filtrate in the lower conical tube was then added to the funnel inside the concentration filter with vacuum pressure to filter the cellular material onto the surface of the membrane filter. The membrane filters were next covered and allowed to dry. Once dry, the center-stained circular area of the membrane was cut out and placed into a small sterile petri dish for DNA processing.

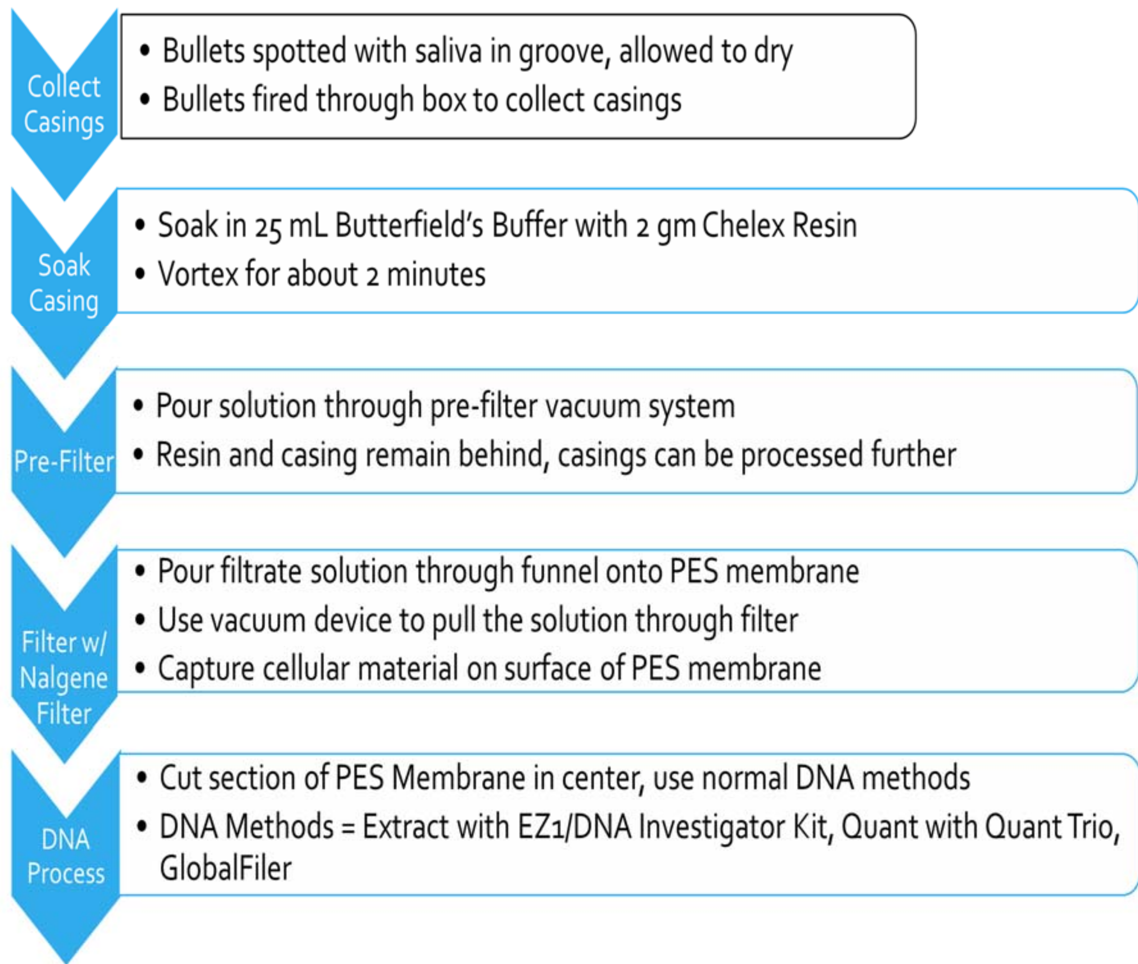

Fig.2.1: Chelation Filtration method outline, produced by author.

RESULTS

Modification of 2-Step Filtration Method with addition of Chelex Resin

In this study, 5 mL of saliva diluted 1:1 was spotted onto each cartridge and two sets of five casings each were processed, one set using the Chelex resin and the other set without the resin, with both sets using the Pre-Filter Device and modified concentration filter. The data obtained (Figure 2.2) did not show a statistically significant difference in the quantity of DNA recovered;

however, after amplification with GlobalFiler, the quality of the DNA profiles was clearly improved with the Chelex resin samples showing reduced degradation and/or inhibition (Figure 2.3). All future work incorporated the use of Chelex resin to help remove metal ions (Chelation Filtration Method).

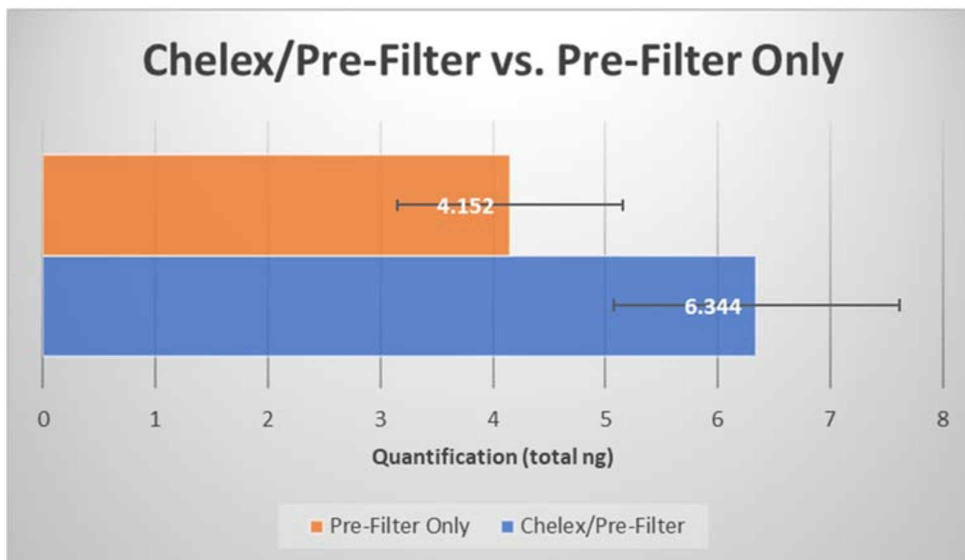

Figure 2.2: Quantification results from Chelex Resin/Pre-Filter samples compared to Pre-Filter alone. N=5, p=0.215 (no statistical significance with the quantity results)

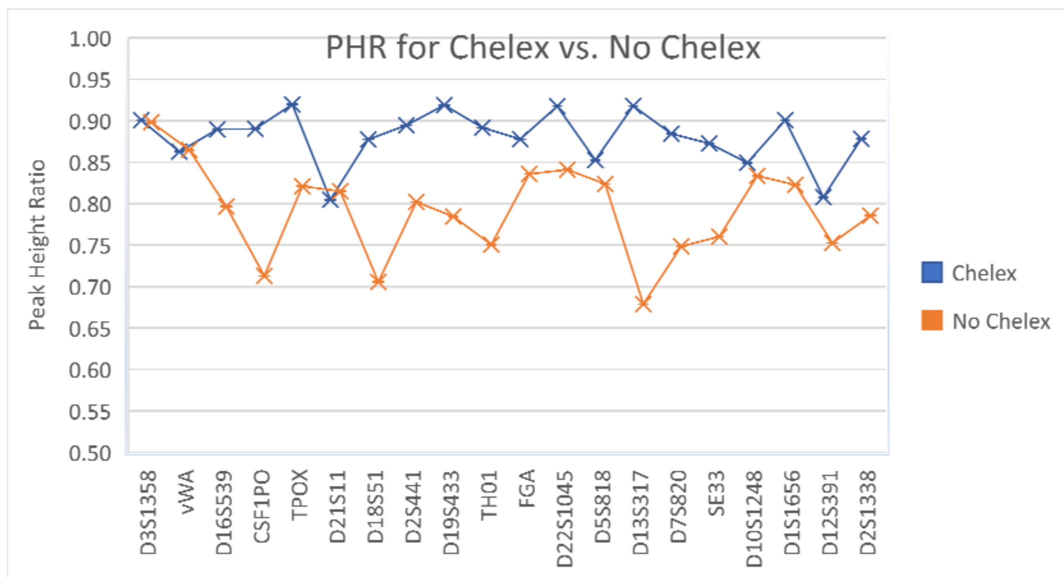

Figure 2.3: Average Peak height ratios (PHR) for each locus from samples processed with Chelex resin and without the Chelex resin. Two-tailed p-value=<0.0001

Comparison of Swabbing Method to Chelation Filtration Method

This study was conducted to perform a larger scale comparison of the traditional swabbing technique to the new modified Chelation Filtration method. The study was set up by spotting 60 cartridges with ~200 buccal cells per cartridge casing, which were allowed to dry, then fired and FCCs collected in a paper box surrounding the gun. The FCCs were processed using the Chelation Filtration method. The results shown in Table 2 are for the samples that were processed using 2 casings per swab or filter. The percentage of profile was calculated by dividing the number of alleles obtained by the expected number of alleles in the donor profile. The percentage of profile obtained for each method was calculated for both two (2) FCCs per sample and one (1) FCC per sample,

as shown in Figures 8 and 9. The results indicated on average, approximately 60% more alleles recovered with the modified Chelation Filtration method when compared to traditional swabbing method. A-NOVA analysis indicated a statistically significant difference between the two sets with a p-value of 2.17×10^{-17} (Table 2.1).

Table 2.1 Results from comparison of two (2) FCCs per sample processed with traditional swabbing vs. Chelation Filtration Method.

	Swabbing				**Chelation Filtration**			
	Total ng	**% Recovery**	**# Alleles Obtained**	**% Profile**	**Total ng**	**% Recovery**	**# Alleles Obtained**	**% Profile**
Sample #1	0.227	9.47	11	24	0.712	29.69	45	100
Sample #2	0.188	7.85	7	16	0.461	19.21	44	98
Sample #3	0.500	20.84	17	38	1.330	55.42	45	100
Sample #4	0.137	5.71	7	16	0.255	10.62	26	58
Sample #5	1.073	44.70	24	53	0.337	14.05	21	47
Sample #6	0.386	16.09	14	31	0.656	27.34	45	100
Sample #7	0.170	7.08	3	7	0.429	17.86	43	96
Sample #8	0.271	11.29	11	24	1.655	68.94	45	100
Sample #9	0.472	19.67	17	38	0.346	14.42	40	89
Sample #10	0.625	26.04	14	31	0.767	31.98	44	98
Average	0.40	16	12.5	28	0.69	29	39.8	88
Std. Dev.	0.29	12	6.1	13	0.46	19	8.8	20

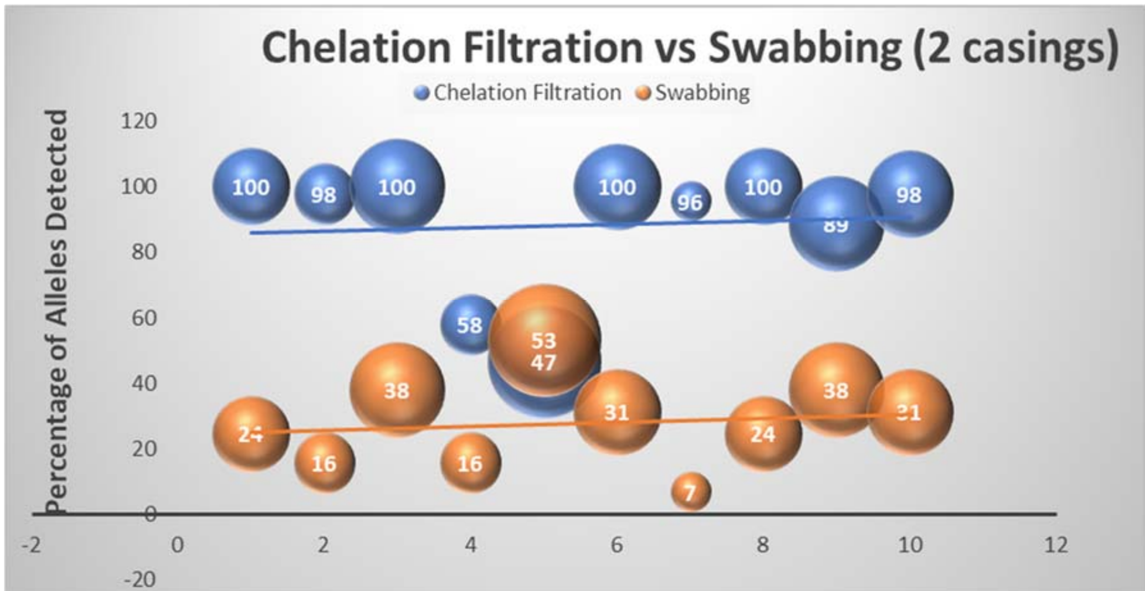

Fig.2.4: Percent of alleles detected with Swabbing vs. Chelation Filtration method (2 casings processed per sample). N=10. A-NOVA analysis indicated a statistically significant difference between the two (2) sets with a p=value of 2.17×10^{-17}.

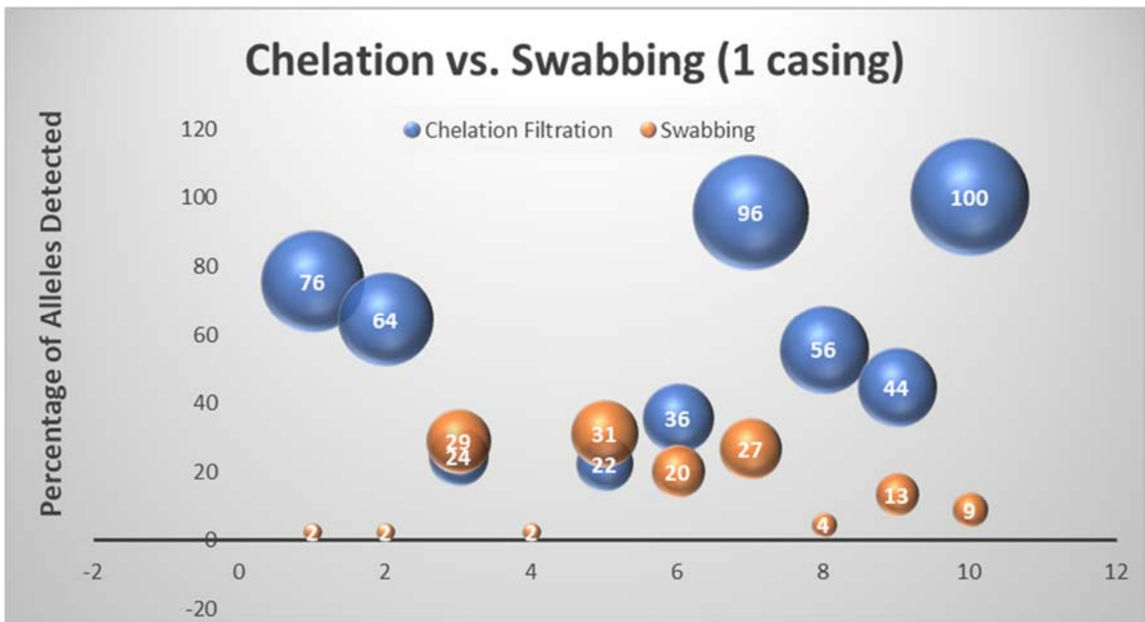

Fig.2.5: Percent of alleles detected with Swabbing vs. Chelation Filtration method (1 casing processed per sample).

DISCUSSION

The greatest improvement in DNA recovery was seen when two casings were processed together using the Chelation Filtration Method and a single concentration filter, as this effectively increased the concentration of cellular material for both FCCs together. The data from the Chelation Filtration method shows that, on average, processing only one casing yielded approximately 26% of the alleles while processing two casings with one filter yielded approximately 88% of the alleles, a 3.3-fold increase. It is noted that two samples (sample #4 and sample #5) showed lower % profiles recovered, 58% and 47% respectively, for this experiment. These data are likely due to the procedural steps where cells can potentially be lost during the experiment to include a) loss of cellular material by handling the cartridges as they are loaded into the magazine prior to firing; b) loss of cellular material during the firing process as the casing is ejected; and c) the loss of cellular material through contact with the cardboard box. Both samples are However, even with samples prepared containing less cellular material (one casing per sample), there was still a significant difference between the two methods. The combining of FCCs according to caliber type and location found increases the chances of obtaining interpretable profiles, these studies showed 52% of profile obtained with one casing and 88% of profile obtained with two casings given all conditions being equal.

CHAPTER 3: OPTIMIZATION OF CHELATION FILTRATION METHOD

ABSTRACT:

The Chelation Filtration method, while already indicating significant improvements of DNA result compared to traditional swabbing, has many variables that could be tested to see if there are other improvements that can be made. The collection filter/membrane comes with a PES membrane which is the surface that collects the cellular material. Different membrane materials exist such as cellulose nitrate (CN) and these membranes can also be purchased for the Nalgene filter unit. In addition, the Chelex incubation protocol utilizes 2 grams of resin. It is possible that more resin may improve DNA recovery results. These two variables were tested separately, and the results show CN filters recovered greater amounts of DNA, while the data does not show any significant difference between the use of 2 grams versus 3 grams of Chelex resin.

INTRODUCTION

The Chelex Filtration method was first tested using the recommended amounts of Chelex 100 resin, according to the manufacturer (Walsh, Metzger, and Higushi 2013) which was 2 grams for every 25mL of buffer. Depending on the level of metal ions on the FCC, it is possible that higher levels of Chelex 100 resin could improve the removal of these metal inhibitors, therefore increasing the quantity of DNA recovered. The type of bullet material (metal composition) may also require different amounts of Chelex resin. The 9mm cartridges used are

primarily composed of brass alloy material, with copper being the largest component metal. The .40 caliber Smith & Wesson cartridges are also made of brass alloy with varying amounts of the same metals, although overall heavier. These are the two most common types of cartridges used in the United States currently.

In addition, the PES filter membrane is typically used for the concentration filter as it is hydrophilic and has a high flow rate along with a low non-specific protein adsorption. The CN filter membranes are composed of cellulose mixed ester recommended for filtering solutions in general applications including buffer filtration. The CN membrane is also hydrophilic, is strong and flexible and has a high flow rate. The membrane filter inside the concentration filter is sitting inside without being secured; therefore, the membrane can be replaced or Nalgene concentration filters with different membrane filters can be purchased. The test will compare PES filter membranes to CN filter membranes.

MATERIALS AND METHODS

Comparison of the Amount of Chelex Resin Used and Different Bullet Types

A total of 100 cartridges, fifty (50) 9mm and fifty (50) .40 cal S&W, were spotted with ~200 cells (using cell counting method) and allowed to dry. These were all fired, and casings (FCCs) were collected in cardboard box and sorted for processing. Within 24 hours of collection, the FCCs were processed using the Chelation Filtration method, in groups of 5 FCCs for each sample. A total of ten (10) samples (50 FCCs in total) were 9mm casings and were processed with five

(5) samples having 2 grams of Chelex resin during the incubation step and five (5) samples having 3 grams of Chelex resin during the incubation step. The ten (10) samples (50 FCCs in total) that were .40 cal S&W casings were also processed in a similar manner, with five (5) samples having 2 grams of Chelex resin during the incubation step and five (5) samples having 3 grams of Chelex resin during the incubation step. Analysis of quantitation data (total ng/sample) was conducted by t-Test.

Comparison of Filter Membrane Materials – PES vs. CN

This study was performed using twenty (20) 9mm cartridges, each spotted with ~200 cells from 1:1 saliva dilution (determined by cell counting) and allowed to dry thoroughly. The cartridges were fired within 24 hours and FCCs were collected in a cardboard box. Samples were processed individually with the Chelation Filtration methodology, ten (10) samples were processed with a PES membrane final concentration filter and ten (10) samples were processed with a CN membrane final concentration filter. The samples went through the normal DNA processing and the Quantification results were analyzed by t-test for statistical significance.

RESULTS

Comparison of the Amount of Chelex Resin Used and Different Bullet Types

There was no statistical significance associated with the DNA recovered from using 2 grams of resin to 3 grams of resin, regardless of the ammunition

tested (9mm vs. .40 cal S&W). Figure 3.1 below shows the results, which were analyzed by t-test for amount of resin as well as the casing caliber types, with no statistical significance for either.

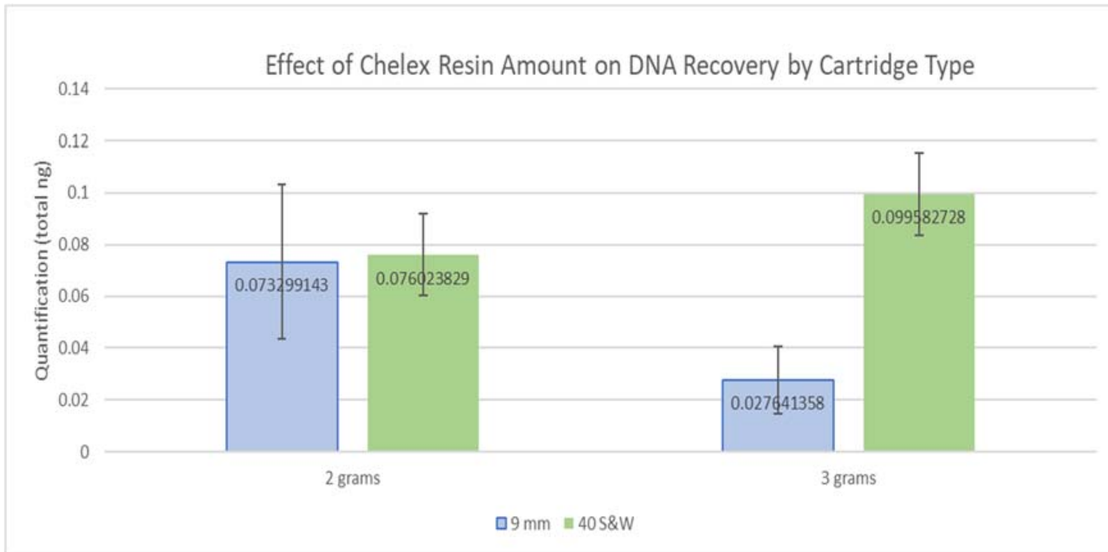

Figure 3.1: Quantification results (total ng recovered) from samples of 5 casings each, 5 samples tested (25 casings in total) for each cartridge type and for each amount of resin. n=5 for each group, total of 20 samples.

Comparison of Filter Membrane Materials – PES vs. CN

The results for this study showed that a greater quantity of DNA was recovered from the samples processed with the CN membrane, and after the t-test was applied, it indicated that this difference was statistically significant, with a p-value of 0.02. See Figure 3.2 below for a plot of the results.

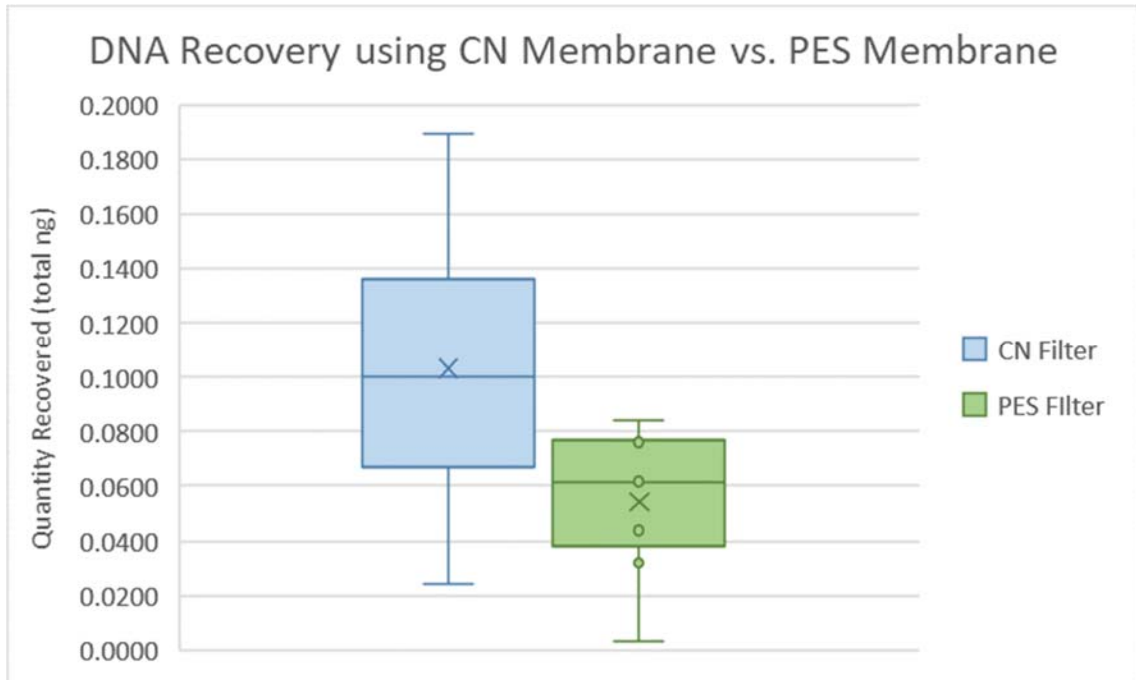

Fig. 3.2: Comparison of the PES membranes to the CN membranes in concentration filters indicating statistical significance, n=5, p-value=0.02.

DISCUSSION

These results show that using the CN membrane filters will provide a greater DNA recovery over the previous PES filters. The Chelex resin does not appear to be a limiting factor in the removal of the metals and therefore changes to the amount of Chelex resin is not warranted. In addition, it is noted that brass alloys of different cartridge types are both compatible with the Chelation Filtration methodology and it is not limited to use on 9mm casings alone.

CHAPTER 4: ANECTODAL STUDY SHOWING RESULTS FROM CASEWORK SAMPLES PROCESSED WITH CHELATION FILTRATION METHOD

ABSTRACT:

Given that the proof-of-concept study demonstrated that the Chelation Filtration method gives 40% more alleles recovered compared to traditional swabbing, this study will incorporate an anecdotal study where the Chelation Filtration method will be utilized on casework evidence in Homicide and Attempted Homicide cases where FCCs were left behind. This study was conducted on 122 cases and resulted in 21 DNA profiles (17% of the case) that were interpretable using STRmix as the deconvolution tool.

INTRODUCTION

The proof-of-concept studies performed using saliva showed that the Chelation Filtration method obtains ~40% more alleles compared to traditional swabbing. Due to the difficulties with controlling how many cells are deposited onto a cartridge when handling it, including the large variability even within one individual who handles multiple cartridges one after the other, leaving varying numbers of cells on each casing. For these reasons real-world “touch” DNA simulations are not possible without testing hundreds of samples.

Anecdotal studies are a way of using large numbers of real casework sample types involving casings that are left behind at a crime scene. These samples can be processed using the Chelation Filtration method to see if the results obtained are better than the swabbing methods. Swabbing methods at

the Broward Sheriff's Office only yielded DNA profiles when the casings had rolled around in blood, and success was less than 1% making it not cost or time effective to process.

MATERIALS AND METHODS

The crime scene unit at the Broward Sheriff's Office was able to implement the Chelation Filtration method utilizing the Chelex resin on at least 122 cases within an 18-month period and the data obtained were tabulated. For each case, the collected FCCs of identical caliber/type were processed together through the Chelation Filtration method. The number of FCCs processed together varied per case, anywhere from one FCC to 27 FCCs. This data included the number of profiles that were interpretable (where statistical calculations could be conducted), how many provided an inclusionary statistic to a potential perpetrator, how many were entered into CODIS, and how many CODIS hits were obtained (Table 3). Historically, with traditional swabbing of FCCs from casework, the percentage of interpretable profiles has been reported to vary somewhere between zero up to 6%, using specialized amplification kits such as Minifiler (Horsman-Hall et al. 2009). Prior to the Chelation Filtration Method, the BSO DNA Unit was not processing swabs from FCCs due to the very low quantity and quality of the data obtained, only obtaining a DNA profile when the FCC had come into contact with blood prior to collection/swabbing.

RESULTS

Table 3.1: Data obtained from cases processed at the BSO Crime Scene Unit with the Chelation Filtration Method over an 18-month period

Cases Worked	Interpretable Profiles	Stats to Suspect	CODIS Entries	Hits
122	21	5	7	5
Proportion of Total	**17%**	**4%**	**6%**	**4%**

STRmix software tool was used for all mixture samples to assist with deconvolution and/or statistical calculations if a person of interest (POI) was submitted for comparisons. In one case, a mixture of two contributors was obtained with data at 12 out of the 22 loci tested. This case had a POI who was compared to the mixture and the STRmix software calculated a likelihood ratio (LR) fo 97,000. This number means that the data is 97,000 times more likely if the POI and an unknown individual contributed to the sample than if two unknown individuals contributed.

DISCUSSION

Anecdotal evaluation of real-life casework samples showed that of the 21 interpretable profiles, ten (10) were single source profiles (48%), seven (7) were 2-person mixtures (33%), and four (4) were 3-person mixtures (19%). While combining FCCs into one sample tube increases the likelihood of obtaining a mixed DNA profile, the anecdotal studies where up to 27 FCCs were processed in the same tube showed that mixtures of no more than 3 people were obtained,

thus allowing for the use of STRmix for assistance with mixture deconvolution which significantly helps with profile interpretations.

The STRmix data shows the power of analyzing mixtures with this tool, which uses mathematical modeling to help deduce contributors and performs statistical calculations on mixtures of up to 4 individuals. In these real-world cases, there were never any mixtures of greater than 3 people obtained, with the majority being either a single source profile or a mixture of two people.

Laboratories may also choose to limit testing to only one (1) FCC per sample tube, as the anecdotal studies show that 3 of the interpretable profiles did come from the processing of only a single FCC using the Chelation Filtration method.

CONCLUSION AND FUTURE WORKS

In addition to the increase in the amount of DNA recovered from these FCCs, the quality of the resulting profiles was also increased with the addition of the Chelex to help remove the metal inhibitors. The level of variability from sample to sample in these studies is expected and can be attributed to various uncontrollable factors affecting the number of cells remaining on the FCC after spotting the cells onto the cartridge, such as the handling of the cartridge when loading into the firearm using gloves, the firing process itself, or even the dropping/falling of the FCC into the paper cardboard box, etc.

Another factor that appears to influence the results, as described by Bille et al. and seen in preliminary testing performed prior to these experiments, was the timeframe of processing. When FCCs were allowed to sit for longer periods of time prior to processing, the DNA became increasingly degraded, presumably due to increased exposure to metal ions. This was the reasoning behind a recommendation for quicker processing, ideally within 24 hours of collection. The Chelation Filtration method is relatively quick, simple, and can be easily performed by crime scene personnel even in the field on the crime scene, without the need for the DNA analyst to first be assigned the case and retrieve the evidence. Although these studies were conducted using an M-VAC system for the vacuum pressure, other vacuum systems should also be effective in assisting with filtration portion. Overall, the method uses common plastics devices and is relatively low cost, allowing for implementation in any crime scene unit. The filters are processed using normal DNA methods, and do not require any special equipment or methodology.

The optional combining of FCCs according to caliber type was shown to improve results, although it does increase the likelihood of obtaining a mixed DNA profile. Laboratories may choose to process only one (1) FCC per sample tube to minimize this risk or use mixture deconvolution tools such as STRmix to further assist with resolving contributors.

This study represents a simple and practical method for optimizing the recovery of fired cartridge casings. The procedure involves capturing cell debris

in a buffer with Chelex to remove metal inhibitors and then processing the sample with a two-step filtration using a vacuum filtration device that isolates the cellular material. The results show an overall 60% increase in sample recovery over traditional swabbing methods, with 17% of actual casework samples providing a usable DNA profile. Processing multiple casings at the same time improved DNA recovery with the potential downside that mixtures may be encountered. To overcome this potential roadblock, many laboratories now use probabilistic software such as STRmix which can be helpful with statistical analysis of any mixtures that are obtained.

In the anecdotal studies, which only captured a period of 18 months, there were not only matches to a POI allowing for the potential arrest of these individuals in some cases, but there were also 5 matches of these unknown profiles to a convicted offender in the CODIS database. These are five cases involving shootings or homicides where the perpetrator would potentially not have been identified without this database match. This information could help identify potential perpetrators of gun violence is crucial to assisting with solving these crimes.

Future studies will focus on expanding the collection material to include cell-free DNA using a DNA capture technology that can be added to the solution prior to filtration through the concentration filter. In addition, work will be done to create a 3-D printed collection container with posts that can prevent the casings

from touching each other or the envelopes and can also serve as a container to incubate the casings in the buffer/Chelex solution.

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PUBLICATIONS AND PRESENTATIONS:

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