
UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

EXTRACTION AND STR AMPLIFICATION OF HAEIII

RESTRICTION CUT, MEMBRANE

BOUND HUMAN DNA

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EXTRACTION AND STR AMPLIFICATION OF HAEIII RESTRICTION CUT, MEMBRANE BOUND HUMAN DNA

INTERNSHIP PRACTICUM REPORT

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas
Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

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Fort Worth, Texas

July 2005

ACKNOWLEDGEMENTS

First of all I would like to thank Dr. Joe Warren for all of his help and guidance during my graduate school career at the University of North Texas Health Science Center. I would like to thank my professors for providing me with the knowledge and support to reach my goals.

I would like to thank Shelly Steadman for allowing me to work on such an interesting research project. Thank you to all of those in the DNA lab at the SCRFSC for showing me that work can be fun. I would also like to thank my family and friends for always supporting and believing in me no matter the endeavor.

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CHAPTER I

INTRODUCTION

The use of DNA in forensics has become widely accepted since its introduction into the field in 1985 with Restriction Fragment Length Polymorphisms (RFLP) by Alec Jeffreys (1). RFLP techniques were utilized in the forensic DNA community until the mid 1990s when less labor-intensive PCR-STR techniques became available. During the transition from RFLP technology to PCR based STR technology a method for comparing RFLP profiles to that of STR profiles was not developed. Currently there have been no published studies where STR profiles have been obtained from membrane bound, restriction cut human DNA. The only way to compare RFLP profiles to STR profiles would be to obtain STR profiles from the bound restriction cut DNA left on the nylon membranes.

Since the shift in technology from RFLP to PCR-STR most labs, including the FBI, have stopped RFLP analysis as of the year 2000 (4). Today many unsolved cases exist that utilized RFLP technology. Due to the nature of RFLP analysis many times all of the biological sample must be consumed in order to obtain an RFLP profile. When this occurs, there is no longer biological sample left for future testing. In these instances the only DNA left from the case is restriction cut and bound to nylon RFLP membranes. The only chance of determining the STR profile of the source of the biological sample found at the crime scene would be to remove the membrane bound DNA and obtain an

STR profile. The experimental hypothesis of this study is that DNA can be recovered from nylon membranes and interpretable STR results can be obtained.

The use of multiple STRs are highly discriminatory being able to generate rare DNA profiles possessing a discriminatory power of 1 in many times that of the earth's population. Due to this discrimination power, profiles are able to individualize the source of a biological sample and aid in criminal investigations. If STR profiles could be obtained from old RFLP membranes numerous cold cases could be reopened and reinvestigated. The STR profiles obtained from the RFLP membranes could be placed into the Combined DNA Indexing System (CODIS). CODIS blends forensic science and computing software into a tool for solving violent crimes. Through CODIS, STR profiles can be entered into the database and searched against possible suspects at the local, state, and national level. Obtaining STR profiles from RFLP membranes would allow for the comparison of these profiles to those found in CODIS for a possible suspect.

This project will employ methods to try and obtain an STR profile from HaeIII restriction cut DNA bound to Magna Graph membranes. Attempts will be made to obtain STR profiles through direct amplification off of the membrane with PowerPlex®16 and separation on the Avant 3100 equipped with GeneMapper® ID. Methods will also be utilized to remove the bound DNA from the membrane prior to amplification and separation. Removal of the bound DNA from the membrane will involve physical means, as well as, the use of various extraction chemicals. If a technique is found successful at removing DNA from Magna Graph membranes, then the technique will be applied to true RFLP membranes donated by the UNTHSC.

CHAPTER II

BACKGROUND

2.1 History of RFLP and STR technology

In the late 1970s it was known that restriction enzymes were capable of recognizing specific DNA sequences in the human genome and cutting the DNA at these sites. The fragments produced by these restriction enzymes may vary between individuals due to differences in the DNA sequences at the restriction recognition site (2). The use of tandem repeat sequences in the human genome to determine inheritable differences arose in a separate study conducted by Alec Jeffreys that involved the characterization of the seal myoglobin gene (2). Jeffreys found a repetitive sequence within an intron of this gene, a minisatellite, that shared a core sequence of other minisatellites that had been previously described (2). A probe was developed that targeted the core sequences and would bind human DNA that had been digested and immobilized on a membrane, allowing Jeffreys to determine that the number of repeat segments varied from person to person (2,3). In 1984, Alec Jeffreys developed the first series of DNA profiles and coined the term "DNA fingerprint" (1). These repeated sequences were termed variable number of repeats (VNTRs), and the method employed for detecting them became known as restriction fragment length polymorphism (RFLP) analysis (4). RFLP analysis was first utilized to resolve immigration disputes (4) and, to assist in criminal investigations (2). Several different RFLP procedures were developed

but all had these basic analytical steps: DNA extraction, endonuclease digestion, electrophoresis, blotting, hybridization, autoradiography, imaging, and interpretation (7).

The use of multilocus probes was highly discriminatory, but could not be utilized efficiently when interpreting mixtures (3). As time progressed laboratories were able to standardize the use of single-locus probes in succession that proved to be highly discriminatory (5). Although nylon membranes allowed for multiple hybridizations and chemiluminescent detection reduced the time required to detect probe signal (6), the RFLP process was labor-intensive and not conducive for automation (4). Therefore, the search began to find a technique that was more sensitive and that could speed up the typing process. PCR soon became discovered and was utilized in the forensic community to determine polymorphisms within the human genome.

The polymerase chain reaction (PCR) is a process developed by K. Mullis that amplifies specific DNA sequences exponentially through the use of primers, polymerase, and nucletotides (4). The technology can be used on a very small quantity of DNA because the process utilizes probes directed to areas flanking stretches of DNA where the repeats occur and amplifies, or makes copies of, these regions (9 and 5). The process was described by K. Mullis (19) and relies upon cycling of the reaction temperature to achieve amplification and the cycle repetition has three basic steps: denaturation at approximately 94°C, annealing at approximately 54°C, and extension at approximately 72°C (20). Following the denaturation of double stranded template, the annealing step allows the 18-22mer primers to bind highly conserved regions of DNA flanking the STR stretches at each locus (9). The extension or elongation step is carried out at the optimal

temperature for activity of the polymerase, usually *Taq* polymerase, which assembles free deoxynucleotide triphospates present in the reaction mixture into DNA polymers complimentary to the target sequence (21). *Taq* polymerase is a thermostable enzyme, a modified form of that isolated from *Thermus aquaticus* (19).

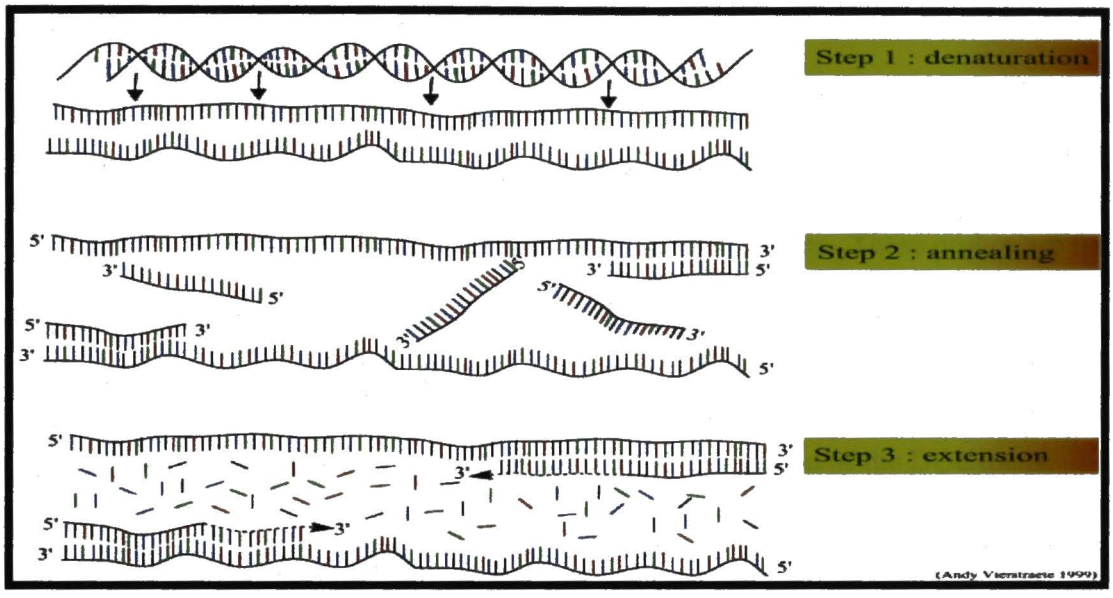

Figure 1: Theory of PCR. PCR is carried out by changing reaction temperature to achieve denaturation of template, annealing of primers, and extension of newly synthesized DNA. This figure illustrates these steps, with colored lines to indicate deoxynucleotide triphosphates (dNTPs) that are assembled to form base pairs of complimentary strands. Adapted from 21.

Because the newly synthesized DNA fragments can serve as template in subsequent PCR cycles, repetition of the cycling process results in exponential increase in the number of fragments of targeted DNA sequence (21). The resulting PCR products can then be separated based on size and fragment lengths. The differences in fragment lengths reflect the length polymorphisms caused by variation in the number of repeats present at a given locus (9).

The polymerase chain reaction was first utilized to amplify a one of the major histocompatibility complex genes, DQα. DQα belongs to the class II antigen genes and is polymorphic, exhibiting 6 common alleles. It exhibits four nominal alleles, Alleles 1, 2, 3, and 4, and the 1 allele subtypes 1.1, 1.2, and 1.3 (23). Thus, 21 genotypes can be detected in the population. The variations detected in these alleles can be found in a 242 base pair region (239 base pairs for Alleles 2 and 4) in the second exon of the DQα gene (23). Primers that flank this region are used in amplification along with allele specific oligonucleotide (ASO) probes that detect each of the DQα alleles. These probes are fixed to nylon membranes known as typing strips, and DQα alleles are identified through hybridization with the amplified DQα DNA. Due to the limited discriminatory power of the DQα locus, short tandem repeats soon replaced amplification of the DQα locus.

Thomas Caskey of Baylor College of Medicine first suggested that short tandem repeats be used in forensics in 1991(3). STRs, are essentially a smaller version of VNTRs described by Jeffreys. While VNTRs range in length from nine to forty bases, STRs utilized in forensic identity testing are usually four or five bases in length, and can be found in the non-coding regions of the gene (8). STRs are identified through the use of the Polymerase Chain Reaction (PCR). Fragments generated from PCR can range from approximately 100 – 500 base pairs; this is much smaller than fragments detected using RFLP analysis (9). Due to the sensitivity of STR typing and the short PCR products, STR PCR technology is suited for forensic applications since crime scene exhibits are often exposed to environmental insult resulting in degraded samples or samples of extremely low quantity (5). Also, since STR PCR products are discrete lengths, the typing process

lends itself well to computer-assisted interpretation because allelic size standards can be constructed for use in determining precise allele calls (9). However, one of the drawbacks of STR technology is the fact that STRs are less polymorphic than VNTRs. Many more STR loci are needed for analyzing in order to obtain the same statistical significance of a few VNTRs. Approximately 10-12 STR loci are needed to yield statistically similar profiles as 4-5 VNTRs (22). Due to the clear advantages over RFLP, STR PCR techniques were rapidly validated and adopted by the forensic DNA community.

2.2 Nylon membranes and binding of DNA

When RFLP techniques were still utilized in the forensic community, nylon membranes were used to bind restriction cut DNA for probing and washing. Binding restriction cut DNA to nylon membranes provided a permanent attachment of the DNA that allowed for repeated washings and probing without substantial loss in probe signal. Few studies have been conducted to assess the ability of removing bound restriction cut DNA from nylon membranes for subsequent STR amplification. Part of the problem for DNA removal from these membranes is the inherent nature of the nylon.

There are many different types of nylon membranes containing different chemical surfaces and adsorption properties (10). Each has different chemical groups on its pore surfaces with corresponding differences in surface charge and chemical behavior (10). Nylon membranes can be positively charged, negatively charged, or uncharged. The type of chemical group and amount of the group present on the membrane determines the charge on the membranes. Most membranes can have their charge attributed to amino and carboxyl groups.

Pall nylon membranes are formed around a nonwoven polyester fiber matrix which exhibits high tensile strength, toughness, flexibility, and resistance to tearing, cracking, and puncture (10). Nylon membranes exhibit little shrink/swell properties with Pall membranes expanding and contracting less than 0.3% when exposed to water and dried (10). Most membranes are extremely hydrophilic and possess superior solvent resistance. Pall nylon membranes are not affected by acetone, alcohols, chlorinated aliphatic hydrocarbons, 100% formamide, 2M NaOH, DMSO, DMF, and most other solvents found in biotechnology labs. The principle exceptions are concentrated formic acid (>50%), HCl (>4 meters), oxidizing acids, and prolonged (days to weeks) exposure to pH <2 (10).

Nucleic acids and proteins bind to microporous membranes primarily through hydrophobic interactions. These hydrophobic attachments of proteins to solid surfaces is well documented (12,13,14,15). Water wettable nylon membranes contain strong hydrophobic components creating microenvironments that encourage association with hydrophobic areas of biomolecules (11). Hydrophobic interactions can be altered depending on the membrane surface chemistry. The presence of hydroxyl groups increases the hydrophilicity of the membrane, stabilizes the layer of hydration around the membrane polymer, and thus significantly decreases the amount of binding (11). The binding of a biomolecule is measured in affinity and bond strength (avidity). Each membrane type possesses a characteristic affinity for biomolecules, so adsorption is then dependent on the affinity and the input biomolecule concentration (16). Avidity is also influenced by the hydrophobicity of the membrane, and to a lesser extent the surface

chemistry (11). Since a complex biomolecule has a large number of potential sites for hydrophobic interactions very high bond strength can be achieved even though individual forces are weak (11).

In hydrophic binding of a biomolecule to a membrane the layers of hydration surrounding the secondary structures of proteins or nucleic acids and membranes are squeezed out when they come into close contact. The biomolecules will flatten out and thus increase the entropy of the system (16), driving the reaction. Desorption can occur when water re-enters the space between the molecule and the membrane (11). The bond strength can be increased by desiccating the membrane-biomolecule complex. This technique is used frequently as a fixation method for DNA detection (11).

Due to the nature of the interaction of the nylon membranes with DNA, the removal of DNA from the membrane can be quite problematic. To remove the restricted DNA the hydrophobic bonds must be broken between the membrane and the DNA. One way to do this would be to re-introduce water between the membrane and DNA. This would be extremely hard due to the baking process (80°C @ 30min) of RFLP membranes to thoroughly dry the membrane and thus strengthen the bonds. DNA can be removed from the membranes by attacking the susceptibility of the nylon membrane, but the membranes are resistant to most solvents. The solvents that would work on the membrane would probably degrade the already restricted DNA to a state completely non-conducive to downstream amplification.

The ability to amplify STR loci directly off of RFLP membranes would alleviate the problem of trying to remove the DNA from the membrane prior to amplification. However the literature in this area is poorly documented and studied. One study was successful at amplifying as little as 5ng of membrane bound DNA off of UV crosslinked Duralon-UV nylon membranes (17). In this study the DNA was not restriction digested as in RFLP analysis. Also, the membrane was not dried through baking, but was instead stored at 4°C in water. The fact that the DNA was not restriction digested prior to binding and that the membrane was not dried through baking could allow for easier amplification of membrane bound DNA. Due to the previously mentioned differences this study cannot be applied directly to RFLP studies.

Storage conditions of RFLP membranes could also affect the ability of DNA to be amplified directly off of a nylon membrane. A study conducted by Giusti and Budowle showed that the storage environment of RFLP membranes affected hybridization and rehybridization of probes (18). They found that membranes could be successfully hybridized and rehybridized if they were stored at –20°C or 4°C, but storage under ambient conditions reduced or eliminated the chance of hybridization (18). It is thought that membranes stored at ambient temperatures for an extended period of time reduces or eliminates a certain degree of hydration from the membrane that is not lost during heat baking. This loss of hydration is thought to be the reason the membranes cannot be hybridized, a term called "hybridization inactivation" (18). This study has direct implications towards the amplification of restriction cut DNA off of a bound RFLP membrane. If ambient stored membranes cannot be hybridized, the chance of amplifying

STR loci off of ambient stored membranes will be unlikely. Thus the storage condition of RFLP membranes for successful STR amplification is crucial.

2.3 Previous studies conducted at the SCRFSC

Prior to the current study, previous attempts were made by Shelly Steadman at SCRFSC to generate STR profiles from restriction cut, membrane bound human DNA. In the studies, HaeIII restriction cut human DNA was bound to MagnaGraph nylon membranes in the same manner as the current project; technique described later.

A study was conducted by S. Steadman to determine if an STR profile could be obtained through direct amplification off of the membrane via a PowerPlex®16 kit. High molecular weight (HMW) S. Steadman DNA was bound to a MagnaGraph membrane in the following concentrations: 10ng, 5ng, 2.5ng, 1.25ng, 0.625ng, 0.3125ng, and 0.15625ng. Each well containing the DNA was excised and placed in separate tubes subsequent to PCR components being added directly to the tubes. Following amplification, the samples were run on a 3% agarose gel and compared to the positive amplification control. No product was observed in any of the samples. The 10ng, 5ng, and positive amplification control were run on an analytical gel to determine if any alleles were present. Several alleles were noted in the 10ng slot sample. These alleles (20,24 at FGA; 17 at vWA, 12,13 at D8S1179; 18 at D3S1358) were consisted with S. Steadman's profile. The 5ng sample yielded a 17 at vWA. The loci that exhibited alleles have proven to be very robust in low copy number situations and are generally the most likely

to yield a profile. To determine if the nylon membrane itself was acting as an inhibitor of PCR (polymerase/primer interaction with the nylon or other non-specific interactions), a positive amplification control was prepared alongside a positive amplification control with nylon membrane added to the reaction. A 3% agarose product gel was run to determine the success of the amplification. From the product gel it was determined that the nylon membrane does cause a decrease in the amount of product present following PCR, indicating possible inhibition of PCR by membrane interaction with PCR components. From this study it was determined that the direct amplification of high molecular weight DNA bound to nylon membranes is not efficient due to the possible interaction of PCR components with the chemical charges on the membranes.

Another study was conducted to determine if HMW, membrane bound DNA could be recovered through the use of a stripping solution or TE. The stripping solution was a solution consisting of .5M NaOH and 1% SDS. Two membrane cuttings of HMW 10ng slots were placed in either 200ul of the stripping solution or TE and heated to boiling for 30 minutes. The solutions were cooled, centrifuged briefly, and loaded onto microcon concentrators. The concentrators were then washed with TE prior to elution. Once eluted, the samples were quantified using Quantiblot®. Neither the stripping solution nor TE yielded any quantifiable DNA. Furthermore, the SDS was not efficiently removed by the microcron clean-up in the sample containing the stripping solution. The washes would not pass completely through the microcon in the sample containing the stripping solution, even under excessive centrifugal conditions. Eluates also caused a color change to the blot loading solution which is an indication that the dye was no longer

at the appropriate pH. It was determined that residual NaOH was likely to be present. If the solution loaded into the blotting well was at an inappropriate pH or contained large quantities of residual SDS, binding would not be optimized. Therefore the absence of signal could have resulted because no DNA was removed from the membrane, or because the samples were inefficiently cleaned/concentrated following DNA removal. Due to the indication that the strip-treated reaction was not efficiently purified by the microcon concentration, it was not seen fit to be amplified via PCR. However, 15ul of the TE treated membrane eluate was amplified via PowerPlex® 16 BIO and yielded two alleles, both of which are consistent with the profile of S. Steadman. (13 at D8S1179 and 17 at vWA). From the results, it appears that heat alone, in the presence of TE buffer served to remove some DNA from the membrane, however the recovery was not substantial. The stripping solution was also determined to be inefficient at removing membrane bound restriction cut DNA.

An alkaline stripping solution was also utilized on a HMW 50ng DNA slot cutting. The solution consisted of two washes. The first wash consisted of 0.4 M NaOH, and the second wash consisted of 1XSSC (0.1% SDS). To the 50ng DNA slot cutting 100ul of step 1 wash was added and then the sample was placed in a boiling water bath for 20 minutes. The wash was removed with pipette and stored. The sample was then treated with 100ul of the step two wash, soaked for 10 minutes at room temperature, and heated in a boiling water bath for 5 minutes. The wash was then removed and combined with the stored step 1 wash. The combined washes from each sample were brought to a volume of 400 ul with TE and extracted with standard ethanol extraction procedures.

The sample pellet was resuspended in 10ul TE, heated at 56°C for 30 minutes, and then 4ul of the sample was quantified on a 2% agarose gel. The sample exhibited no quantifiable DNA indicating the alkaline stripping solution was ineffective at removing membrane bound DNA.

Another study conducted by S. Steadman at the SCRFSC was to test the effect of acid on recovering membrane bound DNA. The following dilutions of acid were utilized in this study: 6M, 3M, 1.5M, 0.75M, 0.375M, 0.1875M, and 0.09375M. Cuttings of seven HMW 50ng slots were taken and one slot was placed in 100ul of each of the previously mentioned acid dilutions. After 15 minutes, the acids were drawn off and placed into tubes containing 300ul TE. To this 500ul of PCIA was added, Samples were vortexed briefly and spun at 21K for 2 minutes. The aqueous phase of each of the samples were then placed on microcons and spun for 20 minutes at 2300 RCF. Samples were then washed with 150ul of NFW followed by a wash with 150ul of TE. The samples were then eluted with 5 ul TE and brought to a final volume of 10ul. 4 ul of each of the samples were then placed on a 2% agarose gel to visualize sample recovery products. All samples exhibited no quantifiable DNA indicating no membrane bound DNA recovery or poor DNA concentration of the micron due to the disruption of the membrane by the acid. Samples were also quantified via Quantiblot® and exhibited no quantifiable DNA. Due to the fact that DNA could be present in the samples below the threshold of quantitation, the samples were amplified via PowerPlex® 2.1 and visualized on a 6% polyacrylamide gel. The acid treated nylon-bound DNA samples yielded no

profiles. This indicates that acid removal of DNA from membranes for STR typing is not an efficient process.

Another test was conducted to determine if DNA could be recovered from a nylon membrane using the SCRFSC's stain extraction buffer (SEB). The stain extraction buffer stock solution (SEBSS) was made by dissolving 1.21 g $C_4H_{11}NO_3$ (Tris base) and 5.84 g NaCl in a total volume of 500 ml of water. The pH was adjusted to a pH of 8.0 with HCl. Then 100 ml 20% SDS and 20 ml 0.5M EDTA pH 8.0 were added and the solution was brought to a final volume of 1.0 L with water. Stain extraction buffer working solution (SEBWS) for this experiment was prepared by dissolving 0.3g DTT in 50ml of stain extraction buffer stock solution. A HMW 50ng slot of K562 DNA was excised from the membrane used in the previous studies and incubated in 400ul of SEBWS overnight. The sample was ethanol precipitated, reconstituted in 10ul of TE, and 1ul of the sample was used for quantitation in both a 1% agarose gel and Quantiblot®. Through visualization of the 1% agarose gel, it was determined that low molecular weight DNA was present in the sample extracted with SEBWS. Through Quantiblot® it was determined that the sample contained 0.3ng/ul of DNA. The extract was amplified using PowerPlex® Bio and 0.5ng of target DNA as a template. Most of the known alleles from the membrane bound DNA were detected indicating successful extraction and amplification of membrane bound DNA. It was therefore determined that the SEBWS extraction would be repeated using various quantities of membrane-bound DNA and microcon concentration (as opposed to ethanol precipitation). Slots containing 50 ng, 30 ng, and 10ng of membrane-bound K562 DNA were each placed in microcentrifuge tubes with

400ul of SEBWS and extracted overnight (18 hours), followed by PCI/microcon purification and concentration. Samples were brought to a final volume of 16 µl in NFW and 1 µl of each was used for Quantiblot® quantification. Through Quantiblo®t, the 50ng, 30ng, and 10ng samples were quantified as 0.3ng/ul, 0.15ng/ul, and 0.10ng/ul respectively. These extracts were amplified, using 0.5ng of target template of each using the PowerPlex® BIO typing system. Typing results were consistent with the known profile for K562, however, signal at TPOX for the 10ng extract was not fully detectable. Furthermore, a substantial amount of imbalance was noted in all samples, and the imbalance worsened as the amount of membrane-bound DNA was decreased. This study showed that amplifiable DNA can be extracted from a nylon membrane once UV crosslinked, and that this extraction method may be a good candidate to be tried on true RFLP membranes.

To determine if HaeIII restriction of DNA had any effect on its amplification a study was conducted to amplify HaeIII restriction digested DNA. Whole blood spotted on a cotton cloth was extracted according to SCRFSC protocol and was determined to be at a quantity of 125ng/ul. To separate tubes, 100ng and 50ng of this extract were added. A Restriction digest was prepared using HaeIII enzyme and digestion was carried out at 37°C for 16 hours. Following digestion, 1ul of the 100ng and 50ng sample were quantified via Quantiblot® and found to be at concentrations of 1.5 and 1.25 ng/ul, respectively. The samples were then amplified via PowerPlex®16 BIO and visualized on a 6% polyacrylamide analytical gel. The profile generated was fully consistent with that of the known individual and data was not available at PentaE. Signal at the TPOX and

TH01 loci were extremely weak, indicating that these loci may have a HaeIII recognition sites within the amp fragment sequence. Partial digestion is suspected in all restriction reactions, and a small amount of undigested sample may have served as template for these loci on a reduced level. Regardless, loss of 3 loci over 16 is considered negligible loss from the restriction process. This study indicates that the restriction process itself will not inhibit the ability to generate a quality STR profile, and that the inability to amplify nylon-bound restricted sample previously was likely related to membrane-binding or other aspects of the RFLP process subsequent to the restriction digestion.

To try and reproduce a previous study were SEBWS was successful at extracting membrane bound DNA for amplification, aliquots containing100ng of DNA extract were blotted to a MagnaGraph Nylon membrane as described in the Materials and Methods Chapter. The 100ng membrane slot was then excised and placed in SEBWS and extraction was carried out overnight at 56°C. Purification and concentration of the sample with PCI was carried out as in the previous study. Sample was eluted to 11ul with NFW. Quantification of the sample was carried out via Quantiblo®t however no band was detected. Sample was amplified in full (10ul) via PowerPlex® 16 BIO typing system and visualized on a 6% polyacrylamide analytical gel. Alleles were detected at 12 of the 16 loci and were consistent with those of the known individual. Alleles were not detected at the following loci: FGA, TPOX, Penta E, or TH01. These loci were not detected due to the restriction of these sites by the HaeIII digestion enzyme. This reproducibility study was successful at demonstrating that membrane bound, restriction cut DNA can be successfully extracted and amplified for STR typing. While 50-500ng of

DNA was generally restricted, electrophoresed, and bound in an RFLP sample lane, amplified product across 12 loci from 100ng of restricted DNA bound in a localized manner is promising for application to RFLP membranes.

To determine if extraction of membrane bound DNA could be applied to a forensic situation, a study was carried out using an RFLP membrane. From an RFLP membrane a lane was excised, diced, and extracted in SEBWS overnight at 56°C. The sample was then purified and concentrated through a microcon 100 device. The sample was then amplified via PowerPlex® 16 BIO and visualized on a 6% polyacrylamide analytical gel. This process did not yield an extensive amount of profile information. One single band was detected at the vWA locus. K562 is homozygous 16 at this locus, so there is reason to believe this is a true result. The primary analyst performing the test did not express this allele. Nevertheless, the amount of information obtained is of no comparative value. Therefore, this initial attempt seemed to indicate that the application of extracting localized membrane bound DNA required further refinement for the application to a forensic situation.

CHAPTER III

MATERIALS AND METHODS

3.1 DNA extraction

Four dried blood stains of approximately 50ul in volume (drawn and prepared on 11/13/03 and 11/14/03, respectively) were extracted via an organic extraction according to Sedgwick County Regional Forensic Science Center (SCRFSC) protocol (Appendix A). After extraction, samples were concentrated on a Microcon100 according to SCRFSC protocol (Appendix B). All four samples were eluted with 7ul of TE and the eluates were combined to one sterile 1.5ml microcentrifuge tube. Tube was labeled "SSDNA1" and was the stock DNA for blotting to nylon membranes. SSDNA1 was quantified via a 1% agarose gel according to SCRFSC (Appendix C) and was found to be approximately 70ng/ul. A 1:49 dilution was prepared and the sample was quantified via Quantiblo®t according to SCRFSC protocol (Appendix D). 1.5ng of DNA was observed in the well indicating the original stock SSDNA1 was 75ng/ul.

3.2 Restriction digestion

SSDNA1 stock was then utilized in a restriction digest to be blotted and bound to a nylon membrane. The restriction digest was prepared as follows, with the intent of preparing 12 blotted wells:

Restriction Digest Component	ul per well	# wells	ul total
NFW	16.25	13	211.25
RE 10X Buffer	2	13	26
Acetylated BSA (10ug/ul)	0.2	13	2.6
DNA (75ng/ul)	1.25	13	16.25
RE (10u/ul)	0.05	13	6.5
TOTAL	19.75	13	262.6

Table 1: Illustrates the various components utilized in the restriction digestion

The digestion was carried out for 5.5 hours, after which 2ul was observed on a 1% agarose gel; it was determined that restriction cutting was efficient. The reaction was then placed at 65°C for 10 minutes prior to blotting. Casework digests did not generally include BSA, but it was added here to ensure restriction efficiency.

3.3 Blotting of DNA to nylon membrane

Restriction cut SSDNA1 was blotted on separate MagnaGraph nylon membranes according to the following protocol, previously utilized by SCRFSC. A well plate was used for sample preparation, along with a slot blot set-up form to designate sample preparation. A quantity of 250 ul 0.5M NaCL/ 0.5M NaOH solution was placed into each sample tube or well. Next, 20ul of HaeIII restricted SSDNA1 was added to each of the sample wells. A nylon membrane was labeled with the appropriate identifier and date and placed in a box containing 2X SSC. The membrane was then soaked for approximately 5 minutes in 2XSSC solution, and placed on a slot blot apparatus. Samples were then added to the membrane according to the slot blot set-up form previously described. Vacuum pressure was applied to the samples to draw them into the membrane. A solution of 400 ul 0.5M NaCl/ 0.5M NaOH was added to each sample for

washing. When all washes were drawn through the membrane, the membrane was removed from the slot blot apparatus and well designations were assigned. Sample wells were marked with an SSC-proof pen by marking dots on both sides of the wells. The membrane was then placed in a box containing enough 2X SSC – Tris HCL, pH 8.0 to cover the membrane and then soaked at room temperature for five minutes. Following the soak the membrane was drained and placed between filter paper and baked at 80°C for 30 minutes. Subsequent to baking the membrane was placed on a piece of filter paper, DNA side up, and UV cross linked (120,000 uJ/cm2). It was then inverted and cross linked with the DNA side down. Following cross linking the membrane was packaged for frozen storage at -20°C. The membrane was blotted according to the following slot-blot chart:

	1	2	3	4	5	6
A	HaeIII-SSDNA1 (93.75ng)	blank	HaeIII-SSDNA1 (93.75ng)	blank	blank	blank
B	HaeIII-SSDNA1 (93.75ng)	blank	HaeIII-SSDNA1 (93.75ng)	blank	blank	blank
C	HaeIII-SSDNA1 (93.75ng)	blank	HaeIII-SSDNA1 (93.75ng)	blank	blank	blank
D	HaeIII-SSDNA1 (93.75ng)	blank	HaeIII-SSDNA1 (93.75ng)	blank	blank	blank
E	HaeIII-SSDNA1 (93.75ng)	blank	HaeIII-SSDNA1 (93.75ng)	blank	blank	blank
F	HaeIII-SSDNA1 (93.75ng)	blank	HaeIII-SSDNA1 (93.75ng)	blank	blank	blank
G	blank	blank	blank	blank	blank	blank
H	blank	blank	blank	blank	blank	blank

Table 2: Slot-blot chart illustrating the orientation of membrane bound HaeIII digested DNA.

3.4 SEBWS with and without PCIA method for nylon bound DNA removal and amplification

Two wells from restriction cut membrane bound SSDNA1 (BSA) were removed with a sterilized razor and placed in separate sterile 1.5ml microcentrifuge tubes. These tubes were labeled SSDNA-PCIA and SSDNA-noPCIA. Both tubes were extracted in SEBWS overnight according to SCRFSC protocol. The SSDNA-PCIA membrane was removed prior to the addition of PCIA. The tube labeled SSDNA-noPCIA did not undergo PCIA addition. Instead it was loaded directly onto a microcon by pipetting the fluid away from the membrane fragment. Excessive additional spin time, approximately 50 minutes, was utilized to draw the sample and wash through the microcon membrane on the SSDNA-noPCIA sample. The SSDNA-PCIA sample was also concentrated on a microcon after PCIA addition. The samples were brought to a final volume of 16ul in nuclease free water (NFW), and quantified via Quantiblo®t according to SCRFSC protocol and amplified via PowerPlex® 16 according to SCRFSC protocol (Appendix E). Samples were then separated on an Avant 3100 and analyzed with GeneMapper™ ID to determine success of procedure.

3.5 SEBWS extraction of membrane with BSA and without BSA in digestion

One well was excised from the membrane containing DNA with BSA in the restriction digest (050527sa), and one well was excised from the membrane with DNA lacking BSA in the restriction digest (050607ss). Both samples were extracted in 300ul of SEBWS overnight on a 56°C heatblock. They were then extracted via organic extraction and concentrated on a microcon according to SCRFSC protocol and amplified

via PowerPlex®16. The 050527sa sample was compromised during the amplification step, so the SSDNA-PCIA sample from the previous study was utilized in its place. After amplification the samples were analyzed on an Avant 3100 via GeneMapper™ ID to determine the effects of BSA in the restriction digest, and if previous work by S. Steadman could be reproduced.

3.6 Electrophoretic removal of membrane bound DNA

One well from previously blotted SSDNA1 was removed and utilized in an experiment to determine if bound DNA can be electrophoresed off of a nylon membrane. The excised well containing HaeIII cut SSDNA was placed in a 2% agarose yield gel with modified combs. Wells 6 through 8 were taped together in order to make a well large enough to hold the excised slot. Then, 3 ul of loading dye was placed in well 1 of the gel to monitor electrophoretic movement. Electric current was then applied to the gel for 15 minutes at 200V. The gel was then stained in ethidium bromide for 15 minutes and visualized on an FMBIO to determine the success of the procedure. The gel was re-electrophoresed at 300V for an additional 10 minutes and visualized on an FMBIO to determine the success of the procedure.

3.7 Preparation and blotting of membrane without BSA in the Digestion

Another membrane was prepared and blotted in the same manner as the previous membrane except acetylated BSA was not used in the digestion process of SSDNA. The reason for this is due to the fact that traditional RFLP digestions did not have acetylated

BSA as a component. The membrane will be blotted according to the following slot blot chart:

	1	2	3	4	5	6
A	blank	HaeIII-SSDNA1 (93.75ng)	HaeIII-SSDNA1 (93.75ng)	blank	blank	blank
B	blank	HaeIII-SSDNA1 (93.75ng)	HaeIII-SSDNA1 (93.75ng)	blank	blank	blank
C	blank	HaeIII-SSDNA1 (93.75ng)	HaeIII-SSDNA1 (93.75ng)	blank	blank	blank
D	blank	HaeIII-SSDNA1 (93.75ng)	HaeIII-SSDNA1 (93.75ng)	blank	blank	blank
E	blank	HaeIII-SSDNA1 (93.75ng)	HaeIII-SSDNA1 (93.75ng)	blank	blank	blank
F	blank	HaeIII-SSDNA1 (93.75ng)	HaeIII-SSDNA1 (93.75ng)	blank	blank	blank
G	blank	blank	blank	blank	blank	blank
H	blank	blank	blank	blank	blank	blank

Table 3: Slot-blot chart illustrating the orientation of HaeIII digested (no BSA), membrane bound DNA

3.8 Extraction using various extraction buffers

Three wells were cut from the blotted membrane that did not have BSA in the digestion. These wells were placed in separate sterile 1.5ml microcentrifuge tubes. Each tube was extracted with a different extraction buffer. To one tube 300ul of Promega digestion buffer, 20 ul of Pro K (20mg/ml), and 80 ul of DTT (60.12mg/ml) were added. To another tube 300ul of SEBWS, 20 ul of Pro K (20mg/ml), and 80 ul of DTT (60.12mg/ml) were added. This tube is considered the "spiked" SEBWS sample since both the ProK and DTT concentration have been increased. To the last tube 400ul of sperm lysis buffer was added (150ul TNE, 50ul sarkosyl (200mg/ml), 40ul DTT (60.12 mg/ml), 150 ul nuclease free water, and 10ul Pro K (20mg/ml). Each tube was then placed on a 56°C heat block overnight and then extracted via organic extraction according to SCRFSC protocol. Prior to organic extraction the samples were sonicated

for approximately one minute. Samples were then quantified via Quantiblot®, and then amplified with PowerPlex®16 followed by separation on an Avant 3100 according to SCRFSC protocol (Appendex E). Samples were analyzed with GeneMapper™ ID to determine success of extraction procedures.

3.9 Scraping of membrane cutting prior to extraction

One other well was cut from the membrane without BSA in the digestion and scraped with a sterile razorblade into a sterile weigh boat. Scrapings were collected via a sterile swab moistened with 50ul of NFW. Swab was then cut and placed into a sterile 1.5ml microcentrifuge tube along with the left over razor scraped membrane well. To the tube 400ul of SEBWS (with 10ul ProK) was added and allowed to incubate overnight on a 56°C heat block and then extracted via and organic extraction according to SCRFSC protocol. Prior to organic extraction the sample was sonicated for one minute. Sample was then quantified via Quantiblot®, according to SCRFSC protocol. The sample was amplified with PowerPlex®16 followed by separation on an Avant 3100 according to SCRFSC protocol. Sample was analyzed with GeneMapper™ ID to determine success of scraping the membrane prior to the extraction procedure.

3.10 Direct amplification from the membrane

To test if restriction cut and bound SSDNA1 can be directly amplified from the membrane, one well was cut from the membrane that contains BSA as one of the restriction components and will be placed in a sterile amplification tube. Prior to the

placement of the membrane into the tube, the membrane was cut into small pieces. To this membrane 20ul of Gold Star 10X buffer was added in an attempt to block the membrane prior to amplification. The tube was then placed on an orbital shaker overnight. Subsequent to overnight soaking, the membrane was washed 3 times with 100ul NFW to remove any residual Gold Star Buffer. The membrane slot was then amplified according to SCRFSC's standard protocol (32 cycles). After the 32 cycles, a 5ul aliquot of supernatant was removed for STR typing via PowerPlex® 16. The membrane and remaining supernatant were then amplified for an additional 8 cycles, making the total cycle number 40. The amplified products where then STR typed in the same manner as the standard 32 cycle amplification.

3.11 Tape lift removal of membrane bound DNA

To one membrane well, clear packing tape was utilized to try and remove membrane bound DNA for extraction. Packing tape was manually pressed on the membrane slot and removed. This was carried out with the same piece of tape numerous times. Upon completion the tape was folded onto itself, cut into small pieces and placed in a 1.5ml microcentrifuge tube. To the tube 300ul of Promega's digestion buffer, 20 ul of Pro K (20mg/ml), and 80 ul of DTT (60.12mg/ml) were added. Sample was allowed to incubate overnight at 56°C. Tape fragments were then placed in a spin basket and spun to remove any liquid off of the tape. Supernatant was then extracted via organic extraction and amplified with PowerPlex® 16 according to SCRFSC protocol. Sample was then separated on an Avant 3100 and analyzed through GeneMapper™ ID.

3.12 Application to forensic sample (RFLP membrane 38719)

A study was conducted to determine if a previous successful technique could be employed on true RFLP membranes from UNTHSC. The most successful technique from a previous study was found to be Promega's Digestion buffer with added Pro K and DTT (1ug/ul ProK, and 12ug/ul DTT). One of the RFLP membranes labeled 38719 was utilized in this study. The exact locations of the lanes on this membrane were not known due to the absence of the autorad. In order to determine a rough location of the lanes, a template was made from a copy of an autorad sent from UNTHSC. This template consisted of wells from an autorad that were highly visible due to the staining process. Through the use of the template, lanes 2 and 3 were cut from the membrane with a sterile razor and placed into separate tubes. Lane 2 from the membrane was noted as a mother, while lane 3 was noted as a child. To each one of the microcentrifuge tubes 300ul of Promega's digestion buffer, 20ul Pro K, and 80ul DTT were added. One of the tubes was labeled "with sonication" and the other was labeled "without sonication". Although one tube was labeled "without sonication," it will be sonicated for 1 minute. Both tubes were placed on a 56°C heat block for overnight incubation. Prior to overnight incubation, the tube labeled "with sonication" was sonicated for 30 minutes. After overnight incubation the tube labeled "without sonication" was sonicated for 1 minute prior to extraction. Both tubes were then extracted via organic extraction and concentrated according to SCRFSC protocol. Samples were then amplified via PowerPlex® 16 according to SCRFSC. Subsequent to this, the samples were separated on an Avant 3100 and analyzed with GeneMapper™ ID according to SCRFSC protocol.

3.13 Application to forensic sample (RFLP membrane 38745)

For reproducibility purposes another UNTHSC RFLP membrane was utilized and extracted in the same manner as the previous RFLP membrane. From this membrane lane 4 (mother) was excised with a sterile razor and placed into a sterile 1.5ml microcentrifuge tube. To this tube 480ul of Promega's digestion buffer, 32ul of Pro K, and 98ul DTT were added. More extraction components were added due to the large amount of membrane pieces in the sample. However, components were added in the same proportion so that the concentration of reagents would be the same as in the previous study. The tube was then placed on a 56°C heat block for overnight incubation. After incubation the tube was sonicated for one minute and extracted via organic extraction and concentrated according to SCRFSC protocol. Sample was then amplified via PowerPlex® 16 according to SCRFSC. Subsequent to this, the sample was separated on an Avant 3100 and analyzed with GeneMapper™ ID according to SCRFSC protocol.

CHAPTER IV

RESULTS

4.1 SEBWS with and without PCIA method for nylon bound DNA removal and amplification

Both samples, SSDNA-PCIA and SSDNA-no PCIA exhibited no quantifiable DNA recovery when visualized on a 1% agarose yield gel. Neither sample exhibited quantifiable DNA when quantified via Quantiblot®. Only the SSDNA-PCIA sample was amplified and analyzed for use in the next mentioned study.

4.2 SEBWS extraction of membrane with BSA and without BSA in digestion

This study was carried out to determine if previous work by S. Steadman could be reproduced and if BSA in the restriction digest had any effect on membrane bound DNA recovery. Results from this study were comparable to the previous study of S. Steadman. As expected from the previous study, Penta E, TH01, and TPOX dropped out in the sample from the 050607ss membrane due to the restriction cut. Along with these loci FGA, D18S51, and Penta D dropped out. These additional loci dropping out are likely not from the restriction cut, but could be due to template recovery and or amplification issues. Many loci exhibited alleles below the 100 RFU threshold, and therefore were not called. However the alleles present were consistent with S. Steadman DNA bound to the membrane. Loci that possessed peaks below the threshold were: D5S818, D13S317, D7S820, D16S539, and CSF1PO. The RFU values for the sample were well below 1000

indicating limited removal and amplification of membrane bound DNA. The following image is the electropherogram for the 050607ss membrane.

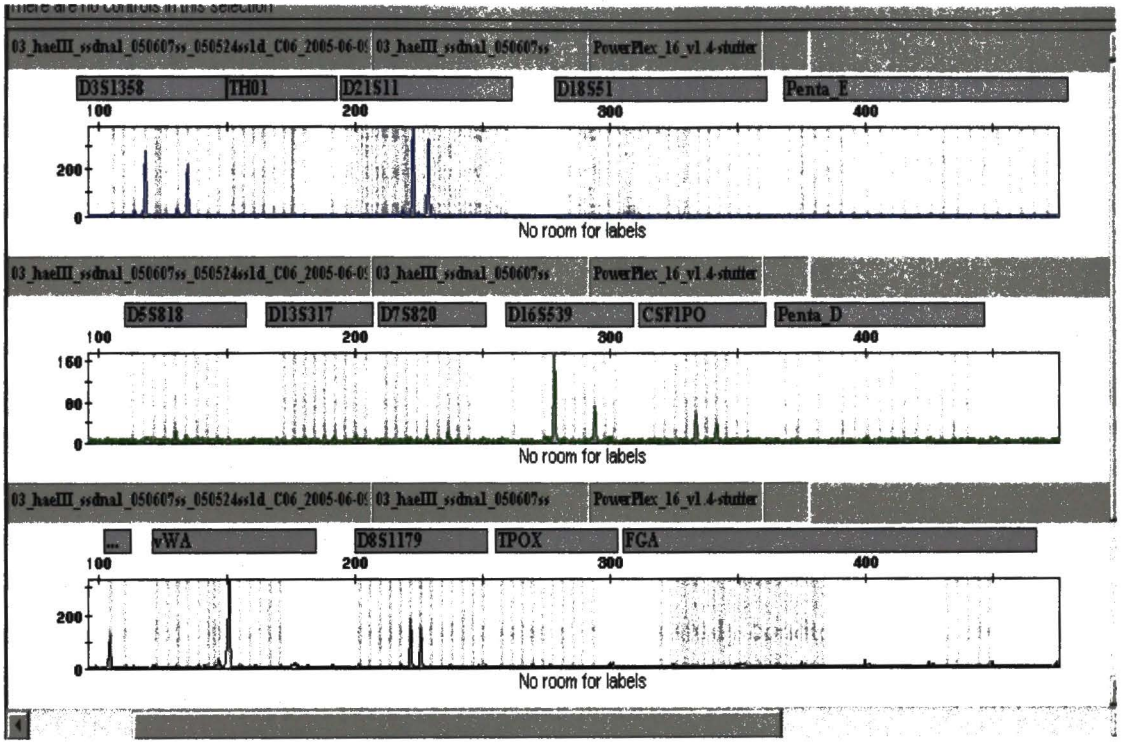

Figure 2: Electropherogram from 050607ss membrane; exhibits loci dropping out as expected and successful extraction and amplification.

SEBWS-extracted SSDNA1 bound to membrane 050527sa (withPCIA) exhibited a mixed STR profile consistent with S. Steadman and S. Andrews indicating contamination by extraneous DNA. All loci exhibited a profile showing the presence of non restriction cut DNA being amplified. Contamination could have entered the sample either through extraction of the membrane bound DNA or in the amplification step. Due to the contamination, this sample cannot be compared directly to the previous study conducted by S. Steadman because it is impossible to discern if membrane bound DNA was removed and amplified (Figure 3).

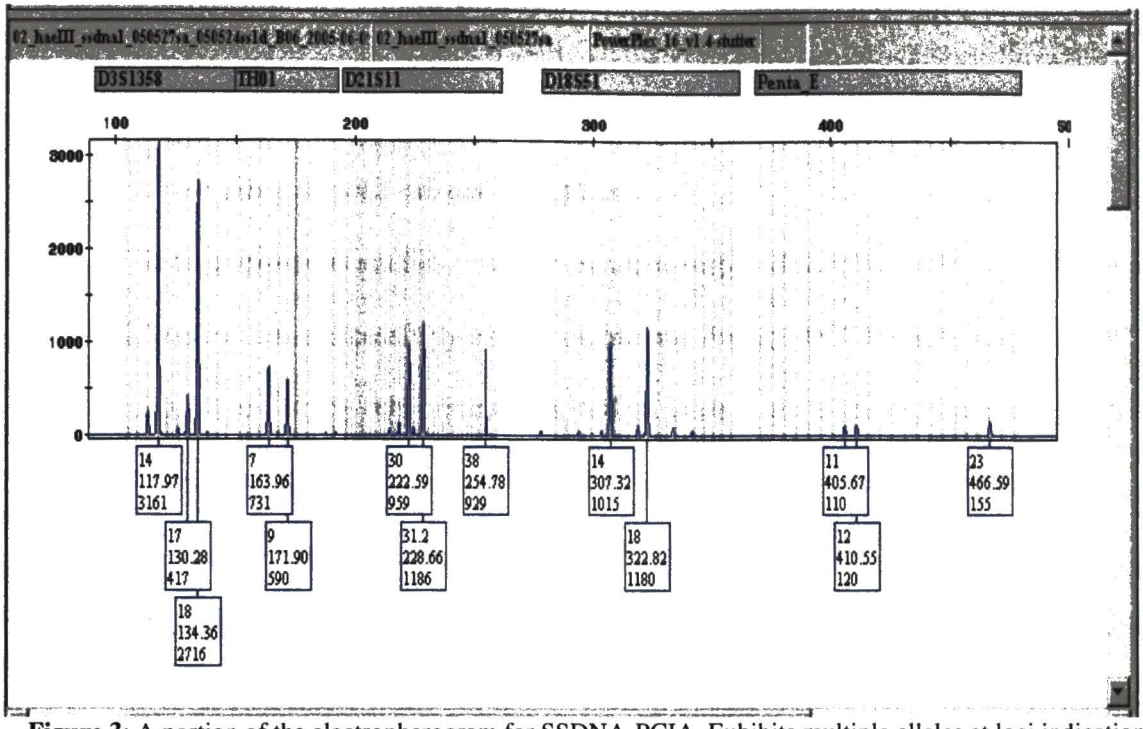

Figure 3: A portion of the electropherogram for SSDNA-PCIA. Exhibits multiple alleles at loci indication extraneous DNA contamination.

Although one sample exhibited contamination, the sample from the 050607ss membrane did not and is comparable to the previous study conducted by S. Steadman. Thus these results support the previous study and show that HaeIII restricted and bound DNA can be successfully removed and amplified to generate an STR profile.

4.3 Electrophoretic removal of membrane bound DNA

Electrophoretic removal of membrane bound DNA proved to be inefficient. After electric current was applied to the membrane and stained with ethidium bromide no detectable DNA could be seen to have migrated off of the membrane. Thus no further steps were conducted on this sample.

4.4 Extraction using various extraction buffers

Through previous studies it was shown that standard SEBWS with the addition of Pro K (20mg/ml) can successfully extract membrane bound DNA for amplification and STR typing. In this study other extraction buffers were tested to determine if they were more efficient at removing membrane bound DNA than the standard SEBWS.

All samples exhibited partial profiles. The sample extracted in Promega's digestion buffer possessed alleles at all loci except for TH01, Penta E, and TPOX. These three loci dropped out due to the restriction cutting of HaeIII, as previously demonstrated. All other loci exhibited alleles above the 100 RFU threshold. Most alleles called were well over 1000 RFUs indicating successful removal of membrane bound SSDNA1. Some alleles were blown out, indicative of too much DNA in the PCR reaction. The alleles that had the lowest RFU values were at D18S51, Penta D, and FGA. This is understandable due to the relatively large size of the fragments being amplified at these loci. Due to the fact that the expected loci dropped out it can be determined that the DNA amplified was in fact the restricted cut membrane bound SSDNA1. The high RFU values of the alleles at the other loci are indicative of the success of Promega's digestion buffer with added Pro K and DTT at removing restriction cut membrane bound DNA (Figure 4 and 5).

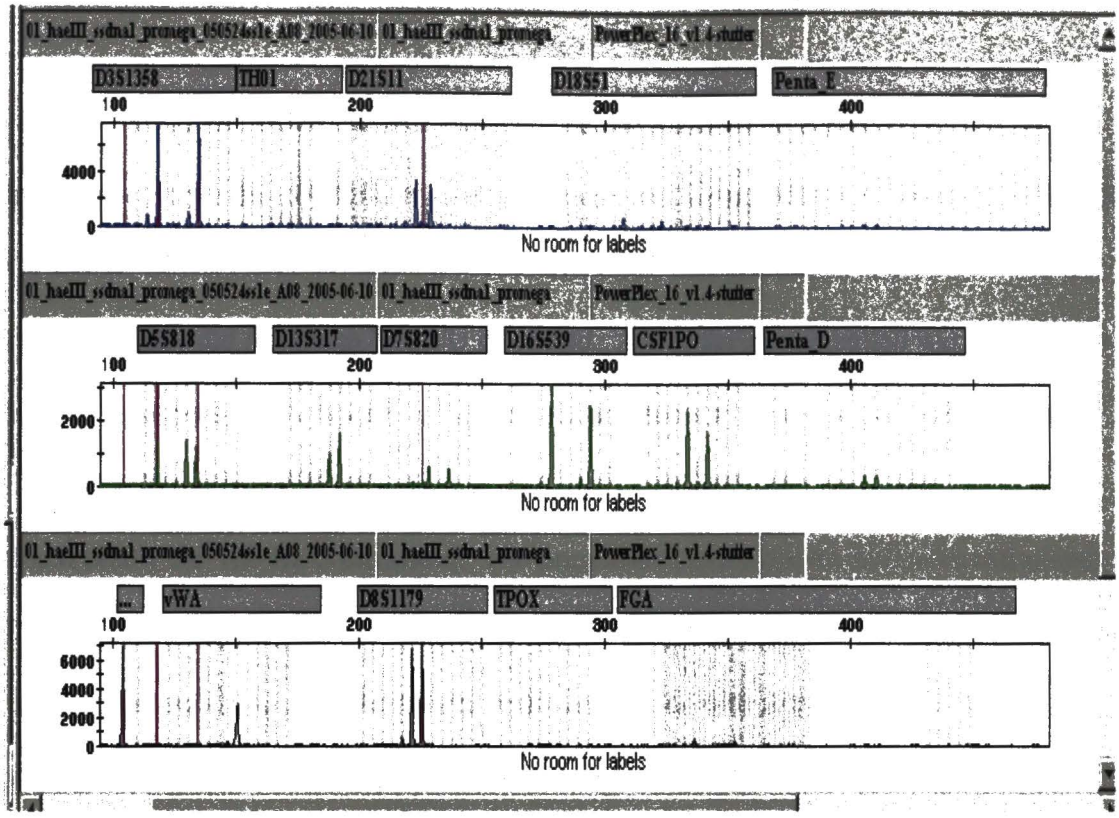

Figure 4: Electectropherogram of Promega's digestion buffer. Exhibits expected loci dropping out and successful amplification of membrane bound DNA

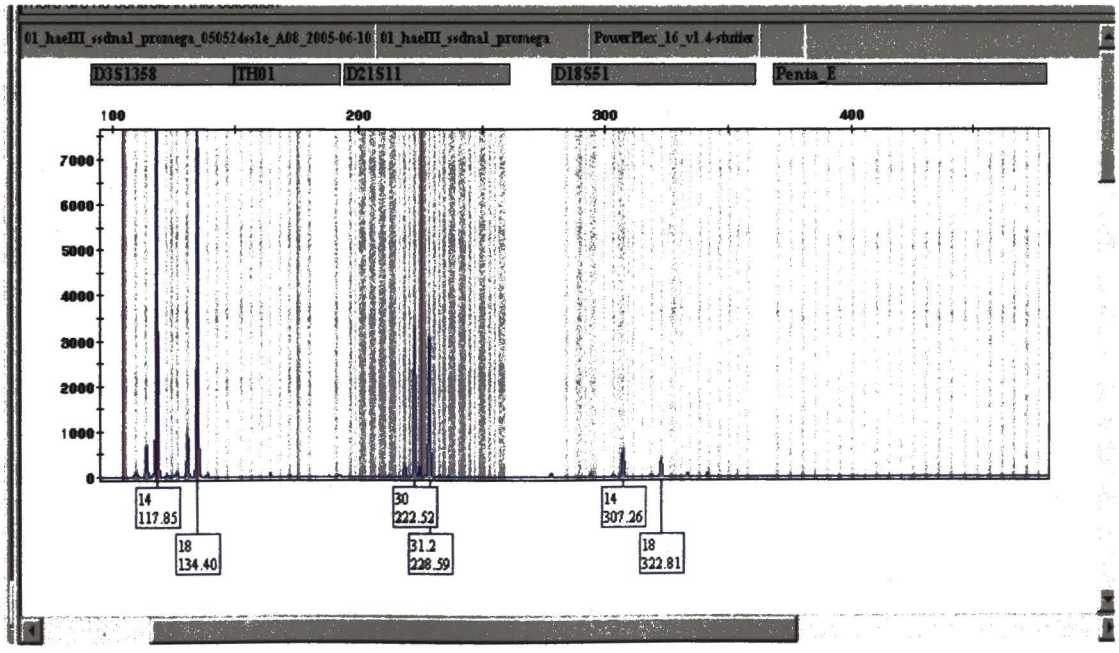

Figure 5: Partial electropherogram of Promega's digestion buffer. Illustrates the expected loci Penta E and Th01 dropping out due to restriction digest.

The sample extracted with "spiked" SEBWS exhibited a partial profile. Six loci had alleles above the 100 RFU threshold. Those loci were: D3S1358, D21S11, D5S818, D16S539, CSF1PO, vWA, and D8S1179. All other loci possessed alleles but were not called for they did not reach he RFU threshold. All of the alleles called were consistent with S. Steadman DNA bound to the membrane. There were no called alleles at the TH01, TPOX, or Penta E loci; however upon closer examination possible alleles were noted below the 100 RFU threshold. The presence of these alleles indicates extraneous DNA other than that bound to the membrane, or DNA amplified from the membrane that was not restriction cut. Furthermore, peaks consistent with the profile of S. Andrews were present at various loci below the threshold. From the data it appears that SEBWS with added Pro K and DTT is successful at removing membrane bound DNA. When compared to the standard SEBWS it appears that the additional Pro K and DTT added proved to be of some benefit to DNA removal from the membrane. The "spiked" SEBWS did allow for two additional loci to be called: D5S818 and CSF1PO. However it must be noted that this could be due to the nature PCR and not the additional Pro K and DTT. The RFU values for the alleles in the "spiked" SEBWS were in many cases double that of the non-spiked SEBWS indicating that the added ProK and DTT could allow for more removal of membrane bound DNA (Figure 6).

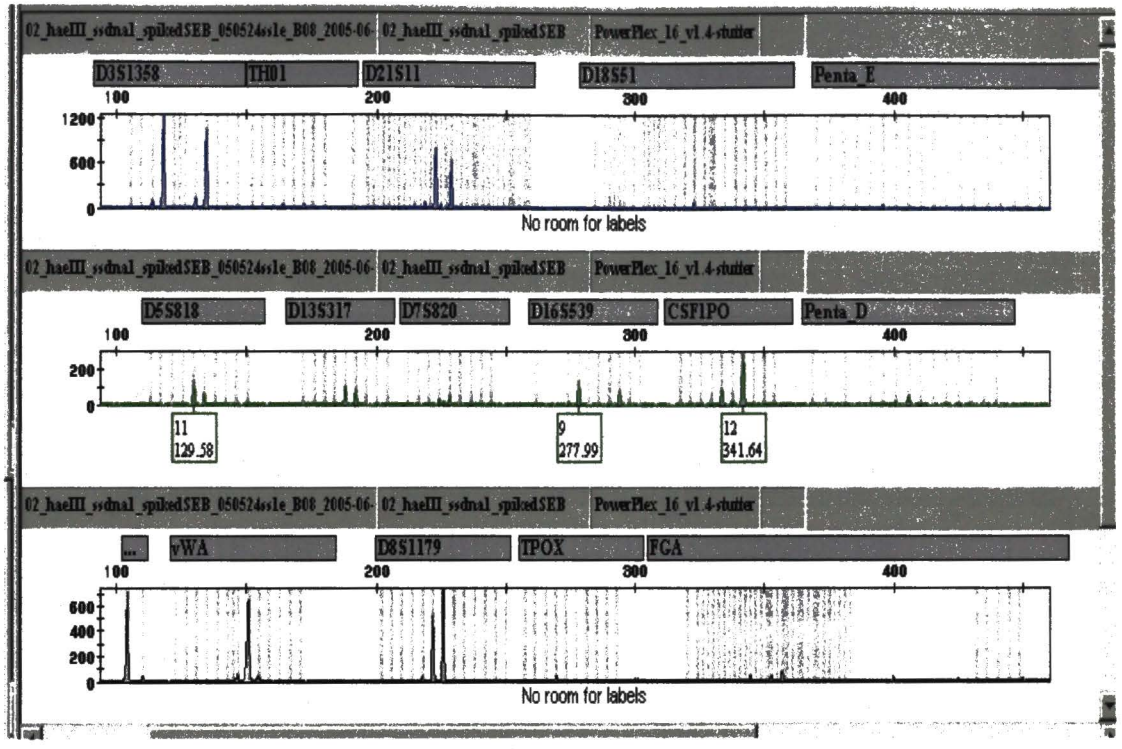

Figure 6: Electropherogram of "spiked" SEBWS. Electropherogram indicates successful removal of membrane bound DNA and the increase in RFU values when compared to the standard SEBWS.

The sample extracted in the SCRFSC sperm lysis buffer exhibited a full profile which is indicative of contamination. Not only were S. Steadman's alleles present, but alleles of S. Andrews were also present. S. Steadman's alleles were present at TH01 indicating contamination by extraneous DNA after restriction digest, or amplification of non-restriction cut DNA off of the membrane. At the TPOX locus alleles were present from S. Steadman and S. Andrews also indicating contamination. Due to the contamination, the success of the sperm lysis buffer on restriction cut, membrane bound SSDNA1 cannot be ascertained (Figure 7).

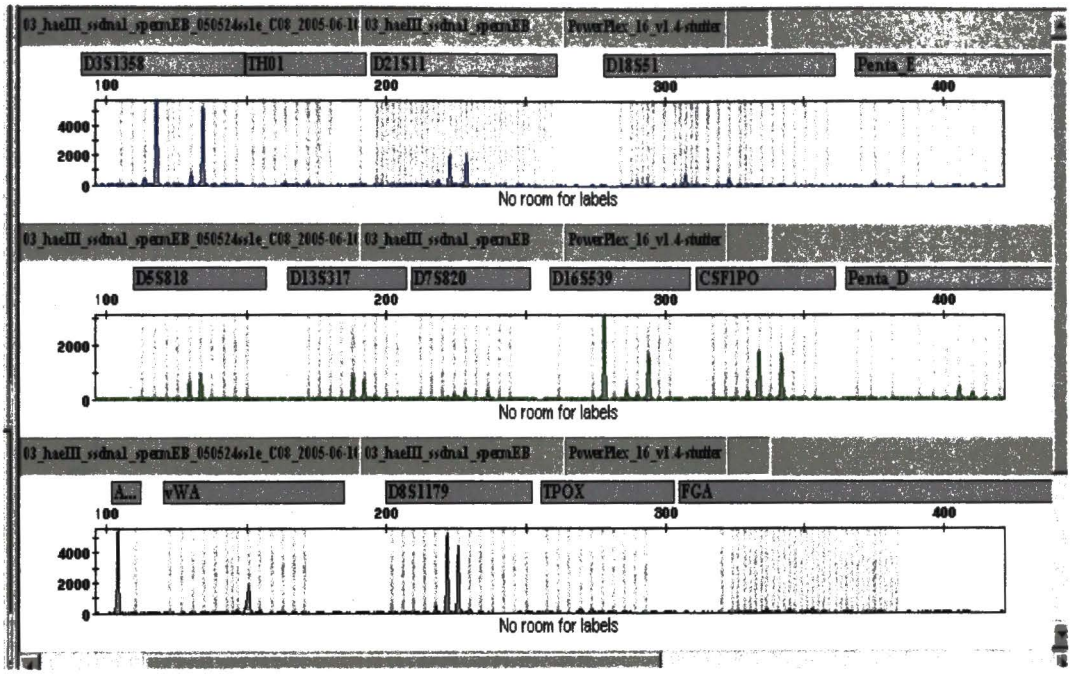

Figure 7: Electropherogram of Sperm lysis buffer. Indicates contamination due to the full profile generated and the extraneous alleles present.

4.5 Scraping of membrane cutting prior to extraction

The sample that was scraped and placed in SEBWS exhibited a partial profile. Loci TH01, Penta E, and TPOX all dropped out as expected. In addition to these loci, FGA also dropped out. All other loci exhibited alleles breaking the 100 RFU threshold. The 11 allele at the Penta D locus of S. Steadman did not break the threshold while the 10 allele did. This could be due to preferential amplification of the10 allele. Upon closer observation S. Steadman's alleles at TH01 and FGA may be present but are below the threshold. This is an indication of extraneous S. Steadman DNA being amplified or non-restricted DNA being amplified off of the membrane. No alleles below the threshold were detected at Penta E or TPOX as expected. Peaks consistent with S. Andrews'

alleles can be seen below the threshold at FGA indicating contamination by extraneous DNA (Figure 8).

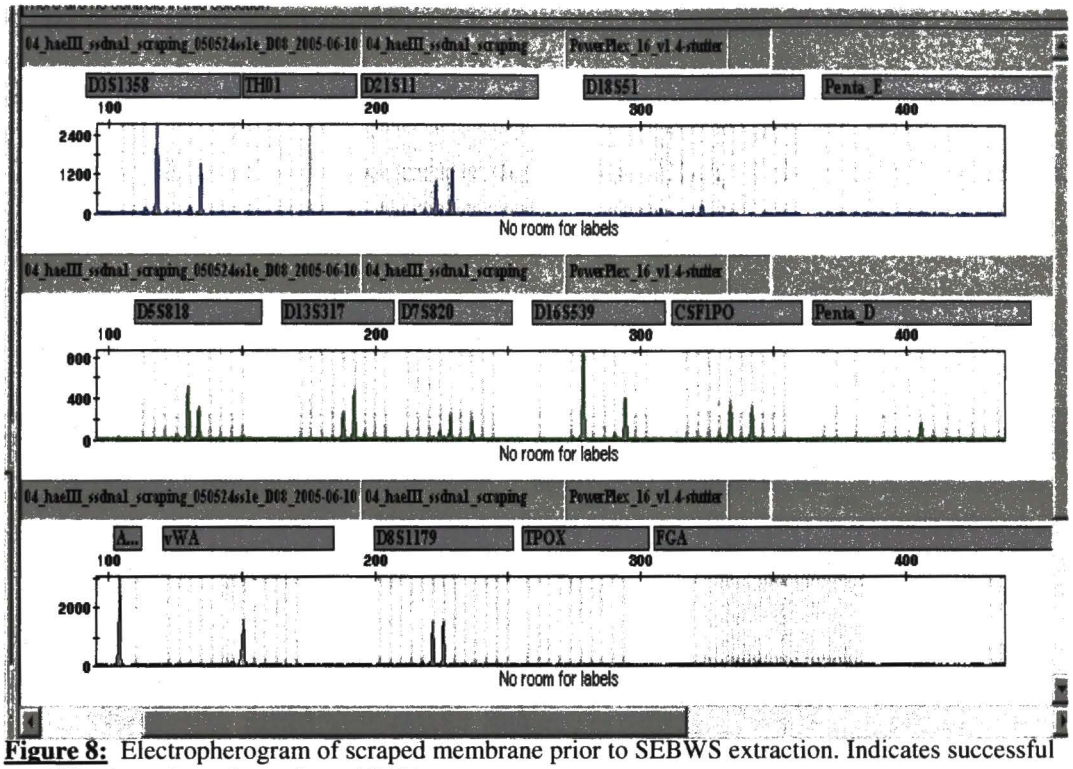

Figure 8: Electropherogram of scraped membrane prior to SEBWS extraction. Indicates successful amplification of membrane bound DNA.

4.6 Direct amplification from the membrane

Through analysis of the electropherograms direct amplification after membrane blocking is not successful at generating STR profiles. In addition, more PCR cycles does not increase the RFUs or success of amplification. In both the standard 32 cycle (figure 9) and extended 40 cycle (figure 10) PCR reaction, no alleles were called by the computer software. Upon closer observation three small peaks can be seen below 50 RFUs. A 14 allele at D3S1358, a 31.2 at D21S11, and a 12 allele at D13S317 can be seen and are consistent with S. Steadman's profile. This study reinforces a previous

study conducted by S. Steadman which indicated that STR amplification from membranes with restriction cut bound DNA was not successful. The inability to amplify STRs from RFLP membranes could be due to the interaction of the PCR components with the charges on the nylon membrane. This interaction may prohibit the successful interaction of the PCR components with the bound DNA. This study also demonstrates that blocking of the membrane prior to amplification has a minimal increase in the success of STR amplification.

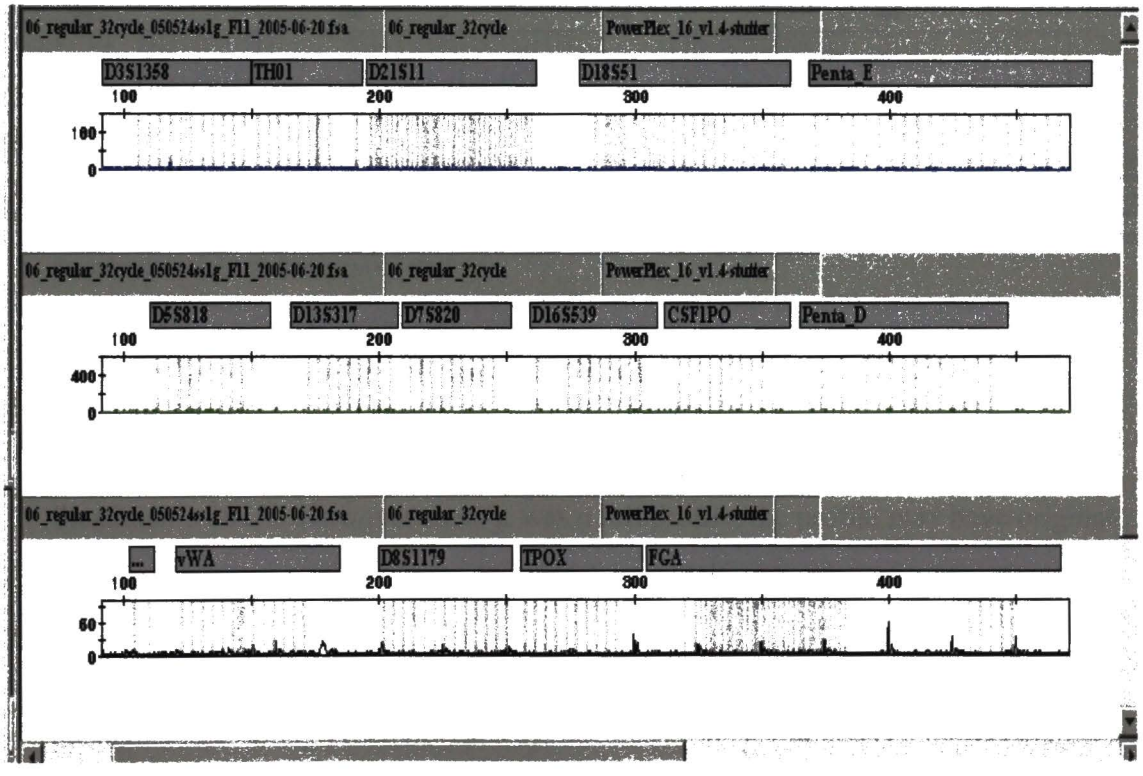

Figure 9: Electropherogram of standard 32 cycle direct amplification. The electropherogram illustrates the unsuccessful amplification of alleles.

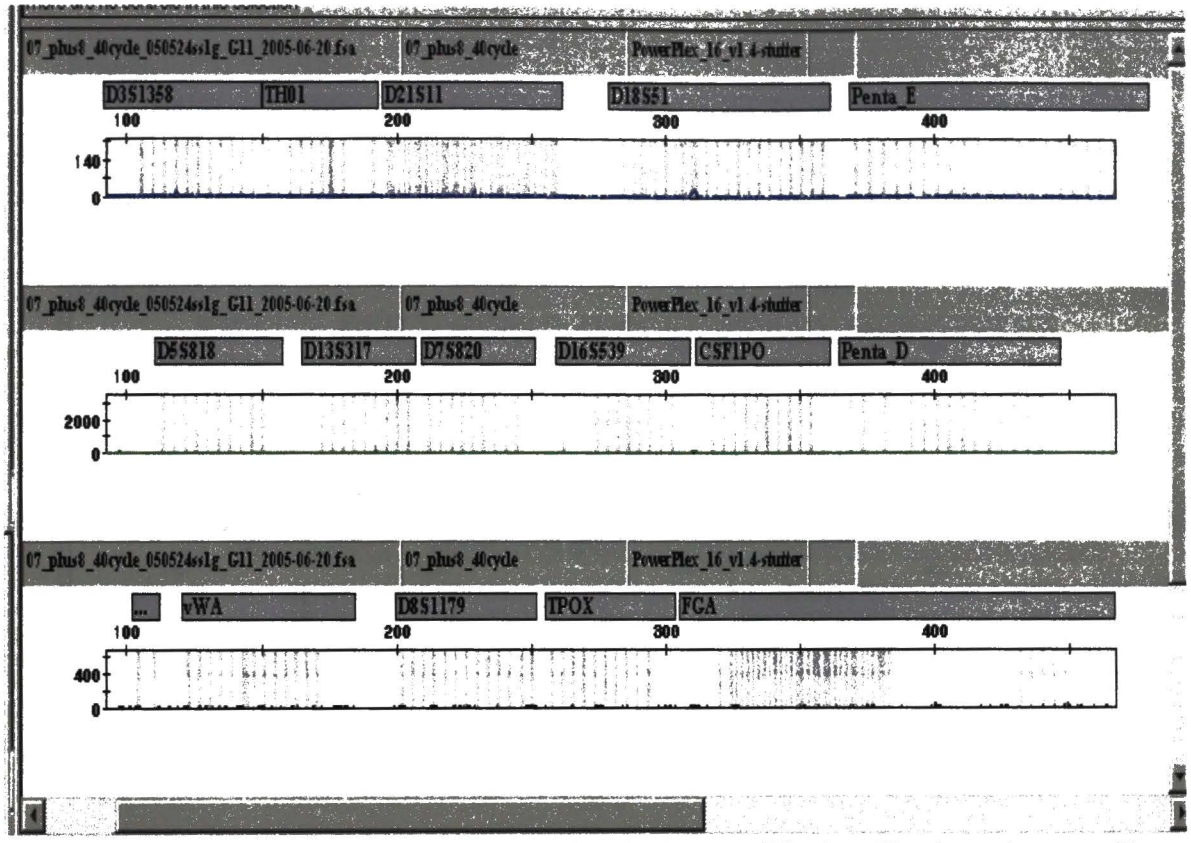

Figure 10: Electropherogram of extended 40 cycle direct amplification. The electropherogram illustrates the unsuccessful amplification of alleles.

4.7 Tape lift removal of membrane bound DNA

The tape lifted sample exhibited a full profile that was consistent with S. Steadman's DNA. However since it was a full profile, the profile may have originated from extraneous DNA. S. Steadman was the person responsible for tape lifting the membrane which is a good indication that the profile obtained probably originated from the actual tape lifting process. Thus the success of the technique cannot be determined (Figure 11).

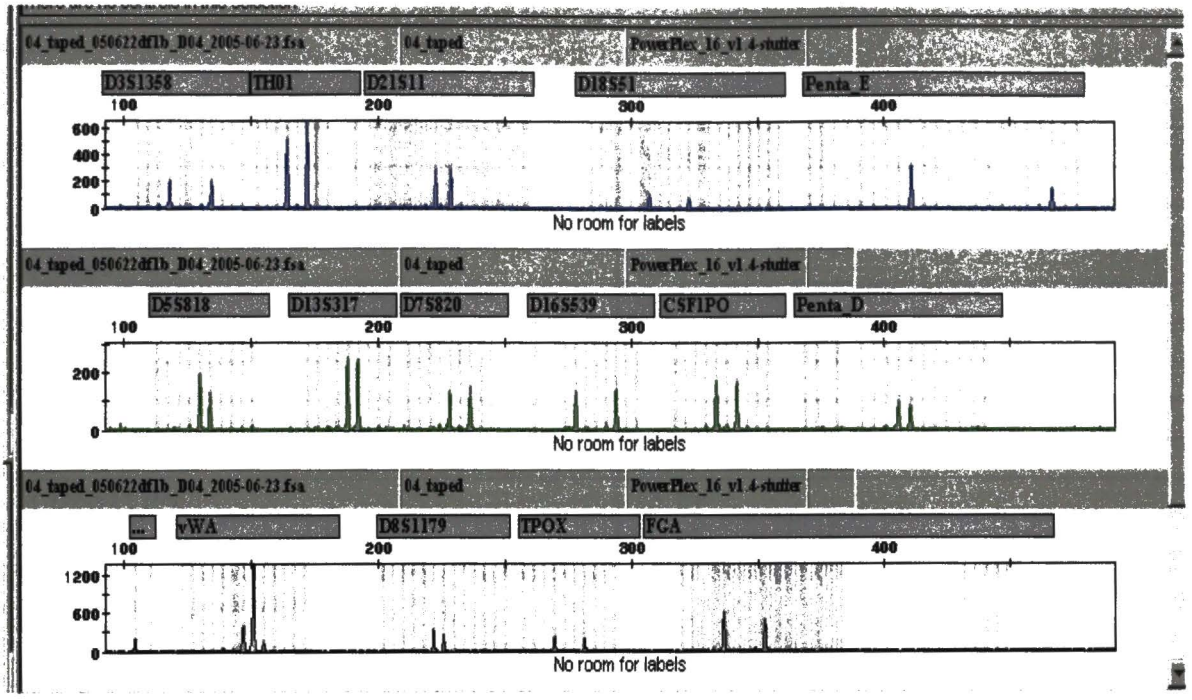

Figure 11: Electropherogram of tape lifted sample showing the generation of a full profile which likely indicates amplification of extraneous DNA.

4.8 Application to forensic sample (RFLP membrane 38719)

The tube labeled "no sonication" exhibited numerous alleles exceeding 100 RFUs. Alleles were called at loci D3S1358, Th01, D21S11, D5S818, D13S317, D7S820, vWA, D8S1179, and FGA. A major profile could be seen at 9 of the analyzed loci (Table 1, Figure 12, and Figure 13). This profile is not consistent with S. Steadman, S. Andrews, or any other analyst present in the SCRFSC lab. The major contributor to the profile observed is as follows:

Locus	Allele
Amelogenin	X,Y
D3S1358	16,17
Th01	7,8
D21S11	30,34.2
D5S818	11,12
D13S317	8,12
D7S820	9,10
VWA	16,17
D8S1179	13,14
FGA	20,21

Table 4: Major profile observed from "no sonication" sample from RFLP membrane 38719 using Promega's digestion buffer.

This unknown profile could be attributed to amplified DNA bound to the membrane, which is the goal of this study. However, it cannot be determined certainly whether this is the case due to the unavailability of the STR profiles from this membrane. This profile could also be attributed to extraneous DNA from outside of the SCRFSC. Extraneous DNA could have come in contact with the membrane during the actual RFLP process by the analyst, or subsequent handling of the membrane by a number of individuals. However, the male analysts from the UNTHSC can be ruled out as contributors of this profile.

Many of the alleles seen in the major contributors profile can also be seen in either S. Steadman's or S. Andrews' profile. However, the unknown major contributor profile cannot be attributed to the combined profiles of S. Steadman and S. Andrews. In order for this to be the case preferential amplification would have to occur over numerous loci for both individuals; this is highly unlikely. Furthermore, alleles are present that

cannot be attributed to either individual such as: allele 16 at D3S1358, allele 34.2 at D21S11, allele 8 at D13S317, allele 16 at vWA, and allele 21 at FGA.

Of concern is the presence of peaks at TH01 which is one of the loci that should drop out due to the HaeIII restriction enzyme. However the presence of alleles at this locus does not positively indicate amplification of extraneous DNA due to the possibility of unrestricted DNA on the membrane. Not all restriction digests completely digest the entire DNA sample. Some unrestricted, high molecular weight DNA, can be found on RFLP membranes below the well. In this particular sample, this could be the case.

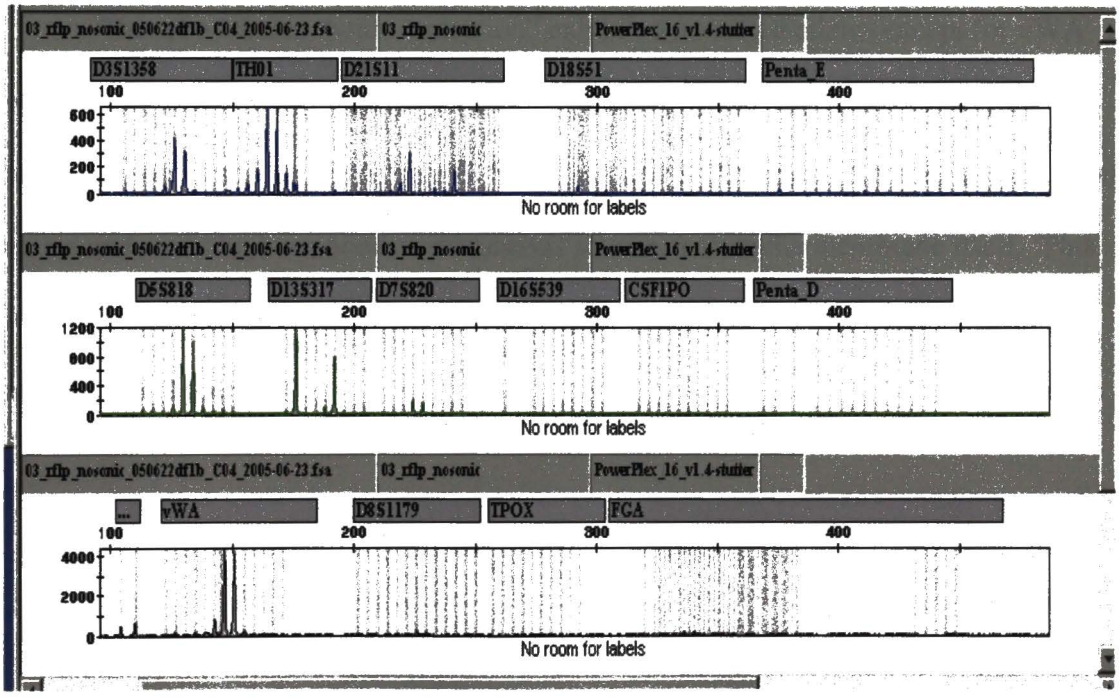

Figure 12: Electropherogram of "no sonication" sample from RFLP membrane 38719 showing the amplification of an unknown partial profile.

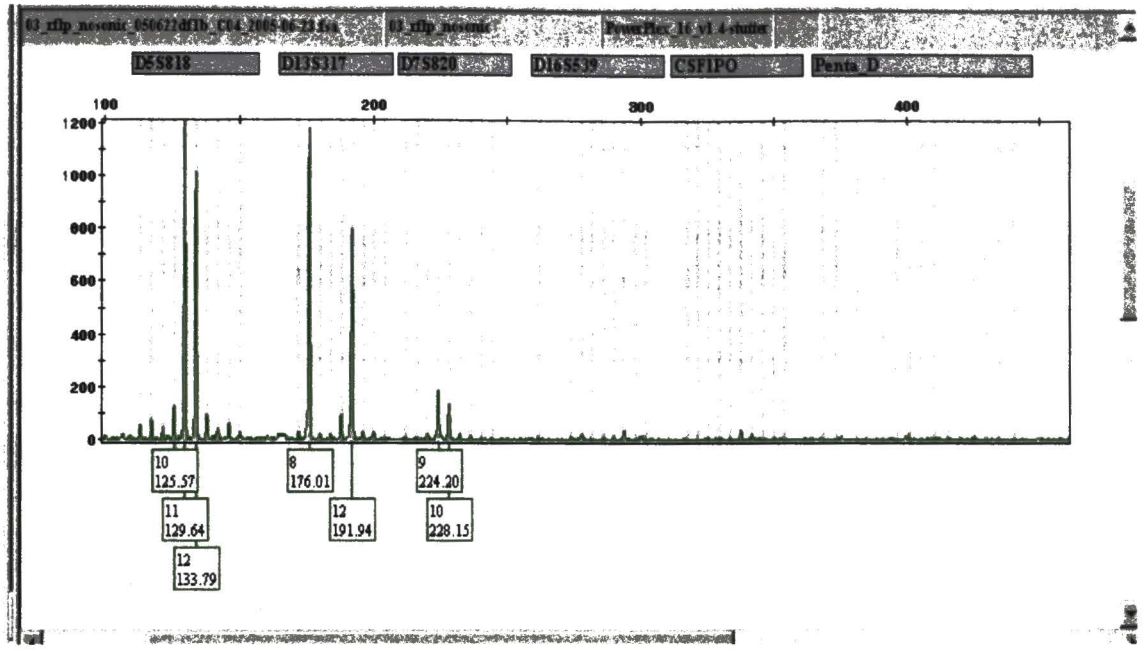

Figure 13: Partial electropherogram of "no sonication" sample showing amplified unknown alleles.

The tube labeled "with sonication" exhibited no called alleles except for vWA where three alleles were present. Two of the alleles, 17 and 18, could possibly be attributed to extraneous DNA from S. Steadman and S. Andrews. Another allele, 16, was called and could be from another extraneous source or from the membrane itself. This technique, with added sonication, proved unsuccessful at extracting DNA from RFLP membranes (Figure 14).

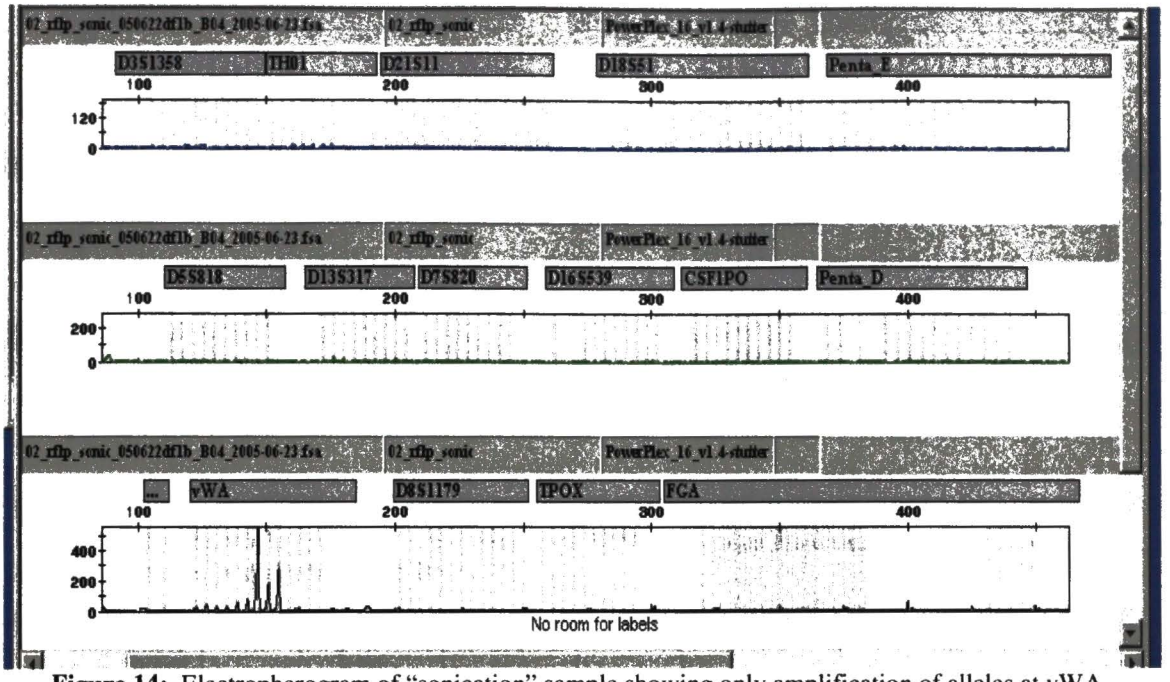

Figure 14: Electropherogram of "sonication" sample showing only amplification of alleles at vWA.

4.9 Application to forensic sample (RFLP membrane 38745)

To try and reproduce the results obtained in the previous study another UNTHSC membrane was processed the same way as the "no sonication" sample. After analyzing the electropherograms it was determined that the technique failed in this instance. No detectable alleles were seen at any of the loci analyzed (Figure 15). This does not mean the technique does not work, but simply the technique did not work on this sample. This could be due to a number of things that will be addressed in the discussion.

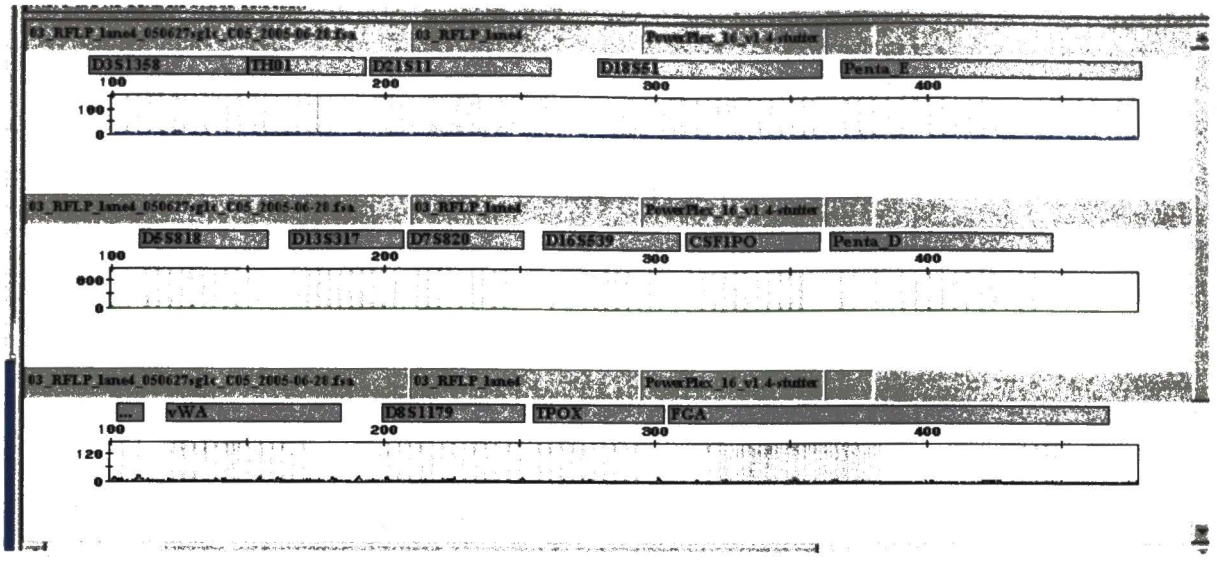

Figure 15: Electropherogram of RFLP membrane 38745 indication the unsuccessful extraction and amplification of membrane bound DNA.

	Known bound DNA	BSA sample	No BSA sample	Promega's Buffer	Spiked SEBWS	Sperm lysis	Scraped sample	Taped Sample	Direct amp 32 cycle	Direct amp 40 cycle
Locus										
D3S1358	14,18	cons	cons	cons	cons	cons	cons	cons	no call	no call
TH01	7,9	cons	no call	no call	no call	cons	no call	cons	no call	no call
D21S11	30, 31.2	cons	cons	cons	cons	28,29,30,31.2	cons	cons	no call	no call
D18S51	14,18	cons	no call	cons	no call	12,14,18,19	cons	14	no call	no call
Penta E	12,23	11,12,23	no call	no call	no call	5	no call	cons	no call	no call
D5S818	11,12	cons	no call	cons	11	cons	cons	cons	no call	no call
D13S317	11,12	11,12,13	no call	cons	no call	11,12,13	cons	cons	no call	no call
D7S820	10,12	cons	no call	cons	no call	9,10,12	cons	cons	no call	no call
D16S539	9,13	cons	9	cons	9	9,11,13	cons	cons	no call	no call
CSF1PO	10,12	cons	no call	cons	12	cons	cons	cons	no call	no call
Penta D	10,11	cons	no call	cons	no call	cons	10	no call	no call	no call
vWA	17	cons	cons	cons	cons	17,18	cons	16,17,18	no call	no call
D8S1179	12,13	10,12,13,14	cons	cons	cons	12,13,14,15	cons	cons	no call	no call
TPOX	8,11	cons	no call	no call	no call	8,9	no call	cons	no call	no call
FGA	20,24	cons	no call	cons	no call	20,22,24,25	no call	cons	no call	no call
Amelogenin	X	cons	cons	cons	cons	cons	cons	cons	no call	no call

Table 5: Table illustrating the control (known) profile bound to the membranes and the profiles obtained from each technique. Abbreviations: cons= consistent with, no call= no called alleles

CHAPTER V

DISCUSSION

Based on the results of this study, it appears that HaeIII restriction cut DNA can be extracted and successfully amplified from membranes. However samples must be subjected to PCIA in order to have successful recovery. Although the SSDNA-NOPCIA sample was not amplified, when it was concentrated on a microcon, the flow-through of the washes was slow. This is an indication that extraction components remained which interfered with purification and concentration. Furthermore, if a sample is not properly cleaned up as in the SSDNA-NOPCIA sample, extraction components would be present inhibiting successful amplification of the DNA.

Due to contamination, it could not be determined if BSA in restriction digests had any effect on the recovery of membrane bound DNA. However since the aim of this study was to extract DNA from RFLP membranes this has little significance because traditional RFLP restriction digests did not have BSA as a component. From previous studies conducted at the SCRFSC it was determined that the TPOX, TH01, and Penta E loci become unamplifiable due to the HaeIII restriction digest. However, due to the nature of restriction digestion, some DNA may not become restriction cut depending on how long the reaction is carried out and the efficiency of the reaction. The fact that some samples exhibited amplified product at loci expected to drop out does not necessarily indicate contamination. While contamination or amplification of unrestricted DNA seemed to be prevalent throughout the study, the amplification of alleles at loci expected

to drop out may indicate the amplification of unrestricted membrane bound DNA instead of extraneous DNA. Some samples did exhibit extraneous DNA being amplified, and this DNA can be attributed to the analysts performing the study. This shows how easy it is for a membrane to become contaminated even with frequent glove changes and the analyst wearing a face mask.

Studies herein demonstrated that membrane bound DNA could not be electrophoresed off of a nylon membrane; at least not in a quantifiable amount under the parameters utilized. Between 200-300 volts were used in this study and may not be a strong enough current to break the bonds binding the DNA to the nylon membrane. It is possible that some residual DNA may have migrated off of the membrane but not enough to be detected by a yield gel. Thus, if the DNA can not be detected then it cannot be excised from the gel for purification and amplification purposes.

In regards to all of the techniques utilized, Promega's digestion buffer was most successful at removing membrane bound restricted DNA from a nylon membrane. It removed so much DNA that the sample was blown out; an indication of too much DNA in the PCR reaction. All loci expected to drop out did in the Promega digestion buffer sample. This is an indication that the DNA was in fact restriction cut and amplified from the membrane. All other loci exhibited alleles above the 100 RFU threshold. The scraped sample with standard SEBWS was also successful in removing membrane bound DNA. All loci expected to drop out did, along with FGA, which may be expected due to the large size of the amplicons produced at this locus. However, S. Steadman and S. Andrews' alleles can be seen at some loci below the 100 RFU threshold. This is an

indication of contamination and/or the possibility of unrestricted DNA. The "spiked" SEBWS technique was somewhat successful at removing membrane bound DNA but not as successful as the prior two techniques. However the "spiked" SEBWS (Pro K 1ug/ul and DTT 12ug/ul) did exhibit an increase in RFU values of alleles and the number of loci with alleles breaking the threshold when compared to the standard SEBWS (5ug/ul Pro K and 6ug/ul DTT). This is an indication that the added ProK and DTT have a desired increase in the removal of membrane DNA. This could possibly be due to their ability to break the bonds holding the DNA to the membrane; thus increasing removal. Due to contamination of the sperm lysis buffer sample, it is impossible to tell if the technique was successful. The sonication of the samples prior to PCIA addition may have helped in the removal of DNA from the membranes. Sonication is known to fragment DNA into small pieces, and was used in this study in hopes of helping break the bonds holding the DNA to the membrane.

All of the previously mentioned techniques were tried on membranes prepared by S. Steadman and S. Andrews prior to attempts on true RFLP membranes to determine which technique should be employed on the RFLP membranes. It must be noted that approximately 100ng of DNA was blotted to each of the slot blot wells. Slot blot wells are much smaller than a lane on a traditional RFLP membrane. In any one RFLP membrane lane between 50 -500ng of DNA is bound. This DNA is spread across a surface that is many times larger than a slot blot well. It would be expected that approximately 100ng of DNA bound to a slot blot well would be easier to recover than 50-500ng of DNA spread across a surface area many times that of the slot blot well.

Also, the binding capacity of a membrane covering a single slot blot well is not known. It could be possible that 100ng of DNA may overload the membrane preventing efficient binding, and thus easier DNA removal from the membrane. The membranes prepared by S. Steadman and S. Andrews did not undergo multiple probing and stripping as did traditional RFLP membranes. This would also allow for easier removal of membrane bound DNA from the "pseudo" RFLP membranes than true RFLP membranes.

Direct amplification off of a slot blot well proved to be unsuccessful even after attempting to block the membrane. In addition, more PCR cycles does not increase the RFUs or success of amplification. In both the standard 32 cycle and extended 40 cycle PCR reaction, no alleles were called by the computer software. Upon closer observation three small peaks can be seen below 50 RFUs. A 14 allele at D3S1358, a 31.2 at D21S11, and a 12 allele at D13S317 can be seen and are consistent with S. Steadman's profile in the standard 32 cycle sample. Based on this study, STR profiles could not be generated through direct amplification off of RFLP membranes. This study reinforces a previous study conducted by S. Steadman which showed no STR amplification off of RFLP membranes. The inability to amplify STRs off of RFLP membranes could be due to the interaction of the PCR components with the charges on the nylon membrane. This interaction may prohibit the successful interaction of the PCR components with the bound DNA. The current study also demonstrated that blocking of the membrane prior to amplification in order to sequester free charges on the membrane has a minimal increase in the success of STR amplification.

Since Promega's digestion buffer was most successful at removing membrane bound DNA from the membranes prepared at the SCRFSC, it was utilized on true RFLP membranes from the UNTHSC. The excision of lanes from the true RFLP membranes was a bit complicated due to the lack of the autorads. This could be one of the greatest limitations to the study since a small deviation from the actual lane could prevent successful extraction of DNA due to no DNA being excised. The best spot for successful DNA extraction on an RFLP membrane would be located directly under the well because this is where the unrestricted, high molecular weight DNA would be found, if any was present. The successful extraction and amplification of DNA on an RFLP membrane lane is difficult due to the small amount of DNA spread across a large membrane surface area. Furthermore, the membranes are designed to permanently bind the DNA so that multiple probing and stripping can occur without the substantial loss of DNA. This is another obstacle that has to be overcome when extracting DNA from RFLP membranes.

When two lanes were excised from UNTHSC membrane 38719 one of the samples exhibited a major contributor profile at 9 loci. The sample that exhibited this profile was the sample labeled "no sonication." The sample labeled "sonication" exhibited the presence of alleles at only FGA. Both samples were extracted the same way except for the sonication sample being sonicated for 30 minutes prior to incubation. The fact that the sonication sample exhibited alleles at one locus were two of the alleles can be attributed to extraneous DNA, may indicate that extended sonication is not beneficial for membrane bound DNA recover. However, the unsuccessful extraction may also be

attributed to the excision of the lane where the excision of the lane was off causing no DNA to be removed.

The major male profile obtained from the "no sonication" sample was not consistent with anyone from the SCRFSC or the male analysts at the UNTHSC. This is a good indication that the profile seen may have originated from the DNA bound to the membrane. However this could not be determined for certain due to the lack of STR profiles for this particular membrane. The profile obtained may also be attributed to extraneous DNA that may have come in contact with the membrane during the actual RFLP process by the analyst, or subsequent handling of the membrane by a number of individuals. Back when RFLP was the DNA analysis method of choice, contamination was not as big of a concern due to the sensitivity limitations of the test itself. Currently, PCR is utilized which is magnitudes more sensitive than RFLP techniques. Due to this, PCR based amplification of RFLP DNA can be extremely hard due to the likely chance of extraneous DNA contamination.

Due to the lack of STR profiles available for RFLP membrane 38719, another RFLP membrane (38745) was processed in the same manner for reproducibility purposes. After STR typing it was determined that no amplifiable alleles were observed. Therefore this study did not support the previous results obtained. However it must be noted that a different membrane was utilized in this study and therefore had a different DNA amount in the lane excised. This study questions the validity of the first experiment. Although a DNA profile was obtained in the first study, it may be attributed to membrane bound DNA or extraneous DNA not bound to the membrane. As in the first study, a template

was made from a photocopy of wells to facilitate in cutting a lane out of the membrane. If the template was off slightly then the lane containing the DNA could have not be excised, or the amount of DNA removed could have been decreased significantly, thus reducing STR typing efficiency. Another possible reason for no alleles being amplified from this membrane could be due to the lack of high molecular weight DNA. High molecular weight DNA can be typed more readily than digested, and thus the amount of high molecular weight DNA could greatly affect the STR amplification process. If little or no high molecular weight DNA was present on the lane excised from this membrane, then STR amplification could have been hindered. The fact that no alleles were amplified from this sample does not mean the technique failed, but that it was unsuccessful on this sample.

The successful extraction and amplification of DNA from RFLP membranes may be dependent on a number of factors. Through a previous study conducted at the SCRFSC it was shown that the actual HaeIII restriction digest does not prevent amplification of STR alleles other than at the aforementioned loci. However, due to the relatively small size of the STR loci, some of these loci or alleles may drop out during the electrophoresis step. Due to the nature of RFLP analysis the smallest fragment size is approximately 1000bp at the bottom of the separation gel. Most STR loci are between 100-500bp. If a HaeIII restriction cut site is within a close enough distance to one of these loci, the locus could be cut and included in a fragment that is smaller than approximately 1000bp. If this occurs the particular locus would be lost due to the electrophoresis process since the fragment would migrate off of the gel under electric

current. The location of HaeIII restriction cut sites in relation to STR loci could cause additional loci to drop out due to the small size of the fragment the locus would be found in. Studies herein indicate that membrane bound, restriction cut DNA can be extracted and amplified from a small localized membrane area (slot blot well) which is promising for DNA extraction from RFLP membranes. The successful extraction and amplification of membrane bound DNA from RFLP membranes will depend on the membrane used, the lane excised, and the storage condition of the membrane. Not all RFLP membrane lanes contain the same amount of membrane bound DNA and this amount can vary considerably. The amount of DNA bound to the excised lane may greatly affect the success of extraction. Membranes must also be stored properly (-20°C or 4°C) if DNA is to be recovered and successfully amplified. Autorads of the membrane need to be available in order to know the exact position of the lanes for successful extraction. To prevent amplification of extraneous DNA present on the membrane it must be washed or boiled prior to extraction to remove extraneous DNA. The membranes in this study were not washed which may have led to the amplification of extraneous DNA.

Future studies at the SCRFSC include the use of Promega's digestion buffer on other RFLP membranes and also the use of isopropanol on membrane bound DNA. Nylon membranes have been shown to be susceptible to isopropanol over an extended period of time. High molecular weight DNA can be recovered after exposure to isopropanol. The theory behind subjecting membrane bound DNA to isopropanol is to attack the integrity of the membrane and thus release the DNA for extraction and amplification. The generation of an unknown STR profile from an RFLP membrane in

this study is promising. However, more extensive studies testing reproducibility and addressing the aforementioned limitations must be conducted to ascertain the success of RFLP membrane bound DNA recovery and amplification.

APPENDIX A

3.1 NUCLEAR DNA EXTRACTION

PRINCIPLE

DNA is extracted from nucleated cells by the combined action of a stain extraction buffer (containing SDS, EDTA, and DTT) and Proteinase K. SDS is present to rupture the cellular membrane to expose the nucleic acids. It also will assist in the denaturation of proteins to be more susceptible to the action of Proteinase K. Proteinase K is a proteolytic enzyme that reduces proteins to their constituent amino acids. The Proteinase K will remove nucleases and histone groups which are bound the DNA strands. It is active in the presence of detergents and is unaffected by metal chelators. EDTA is a chelator that binds divalent cations and serves to lower nuclease activity. Dithiothreitol (DTT) is present to reduce disulfide bonds. The stain extraction buffer solubilizes and denatures protein.

MATERIALS

Stain extraction buffer working solution (SEBWS)
Proteinase K
56°C heat block

WORKSHEET

DNA Isolation and Extraction

PREPARATION OF MATERIALS

Note: Universal precautions regarding personal protection equipment and biohazardous material handling must be employed at all times when conducting this analysis.

Blood or Semen Stains

Cut the stain into small pieces and place the pieces into a microcentrifuge tube. Dime-sized stains are preferable, however, sufficient quantities of DNA can be obtained from smaller stains as well.

Buccal Swabs

Cut one swab from the stick and place it into a microcentrifuge tube. If only one buccal swab is available, half of the swab may be cut for extraction.

Tissues
Using a clean scalpel blade, cut the tissue into small pieces and place the pieces into a microcentrifuge tube. 1 mg of liver tissue yields approximately 15 μg DNA, while 1 mg of muscle tissue yields approximately 3 μg DNA.

Unmounted Hairs
Place approximately 1 cm of the hair root end into the microcentrifuge tube. Rinse the hair with 100% ethanol and NFW if necessary. A plucked hair will yield approximately 250 ng of DNA. Hairs that were not forcibly removed are not likely to generate a DNA profile.

Slide-mounted Hairs
Loosen the coverslip by carefully pipetting xylene around the coverslip edges. If it does not loosen, the entire slide can be submerged into xylene for one or more hours until loose. After removal of the coverslip, remove the hair and rinse thoroughly with xylene.

Swabbings taken from exhibits
Cut the swab from the stick and place it into a microcentrifuge tube.

Tapings taken from exhibits
Remove the outer layer of tape from the roll to avoid collection with a section of tape that has been exposed to the environment. Cut a small piece of tape and collect trace material onto sticky surface. Cut the tape into small sections and place them into a microcentrifuge tube. Fold the tape together (lightly press adhesive sides together), cut it into small pieces, and place it into a microcentrifuge tube.

Tears, Urine, or Other Relatively Weak Stains
Cut quarter to half-dollar sized portions or larger depending on the amount of material in the sample. Cut the stain into small pieces and place the pieces into a microcentrifuge tube.

Envelope Flaps or Stamps
Carefully cut half of the stamp from the envelope or a ~1 cm^2 portion of envelope flap into small pieces and place the pieces into a microcentrifuge tube. Alternatively, carefully open the envelope flap or remove the stamp using steam and clean forceps. Swab the gummed flap or stamp with a swab dampened with NFW. Cut the swab from the stick and place it into a microcentrifuge tube.

Chewing Gum
Carefully cut a ~1 cm^2 portion of chewed gum into small pieces and place the pieces into a microcentrifuge tube. After extraction remove supernatant and save remainder of the gum. Alternatively, carefully swab the surface of the gum with a swab dampened with NFW. Cut the swab from the stick and place it into a microcentrifuge tube.

Cigarette Butts

Cut a ~0.5 cm wide strip from the paper covering the cigarette butt in the appropriate area or cut ~0.5 cm of the tip of the filter that makes contact with the mouth. Cut the paper and/or filter into small pieces and place the pieces into a microcentrifuge tube.

Cell Suspensions

If cellular components are believed to be suspended in larger volumes of liquid (i.e.amniotic fluid or diluted biological samples), place the fluid in an appropriate sterile container and centrifuge at maximum speed for 5-20 minutes. Longer centrifugation may be required depending on the viscosity of the fluid.

Nail Material

Nail material may be extracted in whole by direct addition of extraction buffer. However, in cases where differentiation between the nail donor and the donor of material under the nail is desirable, the following general separation process may be used. Note that reagent amounts for soaking and washing may be adjusted depending on the amount of substrate (nail material) being extracted:

1. Soak nail material in 200 µl PBS for 10 minutes at room temperature. Follow with a 30 second room temperature sonication and allow to soak an additional 5 minutes.
2. Place nail in a filter basket and centrifuge at 6000 RCF for approximately 5 minutes. Discard all but ~50 µl of the supernatent; take precaution not to disturb the pellet. Denote this pellet fraction as "A" (i.e. Q1A).
3. Place nail in a new microcentrifuge tube and wash with 200 µl cold ethanol or reagent alcohol. Allow to soak for approximately five minutes, followed by a 30 second sonication. Draw off the wash with a sterile pipet, discard, and repeat wash, substituting a 30 second vortex for the sonication. Draw off the second wash, discard, and add 200 µl NFW to the nail. Shake vigorously for approximately 30 seconds. Remove the NFW with a sterile pipet and proceed with the extraction process. The washed nail is considered fraction "B" (i.e. Q1B).

Personal Items

Cellular material may be removed from exhibits such as jewelry or other exhibits where cells would expectedly be associated. Place the exhibit in a tube. Add PBS to cover substrate. Sonicate at room temperature for 1 minute. Allow soaking at room temperature for 30 minutes. Place substrate in filter basket (if possible) and pulse spin over extraction tube to remove fluid. Remove substrate to dry, and centrifuge fluid at maximum speed for approximately 5 minutes. Remove all but ~50 µl of the supernatent and place in separate tube. Proceed with the extraction process using the resulting pellet in the original tube.

Other Exhibits

DNA may be extracted from a variety of other items not specifically listed here. Depending on the exhibit, the analyst may choose to employ other scientifically sound collection and/or extraction methods in an effort to isolate and type DNA. If the stain has undergone cell extraction, place swab or cutting back into the centrifuge tube containing the cell pellet.

PROCEDURES

1. Add 400 µl SEBWS and 10 µl Proteinase K to each microcentrifuge tubes. Extraction volume may be increased using the 400:10 ratio for larger cuttings.

2. Mix tube contents and spin briefly to force cutting into the liquid.
   Note: Do not excessively mix the sample after addition of the Proteinase K.

3. Incubate at 56°C for a minimum of 8 hours. For known bloods and oral standards, this incubation may be reduced to 2 hours.

4. Spin tube briefly to bring all of sample and fluid to bottom of tube.

5. Place the cuttings into a microcentrifuge tube recovery basket. Suspend the basket in the respective extraction tube and spin for 5 minutes at maximum speed.

6. Discard the substrate and basket. Proceed to the appropriate purification procedure.

REFERENCES

1. Coomey, CT, *et al.* 1994. "DNA extraction strategies for amplified fragment length polymorphism analysis." *Journal of Forensic Sciences*, 39(5), p. 1254-1269.

APPENDIX B

3.4 MICROCON 100 PURIFICATION AND CONCENTRATION OF DNA

PRINCIPLE

Organic extractions serve to denature proteins and remove proteins and cellular debris. DNA and other soluble substances remain in the aqueous phase, while the phenol denatures and removes protein. Chloroform is added to improve the interface and eliminate traces of phenol. Isoamyl alcohol prevents excessive foaming and improves the interface. DNA is concentrated while salts and other aqueous soluble substances are passed through a Microcon microfiltration device. Microcon 50 and 100 units operate on the same basic principle, however, the nominal molecular weight limits (nucleotide cut-offs) differ. For most forensic applications, the Microcon 100 is desirable so that unamplifiable fragments are efficiently removed. Although identical in theory and application, the Microcon 50 and 100 device protocols have differing centrifugal requirements and will, therefore, be separately described.

MATERIALS

Phenol/chloroform/isoamyl alcohol (P/C/I)
NFW or TE buffer
Microcon 100 centrifugal devices

PROCEDURES

Note: Universal precautions regarding personal protection equipment and biohazardous material handling must be employed at all times when conducting this analysis.

1. Quick-spin each sample tube.

2. Add 500 µl P/C/I to extractions. This step **must** be done in the fume hood.

3. Vortex or shake the tube vigorously to achieve a milky emulsion in the tube. Spin the tube for ~5 minutes at maximum speed.

4. Transfer the aqueous phase (top layer) of each sample tube to an appropriately labeled Microcon. Do not disturb the interface. Discard the old tube containing the phenol into the appropriate evaporative waste container.

5. Centrifuge ~10 minutes at 2300 RCF. Discard filtrate.

6. Add 200 µl NFW or TE, centrifuge ~10 minutes at 2300 RCF. Discard filtrate

7. Repeat step 6 as necessary if excessive discoloration is noted on the membrane or if inhibition is suspected for the sample.

8. Add desired amount of NFW or TE (consistent with washes) and invert the sample reservoir into a clean, labeled microcentrifuge tube. For samples of limited quantity 18-20 µl NFW or TE is recommended. Centrifuge for ~5 minutes at 2300 RCF. Proceed with appropriate quantitation procedure. If limited sample quantity is suspected, the analyst may omit yield gel quantification and proceed directly to slot blot.

Procedure for retentate volume reduction:
Once a sample has undergone the concentration/purification process, reduction of the retentate volume may be necessary in when limited quantities of DNA are present in a given sample. If the DNA has been eluted into NFW, then the sample may undergo vacuum centrifugation to reduce the retentate volume. The sample is placed in the centrivap, with a 45°C heat setting, and centrifuged until the retentate has been reduced to the desired amount. Alternatively, the sample may undergo a second Microcon purification. To the original retentate, add ~200µl TE and load the sample onto a Microcon device. Spin at the recommended pass-thru speed for that device until the sample volume has passed through. Load the desired amount of TE or NFW to the membrane and proceed directly to inversion/elution (washes are not necessary for retentate reductions).

REFERENCES

1. Scherczinger CA, *et al.* 1997. "DNA extraction from liquid blood using QIAamp." *Journal of Forensic Sciences* 42(5), p. 893-896.

2. Millipore Corporation. Microcon Centrifugal Filter Devices User Guide. 99394 (Revision J, 03/00), Bedford, MA.

3. Personal communication with and current protocols from the Kansas Bureau of Investigation and the Virginia Division of Forensic Science.

APPENDIX C

3.5 YIELD GEL QUANTITATION OF DNA

PRINCIPLE

Following DNA purification and concentration, extracts are evaluated for quality and quantity using a yield gel. A small quantity of each extract is electrophoresed through an agarose gel. The gel is then stained with ethidium bromide and sample DNA is then illuminated and photographed. A range of known quantities of DNA is also placed on each gel so that extracts DNA quantities can be estimated by comparison to the known quantities.

MATERIALS

PCR analytical gel bromophenol blue loading solution
Human Genomic DNA (at least 100 ng/µl in concentration)
TAE buffer, 1X
Agarose
Ethidium bromide stain solution
Gel apparatus and power supply

WORKSHEET

DNA Yield Gel Analysis

PROCEDURES

Note: Universal precautions regarding personal protection equipment and biohazardous material handling must be employed at all times when conducting this analysis.

1. Quick-spin sample tubes.

2. For a 6 cm x 8.3 cm gel, mix 0.25 g of agarose with 25 ml TAE in a flask. Pre-weigh the flask, and then microwave to dissolve the agarose completely. Once agarose has dissolved, bring flask and contents to pre-weighed mass with type 1 water. Cool to ~56°C.

3. Level the gel tray. Place the well combs into the gel tray. Pour agarose into the gel form. Let stand for a minimum of 15 minutes to gel. If the gel is to be used the next day, it may be stored in a tightly closed humidity chamber at room temperature. Prior to use, pour 1X TAE buffer into electrophoresis tank. Remove comb(s) and dividers. Use 100-150 ml 1X TAE.

4. Using the Human Genomic DNA, prepare 400 µl of a 100 ng/µl stock concentration. From this stock, prepare 50, 25, 10, and 5 ng/µl concentrations as follows:

Target Concetration	Quantity of 100 ng/ml stock (µl)	Quantity of TE (µl)
50 (ng/µl)	50	50
25 (ng/µl)	25	75
10 (ng/µl)	10	90
5 (ng/µl)	5	95

Store this standard series frozen for up to one year.

5. With the gel submerged, (do not perforate the bottom of the gel with the pipette tip) load the known DNA concentration standards in lanes 1 through 6. Mix 2 µl loading buffer with 2 µl of the 100 ng/ml standard; load into lane 1. Then mix 2 µl of loading buffer with 1 µl of each of the 100, 50, 25, 10, and 5 ng/ml standards and load into lanes 2 through 6. This will result in the addition of DNA in the following amounts: ~200 ng to lane 1, ~100 ng to lane 2, ~50 ng to lane 3, ~25 ng to lane 4, ~10 ng to lane 5, and ~5 ng to lane 6.

6. For each sample, mix 1 µl of extract DNA and with 2 µl loading buffer. Load the 3 µl of combined samples and loading buffer to their respective wells.

7. Set the voltage at 200 volts. For 6 x 8.3 cm gels, approximately 8 minutes is needed.

8. Remove the gel from the tank. Stain the DNA by placing gel in box containing 1X TAE buffer supplemented with ethidium bromide. Staining times will vary; staining with fresh solutions may be accomplished in 1-2 minutes, while older solutions may require 20 minutes of staining. The gel may be destained with 1X TAE to reduce background stain.

9. Examine the gel with UV transilluminator and photograph. Do not expose yourself to the UV light for an excessive amount of time. Always use the appropriate protective devices when working with the transilluminator.

10. Detection of ethidium bromide stained gels may also be achieved using the FMBIO II in a manner similar for product gel DNA detection. Refer to Section 3.8 for scanning parameters and image evaluation guidelines.

INTERPRETATION

1. Assess the quantity and quality of DNA in test specimens by comparison with the DNA standards, thus estimating the concentration of DNA.

2. For further quantitation of each sample, make appropriate dilutions with NFW to yield concentrations suitable for slot blot quantitation and/or amplification.

REFERENCES

1. Current Protocols in Molecular Biology. 1996. 3 vols. Ausubel, *et al.*, eds. John Wiley & Sons, Inc., USA.

APPENDIX D

3.6 HUMAN DNA QUANTITATION (QUANTIBLOT)

PRINCIPLE

Quantiblot analysis is a method for human DNA quantitation. Known quantities of DNA and sample extracted DNA are bound to a membrane and the DNA can then be detected. This colorimetric procedure is performed by using a biotin labeled primate-specific probe (D17Z1). This assay requires the addition of an enzyme conjugate (streptavidin-horseradish peroxidase) to the DNA, which will result in the streptavidin binding with a high affinity to the biotin. After the addition of the enzyme conjugate the complex can be observed by adding a chromogen, which is acted upon by the horseradish peroxidase (HRP) to change it from a colorless to a colored product. Quantitation of DNA in the sample extracts can then be compared to known quantities, and the amount of DNA in the sample can be estimated based on that comparison.

MATERIALS

Slot Blot Apparatus

Biodyne B Nylon Membrane (11 cm x 7.9 cm)
Pre-wetting Solution: 0.4N NaOH, 25 mM EDTA
Spotting Solution: 0.4N NaOH, 25mM EDTA, 0.00008% bromothymol blue
Wash Solution: 1.5X SSPE, 0.5% w/v SDS
1X Citrate Buffer: 0.1 M Sodium Citrate, pH 5.0
Color Development Solution: Citrate Buffer, Chromogen, 3% H_2O_2

Hybridization Solution: 5X SSPE, 0.5% w/v SDS

Enzyme Conjugate: Streptavidin-Horse Radish Peroxidase

Type 1 water
Hybridization boxes
Human DNA Quantitation Probe D17Z1
Quantiblot® Standards A-G or alternate standards
Quantiblot® Calibrator 1 and 2

PROCEDURE

Preparation of Standard Series

1. Vortex the DNA Standard A to mix it thoroughly. Label seven microcentrifuge tubes A through G.

2. Place 120 µl of the DNA Standard A into the tube labeled A. Aliquot 60 µl of TE buffer into each of the six remaining tubes (B through G).

3. Add 60 µl of DNA Standard A (from tube A) into tube B. Vortex to mix. This is considered DNA Standard B.

4. Add 60 µl of the DNA Standard B to tube C. Vortex to mix. This is considered DNA Standard C.

5. Add 60 µl of diluted DNA Standard C (tube C) to tube D. Vortex to mix. Continue the serial dilution through tube G. This will create the following standard series which can be stored for three months at 2-8°C:

DNA Standard	Concentration (ng/µl)	Quantity DNA per 5 µl (ng)
A	2	10
B	1	5
C	0.5	2.5
D	0.25	1.25
E	0.125	0.625
F	0.0625	0.3125
G	0.03125	0.15625

Alternate Standard Series Preparation

1. Alternatively, prepare a series of standards using Promega human DNA mixture stock. Be sure to vortex solutions before opening and after preparing dilutions or mixtures. Begin by preparing a 10 ng/µl standard by diluting the concentrated DNA in TE. From this 10 ng/µl stock, prepare a 1 ng/µl standard by adding 15µl of the 10 ng/µl stock to 135µl TE. Mix well. This standard may then be used to prepare other standards.

2. Label seven microcentrifuge tubes A through G.

3. In tube A, mix 60 µl of the 1ng/µl standard with 20 µl TE.

   In tube B, mix 50 µl of the 1ng/µl standard with 50 µl TE.

   In tube C, mix 25 µl of the 1ng/µl standard with 75 µl TE.

   In tube D, mix 10 µl of the 1ng/µl standard with 90 µl TE.

4. Vortex tubes A through D to mix.

5. In tube E, mix 10 µl the mixture from tube A with 90 µl TE.

   In tube F, mix 10 µl the mixture from tube B with 90 µl TE.

   In tube G, mix 10 µl the mixture from tube C with 90 µl TE.

6. This will create the following standard series which can be stored for three months at 2-8°C:

DNA Standard	Concentration (ng/µl)	Quantity DNA per 10 µl (ng)
A	0.75	7.5
B	0.5	5
C	0.25	2.5
D	0.10	1
E	0.05	0.5
F	0.025	0.25
G	0.01	0.1

Blotting

1. Pre-warm the hybridization solution (~150 ml is required) and the wash solution (~450 ml is required) to 50°C.

2. Determine the number of samples to be analyzed. This should include the seven DNA Standards (A through G) and the DNA Calibrators (1 and 2). RNCs need not be analyzed on the blot. Aliquot 150 µl of spotting solution to each sample tube or well.

3. Mix DNA Standards and samples thoroughly. Add 5 µl of each standard (or 10 µl of each standard if the alternate standard series is employed) to the appropriate tube or well. Add 5 µl of each calibrator to the appropriate tube (this is omitted if the alternate standard series is used). For samples, place 1 µl of each DNA

sample in the corresponding tube containing spotting solution. Note that different quantities of standards or samples may be added to the spotting solution at the discretion of the analyst.

4. Mark the Biodyne B nylon membrane for orientation. Place the membrane into a tray containing 50 ml of pre-wetting solution. Incubate at room temperature for at least one minute, but no longer than 30 minutes.

5. Disassemble and wash the slot blot apparatus with regular water, followed by type 1water prior to use. Rinse the rubber gasket as well. Assemble the base of the blot apparatus, pushing the gasket firmly into the base plate.

6. Remove the membrane from the pre-wetting solution and place it centrally over the slots of the gasket. Place the top plate on the apparatus. Turn the unit clamp knob to ON and the sample vacuum knob to OFF. Turn on the vacuum source and apply pressure to the top plate until the vacuum pup intake pressure reads approximately 10 Hg. Slowly turn the vacuum knob to ON, and then back to OFF, thus securing the apparatus seal.

7. Apply the samples to the wells in the slot blot apparatus according to the designated positions on the slot blot worksheet. Eject samples rapidly to prevent entrapment of bubbles. Avoid touching the pipet tip to the membrane. Turn the sample knob to the ON position very slightly until air movement is heard. Leave the knob on for 30 seconds to 5 minutes. If a bubble is trapped and a sample is not drawn through, pipet the sample up and reapply in order to remove air bubbles.

8. Turn the clamp to the RELEASE position while the sample knob remains in the ON position. Remove the top plate, and remove the membrane and immediately place it in a box containing ~100 ml of pre-warmed hybridization solution. Add 5 ml 30% hydrogen peroxide. Cover and rotate at 50°C for 15 minutes.

9. Turn the vacuum and the sample knob to OFF. Disassemble and wash the slot blot apparatus thoroughly, using mild SDS solution followed by several rinses, the last of which should be done with high quality water. The plates and seal should not be soaked for extended periods of time. Brushes, alcohols, or other harsh chemicals should not be used to clean the plates or gasket. Allow apparatus to air dry (do not bake or force dry). After drying, store the apparatus in the case provided by the manufacturer. Periodically it may become necessary to disassemble the base plate by removing head-cap screws for cleaning.

Hybridization

1. Add 20 µl of the QuantiBlot D17Z1 probe to 30 ml of pre-warmed hybridization solution. Decant the pre-hybe and pour the hybridization solution onto the membrane. Cover and rotate at 50°C for 20 minutes.

2. Decant the hybridization solution and rinse the membrane briefly with 100 ml of pre-warmed wash solution. Rock for several seconds. Decant wash.

Stringent Wash/Conjugation

1. Mix 30 ml of the pre-warmed wash solution with 180 µl of enzyme conjugate:HRP-SA. Add this to the tray containing the membrane. Cover and rotate at 50°C for 10 minutes.

2. Decant enzyme solution and rinse membrane thoroughly for 1 minute in 100 ml of pre-warmed wash solution at room temperature for 1 minute. Decant and repeat with another 1 minute rinse.

3. Decant the second rinse and wash the membrane with a final 100 ml of pre-warmed wash solution. Cover and rotate at room temperature for 15 minutes. During this wait, observe colorimetric detection step #1.

4. Decant the wash solution and rinse the membrane briefly in 100 ml citrate buffer.

Colorimetric Detection

1. When within 10 minutes of the citrate buffer rinse (i.e. during the final room temperature wash), prepare the color development solution. Add the following reagents in the order listed into a glass flask and mix by swirling. Do not vortex. To 30 ml of citrate buffer, add 1.5 ml of Chromogen:TMB solution and 3 µL of 30% hydrogen peroxide.

2. Decant citrate buffer rinse and add the color development solution to the membrane. Cover and protect from direct light. Rotate at room temperature for 20-30 minutes. Check the color development and continue to rotate if the desired level of development has not been achieved. Alternatively, proceed to step 3 or 4, depending on observations made at this juncture.

3. Steps 1 and 2 of the colorimetric detection may be repeated if further band enhancement is desired. A half batch of development solution is suitable for the repeat process.

4. Remove from shaker and decant the color development solution. Stop the reaction by washing the membrane with 100 ml type 1 water.

5. Photograph or scan while wet. The blue color will fade when the membrane begins to dry. The membrane may be placed between protective sheet covers while documenting results.

INTERPRETATION

1. Assess the overall quality of the blot. Standard series should exhibit progressive intensity increases as DNA quantity increases. If loaded in duplicate, standard series should have the same general appearance across the blot. Occasional loading error may render one of the known quantities inappropriate for quantification purposes. Should this occur, the duplicate well for that quantity may be used. If duplicates were not loaded, extrapolation between suitable standards may be employed.

2. With respect to calibrators, bands should fall between the expected standards in the series. If calibrators fall out of the expected window, the analyst should evaluate the possibility of a standard series error and proceed as described previously. If neither calibrator is within the expected window, the standards appear satisfactory, and the discrepancy cannot be explained by noted loading error, then the blot should be repeated.

3. Should all controls perform as expected, the quantity of DNA in test specimens may then be compared with the DNA standards, thus estimating the concentration of DNA.

4. For samples exhibiting quantities of DNA less than that of the least standard quantity loaded in the standard series (generally <0.1 ng), these sample retentates should be incorporated into the PCR reaction in their entirety. If samples exhibit quantities of DNA exceeding that of the greatest standard quantity loaded in the standard series (generally >7.5 ng), the analyst should evaluate if extrapolation is possible. If not, and the sample is of question origin, the sample should be further diluted and re-blotted.

5. Following the calculation of DNA quantity in each sample, make appropriate dilutions with NFW to yield concentrations suitable for amplification. Dilutions

prepared for incorporation into the amplification reaction may be discarded following amp set-up. Any original retentate remaining from casework samples are kept for long term storage and should eventually be stored frozen or concentrated to a pellet using the centrivap and then stored frozen.

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APPENDIX E

3.15 PCR AMPLIFICATION OF SHORT TANDEM REPEATS USING POWERPLEX® 16 CE

PRINCIPLE

PowerPlex® 16 allows the coamplification and three-color detection of sixteen loci (fifteen STR loci and Amelogenin). The loci included in this system satisfy the needs of the FBI for obtaining profiles over the 13 CODIS loci and includes two highly polymorphic pentanucleotide repeat loci which add significantly to the discrimination power of the system. The purpose of the amplification set-up is to prepare a polymerase chain reaction mixture. Key components of the reaction mix includes template DNA (DNA from each of your extracts), a buffer solution containing BSA, dNTPs and magnesium chloride, short-tandem repeat (STR) primer pairs and *Taq*Gold DNA Polymerase. With this reaction mix, particular segments of template DNA are copied multiple times and amplification accomplished. The PowerPlex® 16 System allows co-amplification of the following loci: Penta E, D18S51, D21S11, TH01, D3S1358, FGA, TPOX, D8S1179, vWA, Amelogenin, PentaD, CSF1PO, D16S539, D7S820, D13S317, and D5S818.

MATERIALS

Gold ST*R 10X buffer
PowerPlex® 16 Multiplex 10X Primer Pair
AmpliTaq Gold DNA Polymerase
Nuclease-free water
9947A DNA (diluted to 0.5 mg/ml)
Aerosol resistant pipet tips
Sterile thin-walled 0.2 ml amplification tubes
GeneAmp™ PCR System 2400 or 9700 Thermal Cycler

WORKSHEET

PCR Amplification Setup

PROCEDURE

Notes: This protocol was adopted/summarized from the GenePrint™ PowerPlex® 16 System Technical Manual (Promega Corporation, Madison, WI, Part#TMD012, revised 10/02) and may be considered in accordance with the manufacturer's recommended protocol.

Use the dedicated amplification setup area and supplies for Steps 1-9. Universal precautions regarding personal protection equipment and biohazardous material handling must be employed at all times when conducting this analysis. Change gloves frequently. Use only aerosol barrier tips and change tip with each volume

transfer. The thermal cycler should be turned on at least ten minutes before loading the reaction tubes.

1. Bring samples to room temperature. Be sure all samples are thoroughly mixed prior to pipetting any volume; quick-spin if necessary.

2. Heat Gold ST*R 10X buffer at 37° for 5-10 minutes and vortex to mix thoroughly. Thaw and mix primer pairs by vortexing 5-10 seconds. Extensive high speed centrifugation of these components should not be performed following mixing.

3. Determine the number of reactions to be set up, including extraction and amplification positive/negative controls. Place one sterile 0.2 ml reaction tube for each reaction into a rack and label.

4. Determine the dilution of each sample to be amplified in a total reaction volume of 25 µl; 10 µl of which is PCR master mix, 15 µl of which is template and NFW. Note that if template DNA is stored in TE buffer, the volume of the DNA sample added should not exceed 20% of the final reaction volume. The appropriate amount of water is then added to each pre-labeled sample tube.

   Samples/IPCs: Add DNA extract (approximately 0.5-1.5 ng, based on DNA quantitation methods) to the respective reaction tube. Adjust volume to 15 µl with nuclease-free water.
   Reagent Negative Control: Add 15 µl RNC (or the amount equivalent to that of the sample in the extraction set that has been least diluted by volume) to the respective reaction tube.
   Positive Amplification Control: Add 14 µl nuclease-free water and 1.0 µl 9947A DNA (diluted to 0.5 ng/µl) to the respective reaction tube. Other quantities of the 9947A DNA may be used as determined through general lab observation with any given commercially obtained aliquot.
   Negative Amplification Control: Add 15 µl nuclease-free water to the respective reaction tube.

5. Use the following table to calculate the required amount of each component for the PCR master mix. Once the total number of samples to be amplified has been determined, add one or two extra reactions to compensate for pipetting variation in order to determine the "x Number of Reactions" that will be used to calculate final volume for each component.

PCR Master Mix Component	Volume Per Sample	x Number of Reactions	= Final Volume (µl)
Nuclease-free water	4.2 µl		
Gold ST*R 10X Buffer	2.5 µl		
PP16 Primer Mix	2.5 µl		
AmpliTaq Gold DNA Polymerase	0.8 µl		
Total Volume	**10.0 µl**		

6. Prepare the master mix in a sterile microcentrifuge tube (amber if available) in the order listed above. Mix thoroughly. Add 10 µl PCR master mix to each reaction tube.

7. Add the determined amount of template DNA to each pre-labeled reaction tube. Mix each tube gently and quick-spin. It is important to begin amplification within 20 minutes after addition of master mix to reduce possible PCR artifacts.

   Note: Use a dedicated area for amplification (Steps 9-22). All equipment and supplies should be dedicated to this area and should not be used for any other procedures. In addition, wear clean disposable gloves and a dedicated lab coat in the amplification area. Remove gloves and coat prior to leaving the area. Do not allow any supplies to return to the other laboratories without decontamination.

8. Assemble the 0.2 ml reaction tubes containing the DNA reaction mix and sample into a thermal cycler tray. Record the position of the samples.

9. Place the tray holding the tubes onto the thermal cycler block such that the tubes fit into the wells. The trays are numbered and may be used as a reference when recording the well placement of the tubes on the worksheet. Reaction tubes may also be placed in the thermal cycler without being added to a thermal cycler tray.

10. Slide the heated cover forward and pull the lever down.

11. From the Main menu, select "User" of the thermal cycler by pressing the **F5-User** key. Highlight the appropriate name using the arrow keys and press **F1-Accept.** This returns the screen to the Main menu.

12. Press the **F1-Run** key from the Main menu. This will take you to the Stored Methods Screen.

13. The recommended cycling protocol for the PowerPlex 16 System has been programmed into each thermal cycler and named "16 Protocol". It is as follows:

16 Protocol (2400):

95°C for 11 minutes
96°C for 1 minute, then:

ramp 100% to 94°C for 30 seconds
ramp 100% to 60°C for 30 seconds
ramp 23% to 70°C for 45 seconds
For **10 cycles**, then:

ramp 100% to 90°C for 30 seconds
ramp 100% to 60°C for 30 seconds
ramp 23% to 70°C for 45 seconds
For **22 cycles**, then:

60°C for 30 minutes
4°C soak

16 Protocol (9700):

95°C for 11 minutes
96°C for 1 minute, then:

ramp 100% to 94°C for 30 seconds
ramp 29% to 60°C for 30 seconds
ramp 23% to 70°C for 45 seconds
For **10 cycles**, then:

ramp 100% to 90°C for 30 seconds
ramp 29% to 60°C for 30 seconds
ramp 23% to 70°C for 45 seconds
For **22 cycles**, then:

60°C for 30 minutes
4°C soak

14. Using the arrow keys, select 16 Protocol from the Stored Methods screen by moving the highlight box to "16 Protocol" listed on the screen. Press **F1-Start.**

15. To view the parameters of the method before running, press **F2-View** instead of **F1-Start** on the Stored Methods screen. After reviewing the method, it may be

run by pressing the **F1-Start**, or it may be canceled by pressing **F5-Cancel.** Canceling will return the screen to the Stored Methods screen.

16. Once the analyst has pressed **F1-Start**, the Reaction Volume screen will be displayed. Press **F1-Start** if the reaction volume displayed is 25 µl. If the displayed reaction volume is different from the 25 µl reaction volume, enter 25 µl in the "Reaction Volume" field by clearing an entry with the **CE** key and entering numbers with the numeric keys. When the volume is entered, press **F1-Start.**

17. The method will start running when the heated cover reaches 103°C. At this time, the Run Time screen is displayed. This allows the analyst to chart the progress of the run at any time during the run. The flashing line denotes the current temperature and hold time or if the temperature is being ramped.

18. From the Run Time screen, the analyst may view the method information by pressing **F4-Info,** pause a run by pressing **F1-Pause,** or stop a run by pressing the **Stop** key. The run may be resumed by pressing **F1-Resume.** However, it is not recommended that a run be paused or stopped prior to completion of the program.

19. The PCR amplification program is complete in approximately 3 hours. However, once the program is complete, the samples are held at 4°C until stopped. This allows the analyst to start the program in the afternoon and stop the program the next day.

20. Once the program has reached the last step (4°C), the program may be stopped by pressing the **Stop** key twice. To review the history of the run, press **F1-Hist.** To exit the Stop Run screen or the History file screen, press **F5-Exit** or **F5-Cancel**, respectively.

21. After the amplification process, remove the sample tubes and turn off the thermal cycler. Proceed with electrophoresis. If electrophoresis will not be done at this time, store the samples 4°C or lower. The storage of amplified products **must be separate** from storage of pre-amp reagents or unamplified products. Proceed with a product gel and/or electrophoresis at this time.

FURTHER INFORMATION

If the analyst needs to perform other functions on the thermal cycler (i.e. creating / editing stored methods, adding / deleting users, etc.), refer to the GeneAmp™ PCR System Users' Manuals, which are kept in the PCR room.

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