University of Strathclyde

Department of Pure and Applied Chemistry

Investigation into Methods for Recovery and STR Typing of Limited Quantity and Membrane Bound Forensic Samples

By

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ABSTRACT

Challenging samples presented to the casework laboratory drive advancements in technology for generating DNA profiles from specimens of limited quantity and quality. This thesis describes the sensitivity limitations of current typing platforms and explores recent advancements in technology aimed at generating DNA profiles from difficult samples; this potentially could include DNA preserved on archived membranes from RFLP analyses conducted on cases in the past. Methods investigated include the utility of the GenomiPhi™ whole genome amplification (WGA) kit, PCRboost™ enhancement additive, Restorase® DNA polymerase repair kit, and alternate sample processing (cryogenic pulverization) and extraction (paramagnetic) methods. During the course of these studies, the premise of this thesis was shifted to the usefulness of these methodologies in a notorious serial murder case under investigation in the United States known as the BTK (bind, torture, kill) crimes. The ongoing investigation of this prolific serial killer evolved into the systematic development of a procedure to recover DNA from archived RFLP membranes for subsequent STR PCR typing. Initial studies revealed that the WGA, PCRboost™, Restorase® DNA polymerase, cryogenic pulverization and paramagnetic extraction would offer limited advantage over already validated processes. Therefore, the investigation progressed to testing numerous extraction buffers for removal of DNA from nylon. Lanes excised from non-irradiated membranes had slightly higher yields than cross-linked membrane lanes, while cross-linked lane yields varied between labs. This indicates storage conditions may play a larger role in recovery potential than initially suspected. Anecdotal accounts suggest the existence of other cases where the generation of CODIS compatible profiles from RFLP membranes may be useful. Therefore, demonstrating success in achieving partial STR profiles from archived RFLP membranes opens another possibility in the investigation and prosecution of cold cases.

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1.0
Introduction

1.1 Overview of DNA Typing

The work reported here concerns the analysis of human DNA in the context of special circumstances concerning the linkage of contemporary crimes and old unsolved crimes. While this is a specialist application, it is likely that the same circumstances will apply to a number of very serious crimes. This chapter serves to introduce the scientific principles of DNA which serve as the basis for forensic testing and will explain the progression of forensic procedures that has occurred throughout the last several decades.

1.1.1 DNA Structure and Function

Nucleic acids are made up of nucleotides consisting of a five-carbon sugar (pentose), one or more phosphate groups, and a nitrogenous basic compound simply referred to as a base [Horton *et. al*., 2002]. The sugar residue is deoxyribose, which joins with pyrimidine or purine bases; see Figure 1.1. A 3'-5' phosphodiester linkage of the nucleotide residues form polymers [Horton *et. al*., 2002]. Single polymer strands of DNA bind together by hydrogen bonding that occurs between the pyrimidines (thymine and cytosine) and purines (adenine and guanine) to form double stranded structures [Watson *et. al*., 2004]. This double stranded structure was proposed by Watson and Crick in 1953; a present day illustration is presented in Figure 1.1 [Watson and Crick, 1953; Watson *et. al*., 2004].

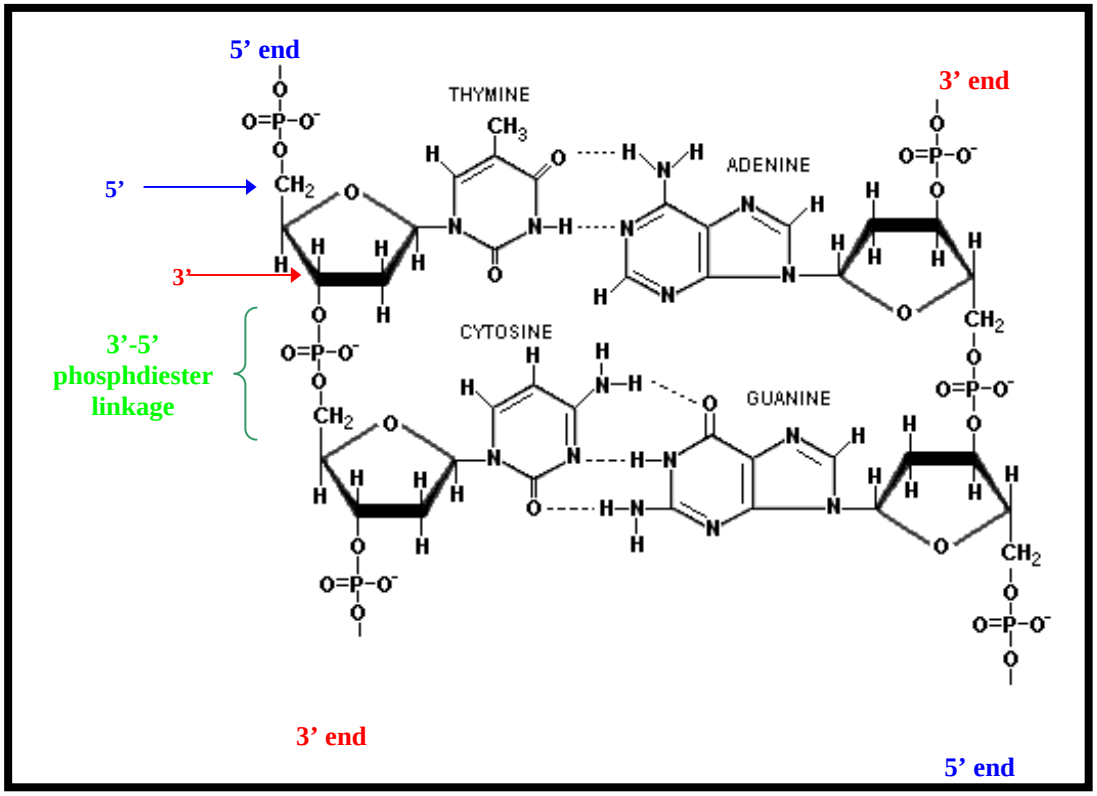


Figure 1.1: Double stranded DNA. The figure illustrates the chemical structure of double stranded DNA; the strands run in opposite directions. Directionality is noted as 5' or 3' based on the hydroxyl groups of the deoxyribose unit that can be phosphorylated; these are indicated by the arrows. The brackets indicate the phosphodiester linkage between nucleotide units, and dashed lines indicate the hydrogen bonds forming base pairs between purines and pyrimidines (thymine to adenine or cytosine to guanine). The covalent interactions are the basis for formation of the double stranded unit of DNA [adapted from Horton *et. al*., 2002; Watson *et. al*., 2004].

There are 3.08 billion base pairs in a single copy of the human genome, and nuclear DNA is located in the nucleus of nearly all cells of the human body [International Human Genome Sequencing Consortium, 2004]. It is nuclear DNA that is most commonly used for identity testing but other types of DNA associated with the various cellular structures also exists [Butler, 2001]. The DNA is packaged into twenty-two matched pairs of autosomal chromosomes and one pair of sex chromosomes. They exist in a diploid state in somatic cells. Germ cells – egg and sperm cells – are haploid in nature, carrying only a single copy of genetic material [Butler, 2001]. When a sperm cell unites with an egg cell, it results in genetic recombination and gives rise to the first cell of the offspring.

1.1.2 DNA Damage and Modification

DNA is one of few macromolecules for which biological systems possess repair systems. This is probably due to the detrimental effect of damaged DNA on the survival of the organism [Horton *et. al*., 2002]. Detrimental changes to nucleic acids can occur within the cell spontaneously and the modifications can complicate the characterization of DNA for forensic applications. Many of the damage mechanisms can be mimicked and induced in the laboratory via chemical treatment or introduction of enzymes that catalyze the alterations. Two of the most common chemical reactions that result in damage of DNA within the cell are hydrolytic in nature, consisting primarily of loss of adenine and guanine bases, termed depurination and/or conversion of cytosine to uracil, termed deamination [Hulbert, *et. al*., 2005]. These reactions are summarized in Figure 1.2.

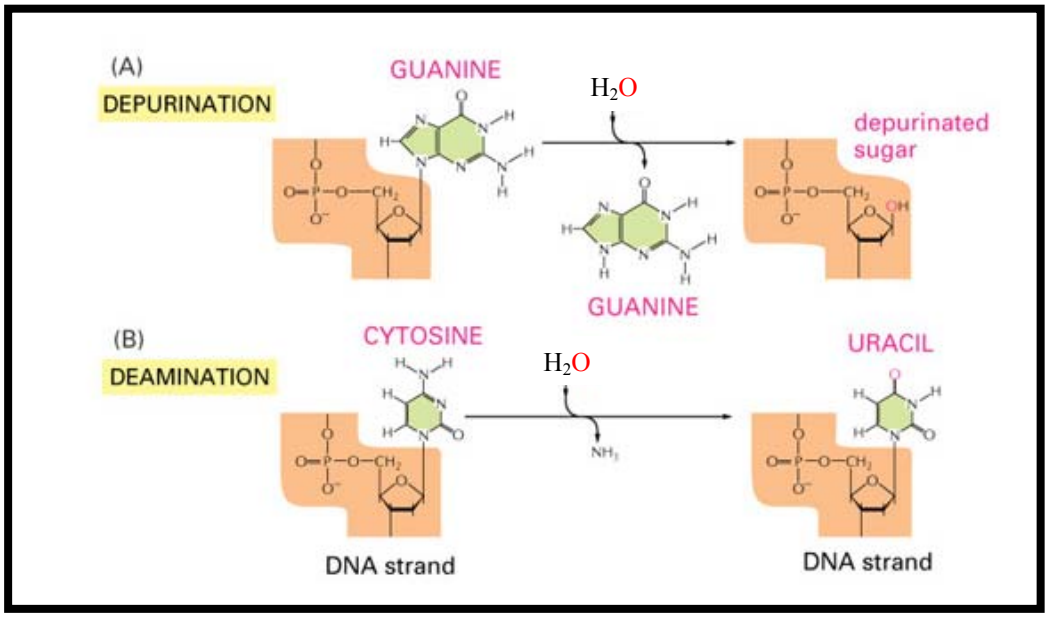


Figure 1.2: Hydrolytic damage of DNA. The figure illustrates the chemical mechanism by which depurination and deamination occur [adapted from Hulbert *et. al*., 2005 (T1Dbase)].

While the cells do possess mechanisms for repair following these reactions, if such damage escapes repair and accumulates this could possibly result in a non-functional translation product. Also critical could be the structural changes imparted upon the DNA since absence of bases and mismatched pairs will inhibit polymerases from replicating the damaged strands. Of particular interest are the structural modifications

known to prevent replication caused by photodimerization, where ultraviolet light promotes covalent linkage of adjacent pyrimidine bases [Hulbert *et. al*., 2005]. Illustrated in Figure 1.3 is an example of thymine dimerization that severely distorts the structure of DNA.

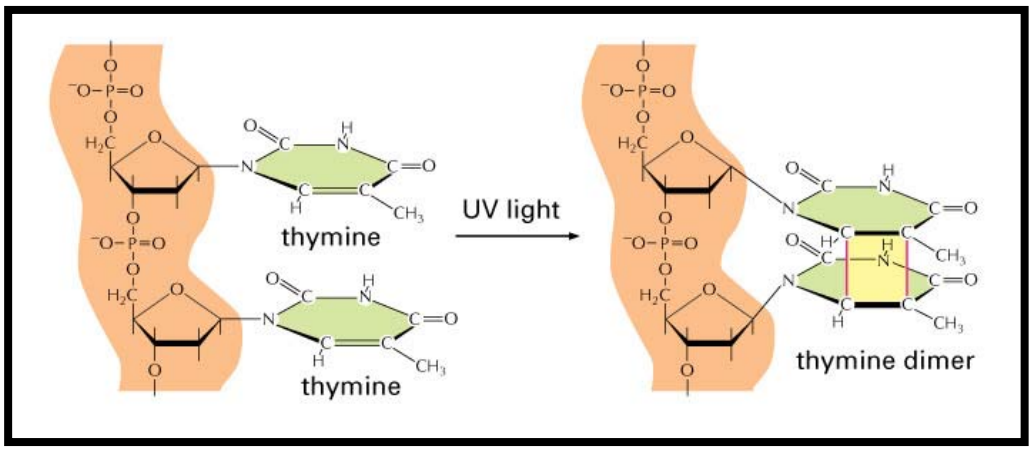


Figure 1.3: Photodimerization of DNA. The figure illustrates the structural changes induced by ultraviolet light due to covalent binding of neighboring pyrimidines to form thymine dimers [adapted from Hulbert, *et. al.*, 2005 (T1Dbase)].

Aside from spontaneous reactions that occur within the organism, the risk of cell and nucleic acid damage once the cell is removed from the organism also exists. Once outside the organism, cells become subject to environmental insult harmful to the DNA harbored within. Basic processes such as photodimerization occur more rapidly and the strand distortion can become so extensive that eventually nicking of the DNA occurs and DNA fragment length is reduced [Horton *et. al*., 2002]. In the laboratory environment, DNA is relatively stable under alkaline conditions but susceptible to chemical (acid) and enzymatic (nuclease) hydrolysis [Horton *et. al*., 2002]. Both treatments can be detrimental to downstream typing but also may be exploited when manipulating DNA for molecular research. Strong acids at high temperatures will degrade the DNA into bases, phosphoric acid, and deoxyribose due to disruption of phosphate ester bonds and N-glycosidic bonds between the deoxyribose and bases [Alberts, *et. al*., 1994].

Single nucleotide damage can also occur by oxidation reaction. As indicated in Figure 1.4, there are several areas of the nucleic acid structure susceptible to free radical attack (reactive sites are indicated by arrows), which ultimately results in unstable or broken DNA complexes.

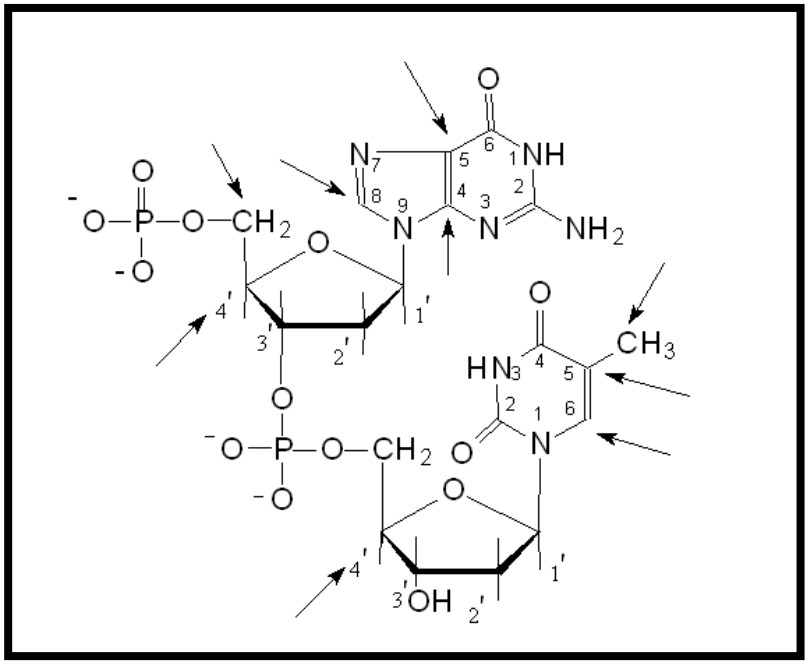


Figure 1.4: Hot spots for free radical attack on DNA. The figure illustrates the areas most conducive to free radical attack within the DNA strand. As shown by each area indicated with an arrow, free radicals can act upon the sugars or the bases of the complex [adapted from Hulbert *et. al*., 2005 (T1Dbase)].

As shown in the diagram, several aspects of the DNA complex are subject to free radical attack. Should the reaction occur on the purines or pyrimidines, the result may be oxidation or reduction and ultimately unstable base structure. More commonly, the attack can occur on the sugar backbone of the complex, resulting in dissociation of the C-O bond between ribose and phosphate at the 3' site of the sugar and this dissociation constitutes a strand break [Von Sonntag, 1987].

Many damage/repair studies conducted in the laboratory make use of enzymes known to hydrolyze the DNA. These are generally either exo- or endonucleases. Exonucleases cleave nucleotides from the 5' or 3' end of the molecule (dependent on specificity of the nuclease), and endonucleases cleave bonds within a DNA strand. They are usually either double- or single-strand specific [Horton, *et. al*., 2002]. It is

important to note that if damage occurs to one of the strands of DNA in the double helix, repair can be easily directed by the other strand, which serves as a template for replacing faulty areas. When both strands are severed (double-stranded damage), returning to the initial state by repair mechanisms may not be possible [Raynard *et. al.*, 2008]. While ligases can join double strand ends together, these are only useful when the breaks occurred such that overhangs exist and sequence recognition facilitates the strand ligation. However, blunt end breaks present special repair challenges since end re-joining would not necessarily result in the recovery of the original sequence order [Cromie *et. al*., 2001].

In addition to possible damage from environmental conditions, the substrate on which the samples are deposited can interfere with the purification of the DNA when the surfaces are not conducive to stain or cell removal or when they contain substances that inhibit purification, concentration, or amplification of the DNA present [Bourke *et. al*., 1999]. For example, some dyes are known to intercalate between the bases of DNA and interfere with replication [Shutler *et. al*., 1999]. These factors, along with bacterial degradation of cellular material induced by a warm moist environment, present conditions unsuitable for stabilizing biological macromolecules. Alone or collectively, the damage mechanisms addressed here can affect the integrity of the DNA and, specifically in relation to this project, the alterations affect the ability to type forensic samples.

1.2 Development of Identity Testing

Human identity testing from body fluids or tissues for medico-legal purposes really began with the elucidation of the ABO blood types by Landsteiner in 1901; this represented genetic testing through gene expression products and the principle has been developed to encompass more immunologically detected marker systems and enzyme polymorphisms [as cited by Gaensslen, 1983]. While these marked an important step in forensic biology, the power of discrimination offered by these systems was still limited and further complicated by the fact that probative markers were not detected in all body fluids since expression was secretor-status dependent [as cited by Gaensslen, 1983].

Analysis of forensic samples based on characteristics of molecular DNA sequence and structure became a reality in the mid 1980's and has continued since with widely validated methods such as RFLP and multiplex STR-PCR typing. This section will provide foundational information on these two major typing strategies in an effort to address challenges with obtaining STR profiles from DNA previously analyzed using RFLP.

1.2.1 Development of RFLP technology

Analyses of the non-coding genome and applications for use in criminal investigations was reported by Alec Jeffreys in 1985-86. The technology, restriction fragment length polymorphism (RFLP) analysis, focused on the detection of variable number of tandem repeats (VNTRs) within the genome, which were hypervariable minisatellite regions. The core sequences range from approximately 8 to 80 base pairs in length but are repeated to comprise segments hundreds to thousands of base pairs in length [Lee and Timaday, 2003]. These initial developments in forensic DNA "fingerprinting" occurred during the same period that the polymerase chain reaction (PCR) methodology was first described for the amplification of DNA, but were based on blotting and hybridization probe methodologies [Jeffreys *et. al*., 1985a/b; Mullis and Faloona, 1987].

The first step in the RFLP process is to cut the DNA of interest with a restriction enzyme. Restriction endonucleases have been one of the most important discoveries for the advancement of recombinant DNA technology, making it possible to cut DNA at predictable sites based on sequence. The enzyme most commonly used to conduct the digest for RFLP applications is *Hae*III, isolated from the bacterium *Haemophilus aegyptius* [Horton *et. al*., 2002]. This enzyme has a four base pair recognition sequence and produces a blunt end cut. More specifically, the action of the enzyme is illustrated as "CC↓GG", because the recognition sequence is CCGG and the cut is made between the two central residues; this produces blunt end DNA fragments from the double stranded molecule. A restriction reaction is carried out for at least an hour, at a pH and temperature optimal for endonuclease action, after which a small portion

of the restriction product is visualized with a product gel to ensure a complete digest has occurred. After restriction cutting, the DNA fragments undergo separation by electrophoresis in a horizontal agarose gel. Once electrophoresis is complete, the gel may be stained with ethidium bromide for sample visualization or photographic documentation and is then soaked in a denaturation solution to produce single-stranded fragments. This is followed by a buffer treatment for pH neutralization. A static support, usually nylon membrane, is then placed on the surface of the gel and topped with absorbent materials that serve to wick the buffer through the gel, transferring the DNA from the gel surface to the membrane through capillary action. This method is referred to as Southern blotting [Southern, 1975]. Following the blotting procedure, which is usually assisted by interactions between the DNA and surface moieties present on the membrane, the fixation of DNA to the nylon is further achieved by baking and, depending on the properties of the membrane, ultraviolet irradiation [Leary *et. al*., 1983; Giusti *et. al*., 1989; Walsh *et. al*., 1992; Inman and Rudin, 1997; Davis, 1997b].

Once the DNA has been fixed to the support, the membrane then undergoes a series of probings. First, the membrane is pre-hybridized to block all sites not bound to DNA. Then probes, designed to match sequences that are characterized as highly polymorphic, are added to the hybridization solution so that sequence specific binding can occur. Once the excess probe is washed away, the probes are detected either by chemiluminescent or radioactive means, resulting in a banding pattern on an exposed piece of film referred to as an autoradiogram (or "autorad"). The bands present within each sample lane are sized in reference to a ladder and the resulting fragment length determinations represent the alleles possessed by the individual at any given locus [Inman and Rudin, 1997]. A diagram describing the RFLP analysis procedure is represented by Figure 1.5.

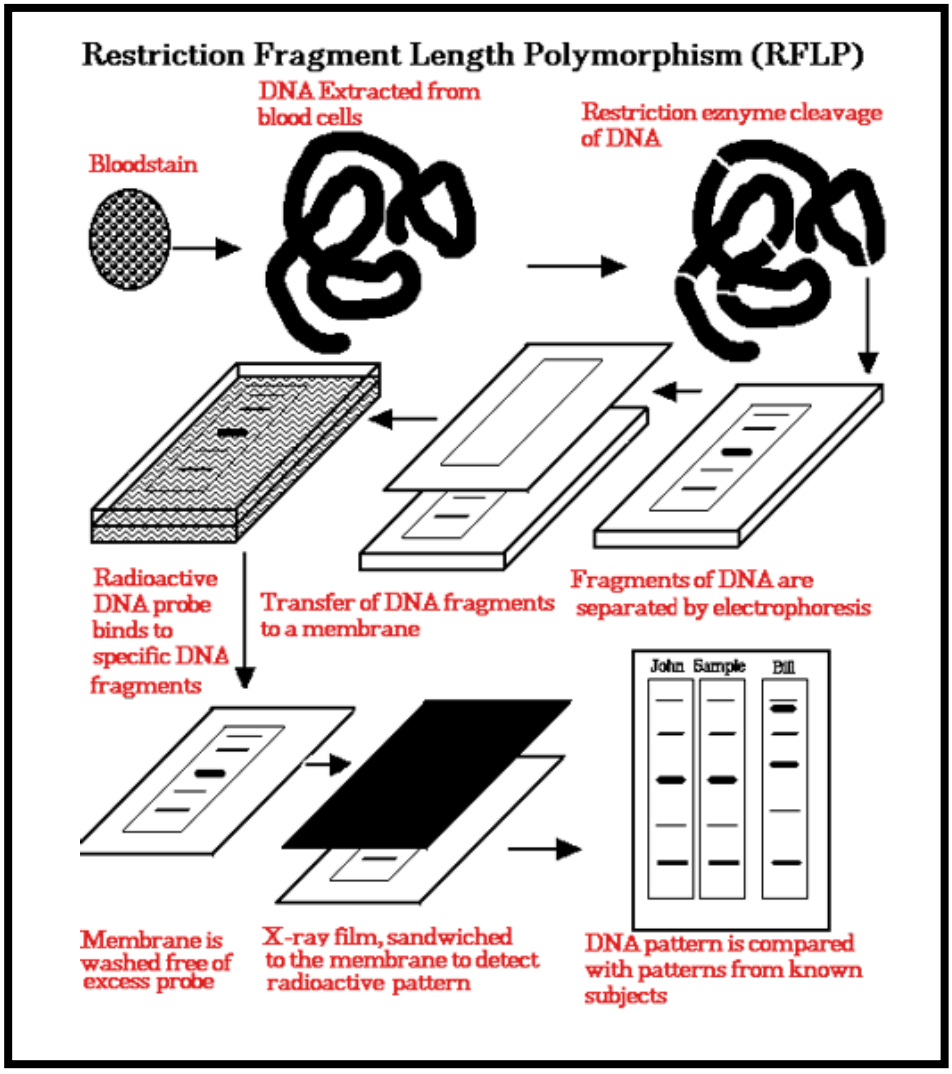


Figure 1.5: RFLP procedure overview. The figure illustrates the basic steps involved in the restriction fragment length polymorphism analytical process [image adapted from Santa Monica College, 2001].

As this technology was broadly adopted around the globe, a variety of static supports, hybridization systems, and stripping solutions were developed and compared for their ability to efficiently bind DNA so that single locus probes could be used and stripped from the membrane sequentially [Evett, 1991]. These systems were optimized for low background, efficient removal of previous probe, and minimal decrease in signal due to dissociated restriction fragments so that repetitive probings (usually four to six) could be conducted to create composite profiles. Of specific interest to this project

are the chemical properties of the various membranes employed for the analysis, the chemistry of which will be addressed later.

1.2.2 Development of Intermediate PCR Technologies

While the use of RFLP became widespread in crime laboratories, methods involving the use of PCR were rapidly being developed because amplification of DNA as a foundational strategy enabled analysis of smaller quantities of DNA. The process was described by K. Mullis [Mullis and Faloona, 1987] and relies upon cycling of the reaction temperature to achieve amplification and the cycle repetition has three basic steps (as shown in Figure 1.6) and has been well-established and described [Vierstraete, 1999; Saiki *et. al*., 1988]. The process is made possible by *Taq* polymerase, a thermostable enzyme, modified from that isolated from *Thermus aquaticus* [Brock and Freeze, 1969; Chien *et. al*., 1976]. 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.

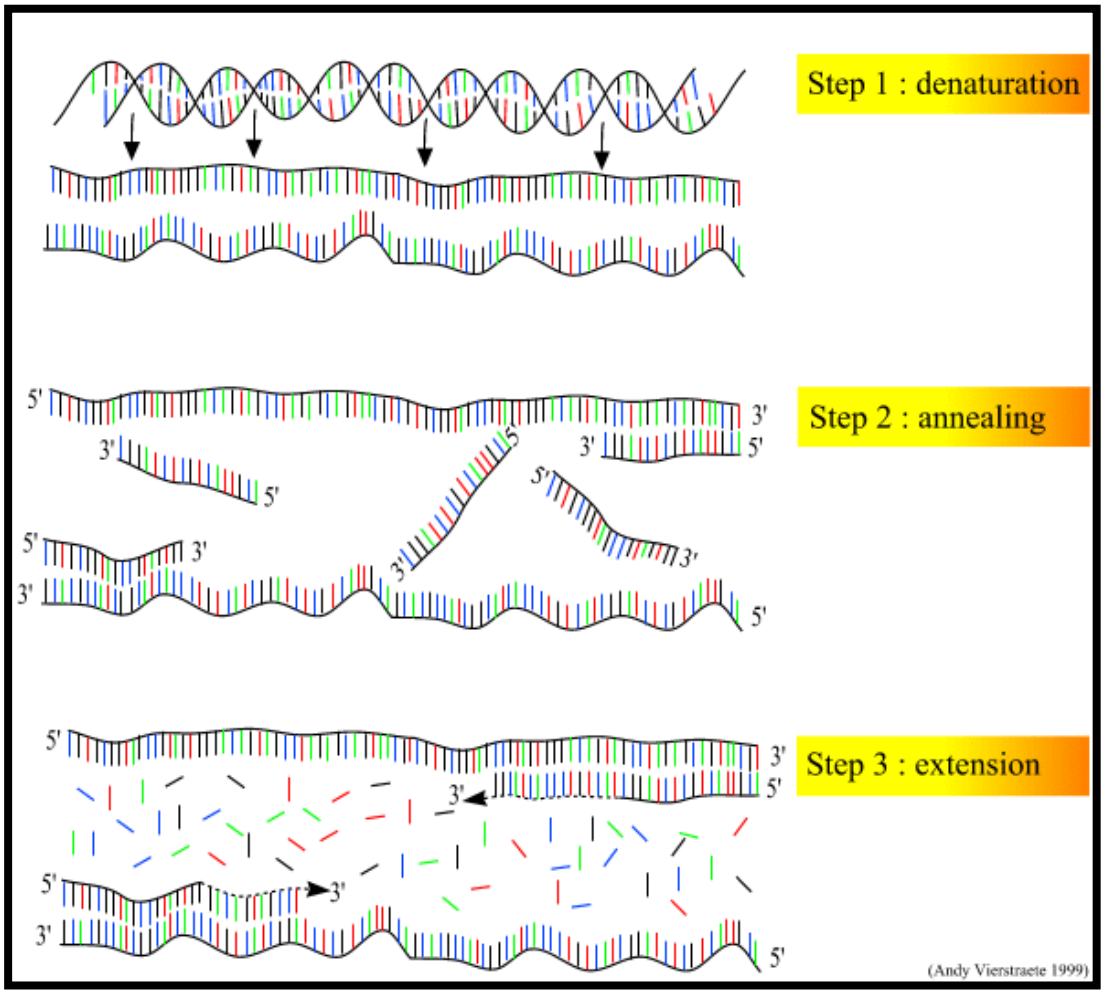


Figure 1.6: Theory of PCR. PCR is carried out by changing reaction temperature to achieve denaturation of template (94°C), annealing of primers (54°C), and extension of newly synthesized DNA (72°C). This figure illustrates these steps, with colored lines to indicate deoxynucleotide triphosphates (dNTPs) that are assembled to form base pairs of complimentary strand [image adapted from Vierstraete, 1999].

Following the description of the PCR amplification of DNA, different approaches to using it in forensic applications were developed. While these met with a certain success the current approach using the amplification of STR regions has prevailed.

1.2.3 Development of STR-PCR Technology

In 1991, Thomas Caskey of Baylor College of Medicine suggested that STRs could be used for forensic applications [Edwards, *et. al.*, 1991]. As previously noted, RFLP technology developed by Jeffreys had focused on the detection of VNTRs with core

sequences 8-80 base pairs in length. While also found in non-coding regions of the DNA, STRs now used for identity testing are four or five bases in length [Butler, 2001]. As with other PCR applications, 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 these regions [Butler, 2001; Jones, 2004]. Resulting PCR products can be separated by size and the product lengths reflect the variation in the number of STRs at a given locus [Butler, 2001]. The amplification products can be detected because the reaction primers, that become part of each newly synthesized strand, are tagged with fluorescent dyes. These are detected with laser-driven fluorescent scanning instrumentation [Butler, 2001]. In general terms, this instrumentation has either consisted of a horizontal electrophoresis gel with subsequent fluorescence detection or, more recently, capillary electrophoresis devices using multiple capillaries with fluorescence detectors at the ends of the capillaries.

The products range from approximately one hundred to five hundred base pairs (bp) [Promega 2001; Promega, 2008b-d]; this is much smaller than fragments detected using RFLP analysis developed by Jeffreys. STR typing is very sensitive and the PCR products are relatively short, so the technology is desirable for forensic applications since crime scene exhibits are often exposed to environmental insult resulting in degraded samples or often yield samples of extremely limited quantity [Jones, 2004]. Moreover, since the STR PCR products are of 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.

1.2.4 Other Relevant Forensic DNA Technologies

The terminal typing process applied throughout the course of this work will be multiplex STR PCR based. However, this is certainly not the most recent technology to emerge within the forensic community.

While autosomal multiplex kits are the most widely used technology in the United States and across the world for the individualization of forensic stains, there are other

specialized kits that have emerged in the last several years. For example, Y STR analysis has emerged in the United States in the last five to ten years and now exists as a prominent analysis conducted in most private labs and many government facilities. Studies in the Y chromosome first emerged with respect to geographic studies and relationship clustering [Hammer *et. al*., 1997; Zerjal *et. al*., 1997; Rolf *et. al*., 1998;]. Development of multiplex systems simultaneously became prevalent, as did database development and introduction of the technology into the courtroom [Prinz *et. al*., 1997a/b]. Meanwhile, efforts continued to expand the number of markers available to the community for typing [White, *et. al*., 1999].

Y STR systems are extremely useful in sexual assault cases where the difference in quantities of male and female DNA would not otherwise allow for the detection of the male component. While autosomal typing kits detect minor contributors down to approximately 10% contributorship, the ability of a Y STR PCR reaction to result in a single source male profile has been demonstrated from mixtures where there are amounts of female DNA hundreds of times greater than that of the male [Krenke, *et. al.*, 2005]. Other technologies for specific case types have also been developed in the last decade, and include kits that amplify shorter allele fragments since they have a greater chance of developing profiles from samples where the DNA is damaged and degraded. In 1994, scientists at the Forensic Science Service (FSS) concluded short fragment loci performed better for the typing of degraded samples from remains [Whitaker *et. al*., 1995]. Prompted by the World Trade Center disaster in 2001, the New York City Office of the Chief Medical Examiner (OCME) requested that efforts with short STRs be accelerated. It was through the OCME's discussions with John Butler at the National Institute of Standards and Technology (NIST), that the term "miniSTR" was born. This expedited initial work published by Butler in 2003 [Butler, *et. al*., 2003] and eventual production and validation of a commercially available miniSTR typing kit [Applied Biosystems, 2007b].

The Y STR and miniSTR technologies are PCR based techniques that examine very similar microsatellite regions of the DNA as those typed with autosomal kits. Very different technologies involving mitochondrial (mito) typing and evaluation of single

nucleotide polymorphisms (SNPs) also have established their own niches within the forensic biology community. While neither of these technologies was available at the host laboratory, the relevance of these applications should not go unmentioned in the course of this discussion.

A standard mitochondrial DNA sequence was reported in 1981 [Anderson *et. al.*, 1981], and nearly a decade later, differences in the sequence between populations [Horai and Hayasaka, 1990] prompted forensic interest. Mitochondrial DNA is maternally inherited, and while far less discriminatory than nuclear DNA typing, this is often the only typing successful for aged samples such as skeletal remains [Gill *et. al*., 1994; Hanni, *et. al*. 1995]. It is also used for database searching between maternal relatives and missing persons; since autosomal nuclear DNA profiles only share a portion of profile data, comparisons between relatives make database searches complicated, even at moderate stringency. Furthermore, due to the high copy number of mito DNA that exists in biological material, this typing is also performed on samples where nuclear DNA is unattainable, such as hair shafts [Wilson *et. al*., 1995].

Single nucleotide differences can also be used to differentiate between individuals and represent the most discriminating of sequence differences detectable on a molecular level. Forensic applications of SNPs were described in 1993 [Syvanen *et. al*., 1993] and have progressed to well validated and highly automated chip-based multi-SNP assays [Dixon, 2006; Musgrave-Brown, *et. al*., 2008]. While STRs remain the gold standard for DNA typing in most forensic labs, specialized applications of SNPs are currently allowing the scientist to produce information as specific as physical characteristics from forensic samples [Frudakis *et. al*., 2003].

1.3 Principles of Human DNA Quantification and Identity Testing

The purpose of this section is to introduce concepts related to quantification and STR PCR methods that will serve as the primary analytical tools for the evaluation of DNA samples related to the project.

In 1998, quality assurance standards set forth by the Federal Bureau of Investigation (FBI) required that forensic laboratories quantify the DNA extracted from samples using a human-specific quantification method [FBI, 1998]. Prior to that, yield gel quantification using agarose gels stained with intercalating dyes were used to estimate sample DNA quantities. High recovery filtration devices, such as the Microcon® centrifugal device, are often used in the forensic laboratory to purify and concentrate DNA samples. Such devices employ anisotropic, hydrophilic regenerated cellulose membranes to bind DNA; following a wash step, the device is inverted and the DNA captured by centrifugal force into a clean sample tube in some desired volume of water or buffer [Millipore, 2000]. A small quantity (1-2 μL) of this extract, also referred to as an eluate, can then be analyzed on either an agarose gel for total DNA yield or by slot blot or real-time quantitation for human-specific quantitation.

1.3.1 DNA Quantification using Agarose Gels

Agarose, derived from seaweed, consists of 1,3-linked b-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose [Labropoulos, 2002]. Following the process of dispersion, hydration, melting, and dissolution, the basic repeat unit forms a matrix upon cooling which allows separation of high molecular weight macromolecules. Known quantities of DNA and sample-extracted DNA are mixed with a dye/glycerine solution (such as bromophenol blue) and loaded into wells formed in an agarose gel [Ausubel *et. al*., 1996]. Upon application of current across the gel, the charged DNA molecules migrate through the agarose complex and become separated based on size. The gel can then be treated with ethidium bromide, an intercalating agent, and DNA fragments are then visible under UV light [Lodish *et. al*., 2004]. Samples of unknown quantity can be compared to known quantities loaded in neighboring wells to estimate the quantity of DNA recovered from any unknown sample. However, the lower end of detection for a standard agarose gel is approximately 2 ng, meaning samples of quantities less than this could not be quantified in this manner. Furthermore, while yield gels do provide information regarding sample quality (degradation), samples containing non-human DNA could not be estimated with any accuracy. Nevertheless, this method is used to estimate DNA quantities recovered from high yield samples prior to dilution for blot analysis and is also used to determine if restriction digestion

has reached completion (digested DNA appears as a smear throughout the lane of the gel due to the abundance of varying length fragments produced by digestion). Figure 1.7 contains results obtained from an agarose gel assay where digested DNA samples were analyzed.

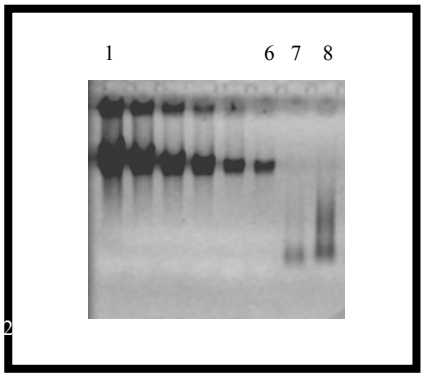


Figure 1.7: Product gel (1%) of digested/degraded DNA samples. Lanes 1-6 (left to right) contain the quantification standard series in the amounts of 200 ng, 100 ng, 50 ng, 25 ng, 10 ng, and 5 ng of DNA. Lanes 7-8 each contain 1 μL of degraded DNA extracts.

1.3.2 Human DNA Quantification with Slot Blot

Following implementation of the FBI standards, the forensic community moved toward the use of Southern blot based technology. A common human-specific DNA quantification kit marketed for the forensic science community is Applied Biosystem's QuantiBlot® Human DNA Quantitation Kit. Known quantities of DNA and sample-extracted DNA are bound to a charged nylon membrane and the DNA can then be detected using colorimetric detection. The detection is based upon DNA interaction with a biotin-labeled probe (D17Z1) that then binds with high affinity to the streptavidin-horseradish peroxidase. A colored complex is then observed by adding chromogen, which is acted upon by the horseradish peroxidase (HRP) to change it from a colorless to a colored product. Quantification of DNA in the sample extracts can then be achieved by sample comparison to known quantities and calibration standards supplied with the kit [Applied Biosystems, 2000b]. Figure 1.8 represents an example of results obtained from a slot blot.

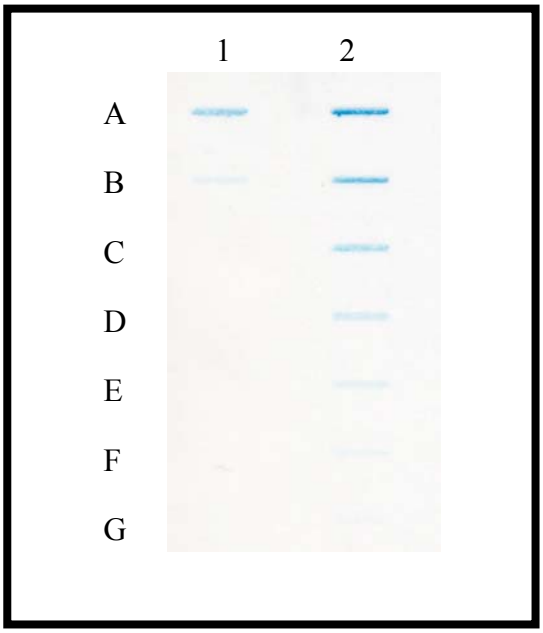


Figure 1.8: Colorimetric detection of human DNA by slot blot. Column 2 contains a human DNA standard series, with wells A through G representing 10, 5, 2.5, 1.25, 0.63, 0.31, and 0.15 ng of DNA per well, respectively. Column 1 contains 1 μL of DNA extracted from forensic samples. The intensities of the forensic sample wells (1A and 1B) can be compared to the standard series and quantity of DNA per μL approximated for the samples.

Slot blot analysis served as the standard quantification method for many years and was the primary method employed during these studies. This method does offer reasonable sensitivity and slot blot quantifications also are rarely subject to failure due to presence of foreign, proteinaceous, bacterial, or other non-human or non-DNA components.

1.3.3 Human DNA Quantification with Real Time PCR

While serving as the standard method in forensic labs for many years and used in relatively recent studies, the blot technology was recently being replaced by real time PCR (RT-PCR) methods also capable of human specific quantification. This technology became available during the later experimental aspects of this thesis. These assays allow for monitoring of PCR product during the amplification process, rather than the analysis of an end-point product after all cycles are completed [Logan *et. al.*, 2009]. This technology, like the predominant STR PCR systems, employs fluorescent detection; reporter dyes become more detectable as product accumulates

and the amount of fluorescence is recorded after each PCR cycle [Research Resource Center, 2005]. This method has many advantages over blot technology. Because the nature of the quantification mimics that of the STR PCR typing assay (both are PCR based), RT-PCR quantification will more accurately reflect the quantity of amplifiable DNA present in a sample. While slot blot techniques are hybridization based assays and not PCR assays, sometimes quantities of DNA obtained were not reflective of the amount of amplifiable DNA present due to sample degradation or the presence of inhibitors.

The commercially available line of real-time quantifications kits most widely used in the United States are manufactured by Applied Biosystems. The Quantifiler™ Kits are *Taq*Man®-based fluorescent assays which are human/higher primate specific and have a fairly large dynamic range of 0.023 to 50 ng [Applied Biosystems, 2005a; Handlesman, 2006]. The *Taq*Man® technology is considered the gold standard for real-time quantification because the patented design is based on a minor groove binding (MGB) probe that enhances the probe melting temperature (Tm) and allows for the use of shorter probes [Afonina *et. al*., 1997]. Furthermore, the technology employs a non-fluorescent quencher (NFQ) which decreases the amount of background in the reaction, thus increasing signal detection specificity and overall reaction precision/accuracy [Applied Biosystems, 2007c; Handlesman, 2006]. A diagram of the *Taq*Man® design comprises Figure 1.9.

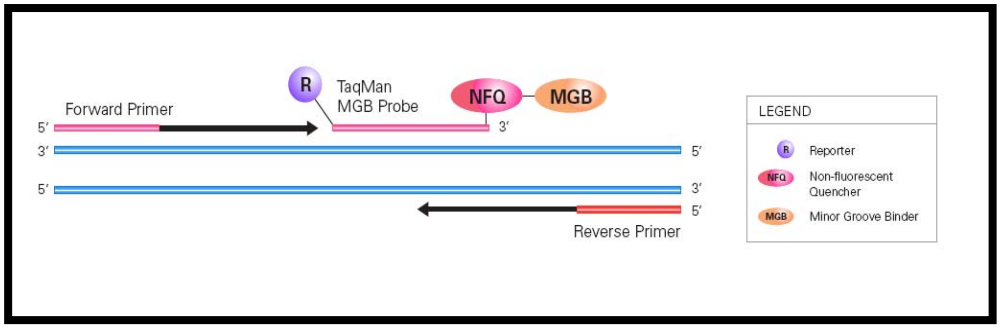


Figure 1.9: Schematic of *Taq*Man® probe technology. *Taq*Man® probes have a reporter dye at the 5' end and a minor groove binder and non-fluorescent quencher (MGB-NFQ) at the 3' end. The 5' nuclease activity of the DNA polymerase during primer extension cleaves fragments hybridized to the template (the probe), dissociating the energy transfer acceptor (NFQ) from the reporter, resulting in emission of detectable fluorescent signal [Applied Biosystems, 2007c].

Primer extension along the template dissociates the probe and signal results as the quencher is no longer in close proximity to the reporter. As product accumulates so does the signal [Lakowicz, 1983]. The Quantifiler™ Human system makes use of a target probe to an autosomal specific region (human telomerase reverse transcriptase gene); if the target-specific assay is for male DNA (Quantifiler™ Y), the target probe is directed to sex determining region Y gene [Handlesman, 2006]. Other target-specific assay components include primers for amplification of the human or male DNA and a *Taq*Man® probe for detecting amplified human or male target sequences. In either case, the target specific assay components are accompanied by internal positive control (IPC) assay components consisting of an IPC template DNA (synthetic sequence not found in nature), primers for amplifying the synthetic sequence, and one *Taq*Man® probe for detecting accumulation of the IPC PCR product [Applied Biosystems, 2006]. The positive control probe binds to a non-human DNA sequence which is included in the reaction mix; therefore amplification occurs in the presence or absence of other template and reflects the ability of PCR to take place in any given reaction well. The presence of inhibitors or competing template in large quantities results in lower IPC amplification efficiency and is useful for predicting appropriate template amounts for downstream multiplex amplifications [Handlesman, 2006]. A passive reference is also included in the primer mix, the level of which remains unchanged throughout the course of the amplification. The detection level of the passive reference dye may be used to indicate differences in

detection from well-to-well or run to run due to instrumental function or user variability (e.g., lamp strength or pipetting error).

Like the blot technology, real-time samples are compared to detection levels achieved for each point along a standard curve. As product accumulates, a threshold is defined within the geometric (exponential) phase of the amplification reaction, and this threshold is the signal intensity where data points are taken. The PCR cycle at which the amplification curve crosses the threshold value, or cycle threshold (Ct), is defined for each standard of DNA in the dilution series. The Ct values are used to formulate the standard curve against which forensic samples of unknown DNA quantities are compared and quantified [Handlesman, 2006; Logan *et. al*. 2009].

Since the introduction of the single target assay kits (human and Y), Applied Biosystems has released a dual quant kit capable of simultaneous detection of human and male DNA components (Quantifiler™ Duo). This has proven advantageous to labs conducting both autosomal and Y STR testing, as the ratio of total to male DNA can be evaluated in a single assay and a better determination made regarding the most appropriate multiplex typing for any given sample. Interesting assays have been documented which assess more than one target within a reaction, and some labs have created in-house real time systems that amplify specific sequence lengths in order to assess the level of template degradation in a sample [Hudlow, 2008]. Since the sensitivity of the real-time PCR assay is greater than that of the blot, often times profiles can be generated from samples that yielded no detectable signal from the blot quantification. Therefore, an obvious advantage to a PCR based quant method is an increase in sensitivity [Kline *et. al*., 2005]. Finally, the 96 well plate format and hands-off methodology of the assay make it more conducive to automation than blot technology.

Undoubtedly, real time PCR has revolutionized the quantification methodology in forensic laboratories across the world. Real time PCR quantitation was not available in the primary laboratory until the very final aspects of this thesis were conducted and could therefore not be used for most of the quantifications. While real time

quantification systems often provide insight about the presence of potential PCR inhibitors, reagents successful in recovery of DNA were those generally used in the lab for extraction and knowingly do not inhibit PCR. Furthermore, one major limitation to blotting is fluctuation in pH, and this is why ethanol precipitation was used when testing acid/base DNA recovery methods throughout this work. The other chemical treatments tested (phenol, chloroform, alcohols, organics) should be removed by the extraction/purification process with the PCI/ Microcon® procedure. Finally, since only a portion of a DNA recovery product is used for quantification, the ultimate test for recovery is multiplex amplification of the majority of the product. Therefore, in light of rapid advances which occurred in the area of quantification assays throughout the course of these studies, the quantification techniques employed herein were used to indicate trends and were not used to draw conclusive inferences nor were they generally applied as end-point experiments.

1.4 Multiplex STR Typing and Interpretation

1.4.1 Overview of Commercially Available Chemistries

Once extraction and quantification has been completed, forensic samples are typed using a commercial multiplex kit. A variety of multiplex systems, which allow for the co-amplification and fluorescent detection of multiple loci have been marketed and validated for forensic casework by the scientific community because these systems enable the laboratory to generate profiles with powerful discrimination.

Two main vendors compete to provide fluorescent STR typing kits to the forensic science community. Applied Biosystems, also referred to as AB or ABI, offers complete systems for human identity testing from quantification and typing kits to the genetic analyzers used to detect amplified STR products. Promega Corporation also provides quantification and typing kits, but does not market fluorescent detection instrumentation. Promega does, however, provide typing kits that are compatible with a variety of detection instruments available to the forensic science community, while ABI kits are compatible only with the ABI PRISM® analyzers marketed by Applied Biosystems [Butler, 2001]. Table 1.1 summarizes commercially available autosomal typing kits that are most widely used throughout the forensic community.

NAME	SOURCE	RELEASE DATE	LOCI	DISCRIMINATION POWER
PowerPlex® 1.1 + Amelogenin	Promega	January 1997	CSF1PO, TPOX, TH01, VWA, D16S539, D13S317, D7S820, D5S818, and Amelogenin	1:1.2 x10^{8}
AmpFLSTR® Profiler™	Applied Biosystems	May 1997	D3S1358, VWA, FGA, Amelogenin, TH01, TPOX, CSF1PO, D5S818, D13S317, D8S820	1:3.6 x10^{9}
AmpFLSTR® Profiler Plus™	Applied Biosystems	December 1997	D3S1358, VWA, FGA, Amelogenin, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820	1:9.6 x10^{10}
AmpFLSTR® COfiler™	Applied Biosystems	May 1998	D3S1358, D16S539, Amelogenin, TH01, TPOX, CSF1PO, D7S820	1:8.4 x10^{5}
AmpFLSTR® SGM Plus™	Applied Biosystems	February 1999	D8S1179, D21S11, D3S1358, TH01, D16S539, D2S1338, D19S433, VWA, D18S51, FGA, and Amelogenin	1:3.3 x10^{12}
PowerPlex® 2.1	Promega	June 1999	Penta E, D18S51, D21S11, TH01, D3S1358, FGA, TPOX, D8S1179, and vWA.	1:8.5 x10^{10}
PowerPlex® 16	Promega	May 2000	D3S1358, TH01, D21S11, D18S51, Penta E, D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta D, vWA, D8S1179, TPOX, FGA and Amelogenin	1:1.8 x10^{17}
AmpFLSTR® Identifiler™	Applied Biosystems	May 2001	D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, VWA, TPOX, D18S51, D5S818 FGA, and Amelogenin	1:2.1 x10^{17}

Table 1.1: Summary of commonly used commercially available STR kits. The table represents a summary of commercially available fluorescently labeled STR kits, indicating the name of each kit, along with the respective vendor and release date. Also included is a list of loci that each kit interrogates and the estimated power of discrimination in the Caucasian population. *Power of discrimination expressed as average match probabilities within the Caucasian population [Butler and Reeder, 2005].

When selecting areas of the genome for forensic STR applications, systems are designed to target flanking regions of the repeat sequence that have low mutation rates (since conservation of sequence is essential in the annealing process of the PCR process) across loci that exhibit high degrees of heterozygosity [Promega, 2001; Promega, 2008b-d]. Primers in multiplex reactions must be engineered so that common buffering conditions and annealing parameters accommodate all primer sets

for loci targeted and result in alleles within a reasonable size range [Schumm *et. al*., 1996]. For example, the PowerPlex® 16 system reactions must accommodate sixteen primer sets, the fluorescent dyes used to tag primers must have limited spectral overlap, and resulting fragment size ranges produced by loci tagged with the same dye must not overlap [Promega, 2008c].

While a variety of platforms may be at the researcher's disposal, the PowerPlex® system products marketed by Promega are well suited for studies herein because 16-plex systems with conserved primer design are available for both detection platforms. The work for this thesis began in 2003, and the slab gel platform was still widely used in the United States. This was the validated method available upon commencement of these studies, and preliminary assays were conducted using the PowerPlex® 2.1 and PowerPlex® 16 BIO kits. Throughout the course of this work, efforts to validate PowerPlex® 16 for the multi-capillary ABI PRISM® 3100-*Avant* commenced. The relationships between the various PowerPlex® chemistries and the progression towards the internal performance verification of the 3100-series analyzers will be more fully described in Chapter 3 of this work. Therefore, introductory material will focus on important differences in data output and interpretation for slab gels scanned with the FMBIO® II versus that of capillary instrumentation.

1.4.2 STR Platforms

Generally, the quality of a multiplex typing kit is evaluated against its ability to produce a complete profile across all loci and the profile should be well balanced between and within the loci [Steadman, 2002b; Steadman, 2005b; Krenke *et. al*., 2002]. Balance is measured in relative fluorescence units (RFU) expressed in peak height using capillary systems, and optical density (OD values) using gel platforms, [Hitachi, 1999; Applied Biosystems, 2003]. Allele quality is assessed based on the shape and intensity of a band (should be bold, clear, and span the width of the lane) or peak (should be sharp and uniformly "triangular"-shaped with a single point and no shouldering on either side). Background may consist of shading and extraneous bands on a gel or high baseline and noise peaks throughout an electropherogram. Such artifacts, including stutter, should be minimal. Finally, since multiplex reactions

currently rely upon fluorescent detection, separation of the fluorophore emissions on primers is critical. Examples of data collected from each platform are illustrated in Figures 1.10 and 1.11.

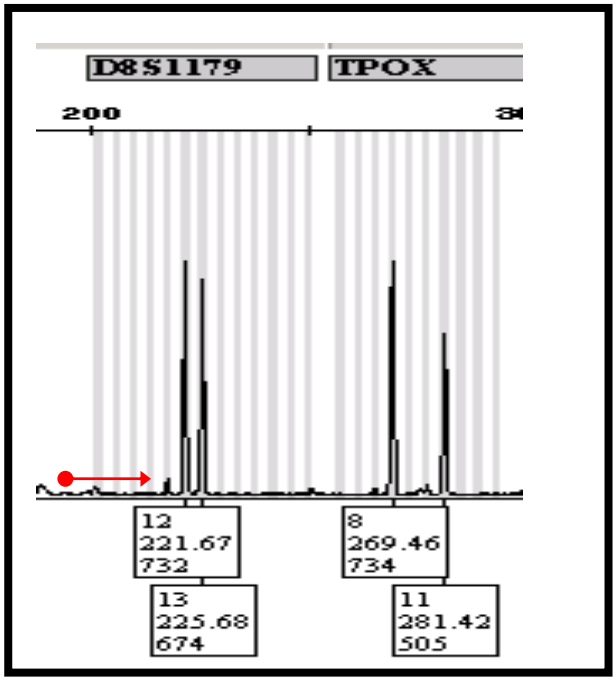


Figure 1.10: Data collected with capillary electrophoresis instrumentation. Data collected with capillary instrumentation is observed in peaks, the RFU value or peak height is proportional to the amount of DNA detected for each allele. The peaks represent PCR products obtained from a known individual at the loci TP0X and D8S1179. The box below each allele indicates the number of STR repeats for the detected allele (the example is a 12,13 at D8S1179 and an 8,11 at TP0X), the number of base residues each peak represents (fragment sizes are 221.67, 225.68, 269.46, and 281.42 bases in length), and the RFU value for each peak (these are 732, 674, 734, and 505). An arrow indicates a stutter peak at the D8S1179 locus [data from Steadman, 2005b].

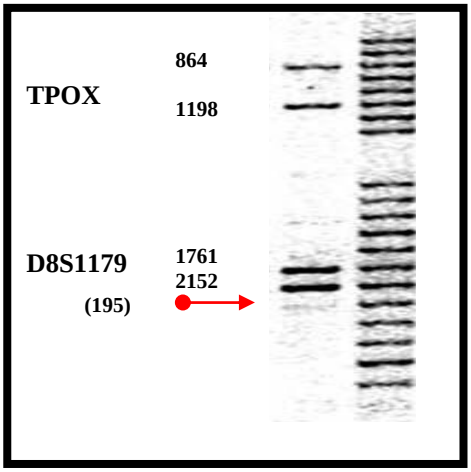


Figure 1.11: Data collected with flatbed gel scanning instrumentation. Data collected with the FMBIO® is observed as bands, the OD value or band intensity is proportional to the amount of DNA detected for each allele. The allelic ladder is in the right lane and PCR products obtained from a known individual at TP0X and D8S1179 are in the left lane. The OD values for each allele are indicated. An arrow indicates a stutter peak at the D8S1179 locus with parenthetical indication of OD value [data from Steadman, 2002b].

These examples can be used to illustrate how profiles are evaluated within and between loci, and this is determined by a ratio comparison of RFU or OD values for the alleles detected [Steadman, 2002b; Steadman, 2005b]. The peak height ratio is measured by dividing the height of the lower quantity peak or band (in RFU or OD) by the height/intensity of the higher quantity peak/band; the value is expressed as a percentage and will always be less than or equal to 100% [Butler, 2001]. One may express the ratio with consideration of amplicon size (dividing the longer allele RFU/OD by the shorter allele RFU/OD) rather than basing the division on peak height or band intensity. This approach was not used when generating data for this thesis since it creates a ratio range beyond 100%, making comparison between loci and samples extremely difficult if mean-based statistics are to be applied to the data. In the capillary example, the RFU value of the less intense allele is 92% that of the more intense allele at D8S1179; this constitutes the peak ratio or allele imbalance for the locus (dividing the lesser intensity by the greater intensity and expressing the value as a percentage). The heterozygote ratio at TP0X, however, is 68% (505/734), indicating more imbalance between sister alleles at this locus. Optimal samples generally yield ratios above 70% [Krenke *et. al*., 2002] and peak heights between the

loci that are fairly consistent, ranging from 500-2500 RFU. For the gel data, the OD value of the sister alleles are approximately 72% and 81% for TP0X and D8S1179 respectively. However, greater imbalance between loci is evident since the total allelic intensity at TPOX is 2062 and the total allele intensity at D8S1179 is 3913. In extreme cases, or situations where very little template is afforded the system, alleles or loci may not be detectable at all; this is referred to as allelic drop-out [Gill, 2001; Wickenheiser, 2002]. It is also speculated that the PCR process can result in detection of untrue alleles, termed allelic drop-in [Gill, 2001; Wickenheiser, 2002; Anjos, *et. al.*, 2006], although a mechanism for this has not been fully described other than sporadic contamination preferentially amplified in the earliest rounds of PCR [Gill, 2001]. Alternatively, in situations where too much template is placed in the amplification reaction, signal, whether band or peak, may saturate the detection system. This results in "bleed through" or "pull-up" due to the difficulty in color-separating fluorophores. These terms refer to non-specific detection of one fluorophore in the panel of another fluorophore due to spectral overlap; this often occurs because the intensity of the signal is so great that complete separation is no longer possible. Saturation also causes artificially elevated stutter percentages, making mixtures extremely difficult to interpret.

Heterozygote ratio, or sister allelic imbalance, is an important consideration for interpreting STR profiles [Whitaker *et. al*., 2001; Kloosterman and Kersberge, 2001; Krenke et al., 2002; Gill *et. al*., 2000a/b]. Levels of acceptable imbalance are determined by each laboratory based on validation studies and are generally established in the 65-75% lower limit tolerance range [Greenspoon *et. al*., 2004a; Krenke *et. al*., 2002; Steadman, 2002b; Steadman, 2005b]. Levels of template DNA required for DNA input is also usually independently performance verified and based on balance within and between loci, although it is well-established that shorter amplicons preferentially amplify, especially in low copy number templates and/or degraded samples [Kloosterman and Kersberge, 2001]. Together, these criteria have persisted as the basis for evaluating multiplex performance and developing interpretation protocols when testing new chemistries and platforms [Vallone, *et. al*.,

2008]. Therefore, STR systems with accurate or predictable heterozygote ratios are important when applied in a forensic setting.

Another aspect of data interpretation involves the proper characterization of PCR artifacts inherent to STR typing. The most common of the artifacts is repeat slippage, referred to as stutter. Slippage during amplification results in a detectable DNA fragment one repeat shorter than the true allele [Weber and May, 1989; Sprecher *et. al*., 1993]. The relative intensity of these fragments in comparison to the true (also termed "parent") allele is almost always included in laboratory performance verification of chemistry. Since the stutters are a product of the PCR process, once expected relative intensities are evaluated, it is usually not necessary to evaluate expected stutter ranges or apply "cut-off" values since they are an amplification artifact rather than a detection artifact. The OD value of the stutter band is indicated in Figure 1.10 as an example of how stutter interpretation occurs [Steadman, 2002b]. The ratio of artifact intensity to that of the parent allele is generally calculated (7.3% in this example). A stutter peak is also indicated by red arrow in the electropherogram example (Figure 1.11) [Steadman, 2005b]. During validation studies, stutter percentages are observed and cut-off percentages for each locus are established based on statistical calculations [Krenke *et. al*., 2002; Steadman, 2002b; Steadman 2005b]. While not as prominent, slippage can also result in the production of n+4 artifacts [Butler, 2001], although this occurs rarely and is generally evaluated on a case-by-case basis.

When a low copy number of the DNA template is provided to the multiplex PCR reaction, stochastic effects present interpretational challenges [Butler, 2001]. Affording the amplification reaction very small amounts of template causes profile variation because limited copies of template are present in the initial rounds of the PCR. When this happens, heterozygote peaks/bands exhibit RFU/OD imbalance and in extreme cases, allelic drop out may occur [Kloosterman and Kernsberge, 2001]. Multiple samplings or amplifications are sometimes useful for verification of profile results when limited quantities of template are at hand [Gill *et. al*., 2000b].

Unfortunately, the very nature of a limited quantity sample is not conducive to multiple analyses, since it is limited to begin with.

An evaluation of system performance using the parameters described is usually achieved by the operational lab through a complete system validation. And while many issues regarding data interpretation can be directly addressed in standard validation assays, situations involving LCN typing, compromised samples, samples containing extraneous or PCR-inhibiting components, and/or degraded samples are more difficult to test in an experimentally controlled manner. Therefore, experience with casework or otherwise challenging samples becomes important in evaluation of typing results either for interpretational comparisons or DNA recovery evaluation.

1.4.3 Statistical Evaluation of DNA Profiles

Statistical calculations in the forensic DNA community are generally put forth in an effort to address the significance of an individual's DNA having not been excluded from a crime scene profile. If two profiles are declared to "match," or an individual cannot be excluded as a contributor to a mixture profile, the scientist is then tasked with explaining the significance of this inclusion to the jury. In other words, one mathematically determines the probability a random individual would be included as a possible source or contributor to the profile of interest. The discriminatory power of current multiplex typing technology is often expressed in the form of a random match probability.

To express the rarity of multiple sets of events occurring simultaneously, the probability of a particular multiple-locus genotype is obtained by multiplying together the frequencies of what is observed at each locus. Therefore, the probability of a random match is determined by multiplying together the frequencies of all the individual alleles and including a factor of 2 for each heterozygous locus; this application is called the product rule [Brenner, 2009]. The frequencies of alleles in any population must be empirically determined by typing numerous individuals from populations of interest. Locus genotype frequency is determined as follows:

$f = 2p_1p_2$
where p_1 = *allelic frequency allele 1* and p_2 = *allelic frequency allele 2*

In the instance where an individual is homozygous at a locus, a correction factor theta (θ) is included in the genotype calculation per locus to account for population substructure:

$f = p^2 + p\,(1-p)\theta$
where p = allelic frequency and $\theta = 0.01$

The National Research Council (NRC) recommends applying a θ value of 0.01 or 0.03 [NRC, 1996], which offers a conservative random match probability in response for the need to produce probabilities for potentially isolated ethnic groups. These values are examples that have been adopted by laboratories in the United States and although the value of θ may vary from lab to lab, it is usually guided by recommendations set forth by the NRC. While the co-ancestry coefficient (sometimes referred to as kinship coefficient) value can be described many ways, it is a relative measure and the effects of the correction factor are not substantial [Evett and Weir, 1998].

The product rule can only be applied in situations where the population and the markers typed conform to certain criteria. The first is that the population in question be in Hardy-Weinberg equilibrium (HWE); this involves a number of assumptions which result in frequency estimates being conserved from generation to generation [Inman and Rudin, 1997]. Frequency data are generally subjected to numerous analyses to determine HWE conformance, and it has been accepted that the assumption of HWE is a reasonable approximation for the genetic markers used in forensic human identity testing [Budowle *et. al*., 2000].

The current multiplex typing systems (15 STR loci) achieve random match probabilities that are often in the magnitude of quad- , quint-, or sextillions [Promega, 2008b/c; Butler, 2001], meaning that the chances of selecting an individual at random

that would have the same profile as any given single source crime scene stain profile would be approximately 1 in 10^{27}.

Forensic DNA analysis relies on statistical methods far beyond considerations of straightforward single source profiles to include mixture profile interpretation and the myriad of approaches that are required for highly specialized DNA typing systems (Y STR, mitochondrial, SNPs). However, for the purposes of this thesis, the relative uniqueness of a profile as it relates to the number of loci that are capable of being typed will be of most interest.

1.5 Databases and Quality Assurance Standards in the United States

1.5.1 The Combined DNA Indexing System (CODIS)

The ability to discriminate between individuals is not only important to consider when expressing the probative value of profile concordance to a jury, it is also an important consideration when developing standards for database entry and searches in order to generate meaningful database matches or "hits." DNA database standards must achieve two things. First, assurance that the quality of the data that is entered by participants is reliable and comparable. Second, that the amount of data required/allowed and search parameters employed achieves minimal advantageous hits without bypassing potentially probative associations between entries. These issues, as managed within the United States, will be discussed in this section.

In 1998, three years after the FSS began using the UK DNA Database, the FBI launched the CODIS. Primary guidance for the system and standards was the Technical Working Group for DNA Analysis Methods (TWGDAM), which has since evolved into the Scientific Working Group for DNA Analysis Methods (SWGDAM). This group of individuals was appointed by the FBI to develop standards of practice in the forensic DNA community. The legislative foundation for databasing DNA profiles preceded the introduction of database software in labs across the country by nearly a decade, as did the planning for acceptable entries, search parameters, and indices by the working group.

CODIS is a hierarchal database which accommodates the maintenance of separate databases at the local, state, and national level. The architecture is illustrated in Figure 1.12.

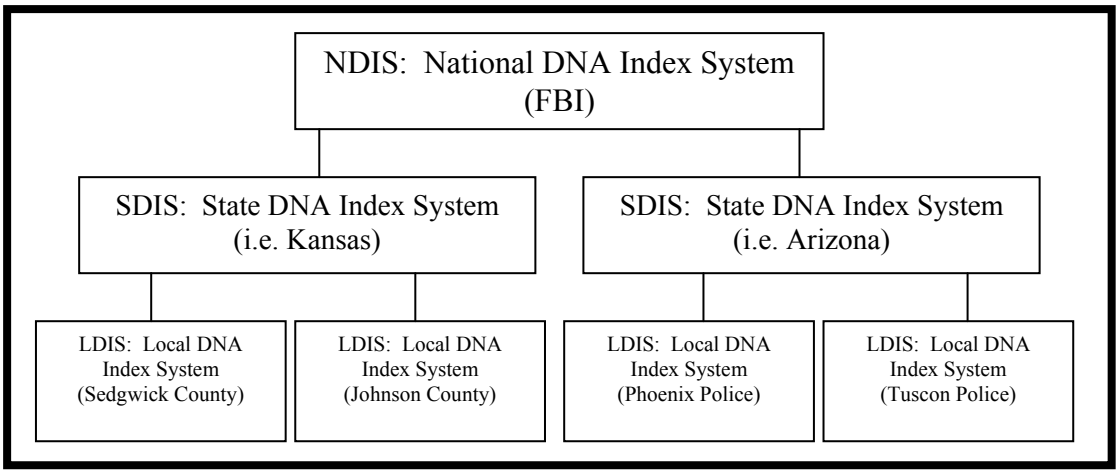


Figure 1.12: Architecture of CODIS. The National Index is housed at the FBI, and accepts data from the 50 states, which in turn accept data from local laboratories and agencies within each state. The data is uploaded from local to state, and then from state to the national level. Local databases can be maintained according to state law, but only certain profiles are eligible for NDIS entry and searches. For example, suspect profiles may be kept at the local or state level, but are not allowed at NDIS unless the State is authorized by law to collect and database suspect DNA records.

DNA records accepted by NDIS are arrestees, relatives of missing persons, convicted offenders, missing persons, forensic unknowns, legal references, and unidentified human (remains). These are the records that can be stored and searched according to the DNA Identification Act [DNA Identification Act, 1994]. Each profile is categorized within a specific index; the indices at NDIS are listed and defined in Table 1.2.

INDEX	DEFINITION
Arrestee	DNA reference standards from persons arrested or indicted
Convicted Offender	DNA reference standards from persons convicted of qualifying crimes, qualification determined by state
Forensic	DNA records originating from a crime scene, criteria set forth by FBI
Legal	DNA reference standards collected under legal authority
Missing Person	DNA records from missing persons and deduced missing persons
Relatives of Missing Person	DNA reference standards from biological relatives of missing persons
Spouse	DNA reference standards from presumptive parents of a common child of a missing person
Unidentified Human (Remains)	DNA records of individuals who refuse or cannot identify themselves (remains)

Table 1.2: DNA Indices at NDIS. The table lists the categories into which DNA profiles are organized, which ultimately dictates against which profiles any given record will be searched [DNA Identification Act, 1994].

The index in which a profile is housed dictates how it will be searched. For example, Relatives of Missing Persons are not searched against crime scene profiles. Therefore, a person who has committed a crime (and left blood at the scene of a burglary) would not be linked to that offense upon submitting a DNA sample for the purposes of identifying his/her missing child. The most probative searches for criminal investigative leads currently occur between convicted offender and forensic indices, as these contain the most profiles. There are qualifying considerations within each index, some of which will be discussed later in this work.

1.5.2 Quality Standards for DNA Testing Laboratories

Along with the database came guidelines for laboratory procedures, to which strict adherence was required for laboratories participating in the database. The second group primarily responsible for development of testing standards in the United States, appointed by the FBI, was the DNA Advisory Board (DAB); this board was created in 1995 and was assigned the task of developing a set of quality standards (then referred to as the "DAB Standards"). The standards developed by this group were issued in

the form of the FBI's Quality Assurance Standards for DNA Testing Laboratories (Forensic Laboratories) [FBI, 1998] and took effect on October 1, 1998. A year later, a similar set of standards were issued specifically for convicted offender laboratories [FBI, 1999; Cormier, 2005]. Both documents address organization and management, personnel, facilities, evidence control, validation, analytical procedures, equipment calibration and maintenance, reports, review, proficiency testing, corrective action, audits, safety, and subcontracting. Significant updates since that time have included the release of a combined (forensic and convicted offender) document in July 2004 [FBI, 2004] entitled "Quality Assurance Audit for Forensic DNA and Convicted Offender DNA Databasing Laboratories" and is in accordance with the previously issued standards. Most recent updates to the document have been issued with an effective date of July 1, 2009 [FBI, 2008].

Laboratories are required to conduct annual audits, which must be performed by an external entity every other year. Results of the external audit are forwarded to the FBI and are assessed by a review committee tasked with the evaluation and resolution of findings cited during audits [FBI, 2008]. Labs that are not in compliance with the FBI's quality assurance standards are not eligible to be CODIS database participating labs. Given that many FBI quality assurance standards and laboratory certification standards are the same, a laboratory found non-compliant with the FBI standards would also be ineligible for accreditation. In October 2006, the United States federal government enacted amendments that require laboratory systems to achieve accreditation for National Institute of Justice grant funding awards, thus making quality standard adherence a funding-dependant operational requirement [DNA Identification Act, 1994]. With rare exceptions, accreditation of laboratories is performed by the American Society of Crime Lab Directors Laboratory Accreditation Board (ASCLD/LAB) and Forensic Quality Services (FQS).

1.5.3 Database Acceptance Standards

A laboratory that has demonstrated adherence to the Quality Assurance Standards may wish to participate in the CODIS database system and must then comply with specific database operational procedures as set forth by the FBI. While state and local

laboratories may house DNA profiles of any nature, those that are uploaded to the national level for inter-state comparison must be in compliance with quality assurance and privacy requirements specified by Federal law [DNA Identification Act, 1994]. Two critical acceptance criteria shall be addressed within the scope of this thesis.

One of the acceptance criteria is related to which typing kits are acceptable for generating profiles that are to be entered into CODIS. This is to prevent the use of in-house amplification primers that have not been fully tested for concordance to other kits. Discordance can result from selection of poor primer binding regions or development of substandard allelic ladders and/or calling software. A list of acceptable typing kits at the time of this work is presented in Table 1.2; the multiplex kits, paired or individually, aim to generate profiles across the 13 core loci: CSF1PO, FGA, TH01, TPOX, VWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, and D21S11.

NAME	SOURCE
AmpFLSTR® Profiler Plus™	Applied Biosystems (Part #4303326)
AmpFLSTR® COfiler™	Applied Biosystems (Part #4305246)
AmpFLSTR® Profiler Plus™ and AmpFLSTR® COfiler™	Applied Biosystems (Part #4305979)
AmpFLSTR® Profiler Plus™ ID	Applied Biosystems (Part #4330284)
AmpFLSTR® Profiler Plus™ ID and AmpFLSTR® COfiler™	Applied Biosystems (Part #4303326)
AmpFLSTR® Identifiler™	Applied Biosystems (Part #4322288)
PowerPlex® 1.1	Promega (Catalog #DC6091/6090)
PowerPlex® 1.2	Promega (Catalog #DC6101/6100)
PowerPlex® 2.1	Promega (Catalog #DC6471/6470)
PowerPlex® 16	Promega (Catalog #DC6531/6530)
PowerPlex® 16 BIO	Promega (Catalog #DC66541/6540)
PowerPlex® Monoplexes D5S818, D7S820, D13S317, D16S539, TH01, TPOX, CSF1PO, vWA	Promega (Catalog #DC6161, DC6141, DC6151, DC6131, DC5081, DC5111, DC5091, DC5141)

Table 1.3: NDIS acceptable typing kits. This table describes the chemistry that may be used to generate profiles deemed acceptable for NDIS. Monoplexes are all fluorescein-labeled and have the same primer sequences as the 16-plex Promega kits.

In order for any particular chemistry to be added to this list, developmental validation data must be presented to the FBI which demonstrates compatible results. Furthermore, any modifications made to an acceptable kit (i.e., primer add-ins), must also be presented with justification for the modification. The criteria used in

reviewing requests for additions/modifications include concordance studies, mixed samples, non-probative casework samples, population studies, precision studies, proficiency sample data, reproducibility, sensitivity assays, scientific manuscripts relating to validation studies, and any other information or supporting documentation deemed necessary or requested by the FBI [FBI, 2008]. Given the laborious nature of the process, very widely accepted kits have been developed and implemented for use in laboratories which were not yet added to the list at the time of this work (such as AmpFLSTR® MiniFiler™, AmpFLSTR® Y-Filer™, and PowerPlex® Y). While Y STR data certainly does not possess the discriminatory power necessary for meaningful autosearches between forensic/offender indices, the data may be determined useful for searches within the missing person indices and is being maintained by some laboratories on the local level at this time; certainly accommodation of the data within the software indicates there is a good chance for future acceptability. The most useful and discriminating addition, especially related to the goals of this work, would likely be that of a miniplex which interrogates the same core loci using primers designed to generate shorter amplicon fragments since these have been shown useful for typing degraded and low copy number samples [Chung *et. al*., 2004]. At the time of this work, additional MiniFiler™ product data was pending approval by the FBI; therefore the chemistry was not considered useful for generating data that could potentially be uploaded to NDIS [Applied Biosystems, 2007b].

Another acceptance standard with direct applicability to this work is the number of loci required for NDIS profiles. These standards are index specific and are dependent upon the nature of the record's source. For example, convicted offender sample records must be complete across the 13 core CODIS loci. However, since forensic evidence can result in mixtures and/or incomplete profiles, more specific guidelines exist for entry of these profiles. For crime scene samples, the laboratory must attempt to obtain a profile across 13 loci and data must be entered for at least 10 loci to be searched nationally. Furthermore, in the case of mixtures, no more than four alleles can be entered at any single locus, and in the event four alleles are entered as such, no more than two alleles may be entered at any of the remaining loci. These parameters

have been set forth to reduce advantageous hits. Since all hits must be appropriately dispositioned by each of two labs, a considerable amount of effort must be put forth to sort through routine hits and determine which may provide investigative information. This is especially true since NDIS conducts moderate stringency searches with some tolerance of mismatched loci.

Sedgwick County Regional Forensic Science Center adheres to the entry requirements set forth by NDIS and has not, up until this time, elected to maintain indices beyond that defined by NDIS. However, there are a limited number of profiles that are partial in nature which reside at the County level that have not been uploaded further because they do not meet the minimum number of loci requirements set forth by NDIS. While a profile may not be allowed at NDIS, special circumstances may allow for keyboard searches of partial profiles.

1.6 Project Overview

1.6.1 Historic Relevance

To date, a method for comparing RFLP profiles to STR profiles has not been developed because the two systems interrogate different regions of the DNA sequence and use fundamentally different analytical platforms. Since the shift in technology from RFLP to STR-PCR, most laboratories, including the FBI and the FSS, have discontinued RFLP analysis. Moreover, the commercial reagents used for RFLP analysis are becoming scarce, further restricting the opportunity for the forensic community to maintain the technology, even in the private sector [Beckwith, 2005]. Because results cannot be compared between systems, and RFLP analysis has become uncommon, it is more difficult to continue investigations where the primary methodology employed was RFLP. This is especially true in cases where the DNA from evidentiary items was consumed to generate a profile. This was often the case because RFLP technology required large quantities of DNA and evidentiary DNA samples were often entirely used in an effort to obtain a profile for comparison. It is possible that DNA left from the exhibit in an RFLP case would be preserved in a restriction-cut form bound to a nylon membrane. Where post-analysis membranes have been archived, an opportunity may exist for the sample DNA to be recovered

from the membrane and used as a template for STR-PCR analysis. Should a procedure be developed that would allow for STR typing of membrane bound DNA, it is possible that the technique could be validated in the forensic laboratory so that profiles generated in this manner could be searched against current criminal intelligence databases like CODIS. Additionally, should membrane bound template be found viable for additional testing, technology may be applied to forensic exhibits that was not available in the past, such as SNP or YSTR testing.

The idea to attempt recovery of DNA from archived membranes was born from a serial murder case in the United States. The killer, self-named "BTK" (bind, torture, kill), terrorized the Wichita Kansas community for 17 years (1974-1991) during which he took the lives of 10 victims. Just prior to the anniversary of the first killings (December 2003), the laboratory began revisiting evidence from the historic crime scenes in an effort to generate a profile that could be entered into and searched against the national database. Shortly after the items were received by the lab, BTK resurfaced and re-established written correspondence with police. Some of the communications contained personal items from the victims, which could easily be verified as authentic. As the need to capture the killer increased, the lab began to explore alternative ways of generating an STR profile, one of which was to further test an RFLP membrane lane containing DNA from the seminal fluid found at one scene. While the case was resolved and prosecuted prior to completion of this work, it is of note because it did inspire this work. Furthermore, the case illustrates a true application for such a method and could provide useful investigative genetic information for other cold cases where RFLP testing was previously performed and legitimizes the work here in as a significant contribution to the field of forensic DNA analysis.

It was recognized that handling archived membranes raises concerns with respect to historical handling procedures that may have been more conducive to the introduction of extraneous DNA. Today's technology requires strict adherence to decontamination procedures and protective gear while RFLP analysis did not. While historical processes cannot be specifically addressed, some effort was made throughout the

course of this research to address laboratory contamination issues and these efforts are specifically discussed in the final chapter of this work.

1.6.2 Project Goals

The purpose of this project is to investigate strategies that can be applied to the existing forms of DNA testing to improve the ability to obtain useful DNA profile information from challenging samples. The ultimate goal, however, was to develop a method for DNA recovery from static supports and generate an STR PCR profile. This goal had to accommodate the need to be compliant with CODIS acceptance criteria and also the rapid pace of technical development that occurred within the Sedgwick County laboratory during the period over which the studies reported here were conducted. Chapter six represents the culmination of the various aspects of the study by directly addressing the primary goal. However, in order to test different approaches and also to demonstrate the compatibility of results generated at different stages of the work using different genetic analysis equipment, the overall goal of this project was achieved step-wise through a series of specific aims, to follow.

The growth and development of the operational forensic laboratory in which the work had been conducted had resulted in early results having been produced using a different procedure and equipment than later results. Therefore, a performance verification for a capillary-based detection system was conducted to demonstrate the limitations of current STR systems for typing limited quantity samples. This verification included some comparative studies to flat-bed detection instruments since both platforms were used to generate data throughout the course of the project. These studies comprise Chapter 2 and provide the foundation for interpretation and evaluation of downstream profile enhancement assays. Note also that many of the fundamental techniques employed throughout the work reported are described in Chapter 2. Specifically, these were the conduction of the multiplex STR amplifications and the electrophoretic analysis of the products of these tests. The performance verification considered the aspects of concordance of results produced from different tissues and body fluids, and concordance of genotypes produced on the 3100-*Avant* and FMBIO® II instruments. In addition, threshold limits, stutter cut-off,

species specificity, mixture analysis, precision, and sensitivity studies were all conducted. Chapter 2 introduces interpretation and mathematical approaches to evaluating profile quality and these analyses are used throughout the thesis when comparing enhancement techniques. The chapter also presents a strong body of data representing the capability of current STR typing techniques for comparison to data collected following enhancement assays.

In Chapter 3, whole genome amplification (WGA) techniques were investigated to determine if the technique would be valuable for generating additional template from an exhibit. This technology was tested on both purified DNA extracts and whole cell lysates in an effort to obtain increased genomic DNA template from low quantity samples that could then be typed with current STR techniques using commercially-obtained, CODIS-accepted chemistry.

In addition to the WGA assessment, products marketed for enhancement of damaged or limited quantity samples during the PCR process were also tested for this work and are explored throughout Chapter 4. These are referred to as PCR enhancement techniques since they are applied at the amplification stage of analysis and specifically refer to PCRboost™ (a proprietary mixture of amplification enhancers) and Restorase® (a mixture of DNA repair enzymes). Thus, assays were designed to evaluate the usefulness of additives designed to increase the amount of detectable amplified end product when added to standard STR PCR typing kit reactions. Also evaluated was the use of a repair enzyme system for restoration and typing of damaged/limited template when used in conjunction with NDIS-approved STR chemistry. Furthermore, alternative sample processing extraction approaches are explored in Chapter 5 in an effort to determine if enhancement can be achieved at the earliest possible steps of the profiling process. These were the use of the Maxwell® 16 LEV for automated paramagnetic DNA extraction and the Spex 6770 freezer mill for pre-extraction sample processing. The former was considered for the possibility of enhancing results by improving extraction yield and the latter for improving sample surface area prior to extraction, also to ultimately improve yield.

The ongoing investigation of a serial homicide case in the laboratory prompted a focus on modified extraction approaches that may be useful for enhancing total DNA recovery from samples with unique substrate binding characteristics, specifically the removal of DNA bound to nylon membranes which had previously undergone RFLP testing. Multiple techniques, including newly developed procedures conducive to automation, were evaluated for the capability to improve DNA recovery. Beyond generally accepted extraction methods, a variety of techniques for removal and/or typing of DNA bound to nylon supports were explored, including unconventional extraction reagents and direct amplification with standard STR chemistry. A variety of approaches are introduced and explored in Chapter 6 of this work and select enhancement products investigated in previous chapters were also revisited for this application. Removal approaches tested on membrane bound high molecular weight DNA were alkaline extraction, acidic extraction, disruption by organic chemicals, and casework buffer extraction. Recovery of restriction digested bound DNA was attempted using standard and modified casework extraction buffers and tests were also conducted using novel approaches such as direct STR amplification, whole genome amplification, and electrophoretic force. The results of these pilot studies were used to formulate an approach to be applied to archived membranes. The membranes that were available for experimentation constituted a very finite resource, analogous to the problems frequently confronting forensic scientists, and therefore a conservative approach was required. Ultimately, the techniques determined most promising for removal of bound DNA were tested on actual archived membranes and evaluated for the ability to generate STR profiles that could be searched against the CODIS database.

2.0
Platform Performance Verification

2.1 Platform Performance Verification

Throughout the course of this work, different typing chemistries and detection platforms were used and a discussion of these systems is required to recognize the differences between them and specifically to address how they may affect the interpretation of profile quality and overall sensitivity.

Furthermore, demonstrating performance of any newly implemented detection platform is required for compliance with the FBI's Quality Assurance Standards for DNA Laboratories [FBI 1998, 2004, 2008]. Therefore, while the flat-bed detection system had already undergone internal validation, the capillary-based platforms were introduced into the host lab throughout the period during which this project was conducted. This constitutes a significant platform change and required verification beyond the scope of a simplified cross-over study.

The immediate aim of this chapter is to demonstrate comparability of results from flat-bed gels and capillary systems. Additionally, the limitations of any chemistry and platform should be fully introduced for the purposes of this particular thesis since the underlying challenges are related to low template sample enhancement. This chapter specifies the reagents and instruments used and it describes the methods employed in the human identification tests used to assess the various experimental procedures described in other parts of this thesis. The work described here therefore underpins the rest of the thesis.

2.1.1 PowerPlex® 16 Kit Development and Design

The loci included in Promega's PowerPlex® systems were selected because they meet requirements for testing set forth by a number of standardization bodies worldwide [Promega, 2001; Promega, 2008 b-d]. The kits were designed to create profiles across the 13 core STR loci required for profile upload into CODIS. With respect to Promega's PowerPlex® system product line, the need to obtain genetic information across the 13 core loci was initially met by a dual amplification system designed for a gel-based fluorescent detection system. The PowerPlex® 1.1 system allowed co-amplification and three-color detection of eight STR loci: D16S539, D7S820,

D13S317, D5S818, CSF1PO, TPOX, TH01, and vWA [Promega, 2008d]. Amelogenin primers were generally added to the 1.1 system reactions so that information regarding the sex of the DNA source could also be simultaneously collected [Promega, 2008d]. The PowerPlex® 2.1 system allows co-amplification and three-color detection of nine loci (Penta E, D18S51, D21S11, TH01, D3S1358, FGA, TPOX, D8S1179, and vWA); three of the loci, TPOX, TH01, and vWA, overlap with the 1.1 system [Promega, 2001]. With the wide spread adoption of capillary electrophoresis fluorescent detection instruments came the release of the PowerPlex® 1.2, a system that interrogates the same loci as the 1.1 system [Promega, 2007].

Recognizing the need to streamline the typing process, vendors produced multiplex systems that could meet the needs of international standards in a single PCR reaction. In 2000, Promega released the first 16-plex typing system, PowerPlex® 16, which was designed for use with ABI PRISM® detection instruments [Krenke *et. al.*, 2002]. The ABI instruments separate amplified products along the course of a capillary, which passes through a detection window where the fluorescent signal is collected. These instruments may be single or multi capillary; the latter of which can electrophorese and collect data from numerous capillaries in an array simultaneously. Shortly thereafter, a comparable 16-plex was released for use with gel-based detection instruments, specifically the FMBIO® fluorescent scanner. This platform separates amplified products through a polyacrylamide gel that is cast between two glass plates. Samples are loaded at the top into wells and an electrical current is applied across the slab gel so that shorter fragments migrate towards toward the bottom more rapidly than do longer fragments. The gel is then suspended within a scanning instrument, which detects fluorescently tagged bands within the lanes of the gel. To clarify, capillary based chemistry is denoted as PowerPlex® 16 and gel based chemistry is denoted as PowerPlex® 16 BIO to differentiate between detection platform specifications for which the kits were optimized. Release of the PowerPlex® 16 was groundbreaking because it was the first system capable of typing the 13 core CODIS loci using a single amplification reaction. Release of the PowerPlex® 16 BIO system was equally important since it is the only 16-plex commercially available for use with the flatbed detection platform.

2.1.2 Multiplex Platform System Specifications

Anticipating studies that will address enhancement of STR profiling success on low copy number samples, it became necessary to fully understand the sensitivity ranges of STR systems selected for these studies. Two common fluorescent detection system platforms, the slab gel-based FMBIO® II and capillary-based ABI PRISM® 3100-*Avant* were to be evaluated and compared for capability to produce reliable STR PCR profiles using Promega's PowerPlex® 16 multiplex kits.

While primer design differed slightly between the 1.1/2.1 and 16-plex systems, the primer sequences used in the PowerPlex® 16 and PowerPlex® 16 BIO systems are conserved [Butler and Reeder, 2005]. These primer sequences are described in Figure 2.1.

LOCUS	ORIENTATION	OLIGONUCLEOTIDE SEQUENCE
FGA	Forward	GGCTCGAGGGCATAACATTA
	Reverse	ATTCTATGACTTTGCGCTTCAGGA
TPOX	Forward	GCACAGAACAGGCACTTAGG
	Reverse	CGCTCAAACGTGAGGTTG
D8S1179	Forward	ATTGCAACTTATATGTATTTTTGTATTTCATG
	Reverse	ACCAAATTGTGTTCATGAGTATAGTTTC
vWA	Forward	GCCCTAGTGGATGATAAGAATAATCAGTATGTG
	Reverse	GGACAGATGATAAATACATAGGATGGATGG
Amelogenin	Forward	CCCTGGGCTCTGTAAAGAA
	Reverse	ATCAGAGCTTAAACTGGGAAGCTG
PentaE	Forward	ATTACCAACATGAAAGGGTACCAATA
	Reverse	TGGGTTATTAATTGAGAAAACTCCTTACAATTT
D18S51	Forward	TTCTTGAGCCCAGAAGGTTA
	Reverse	ATTCTACCAGCAACAACACAAATAAAC
D21S11	Forward	ATATGTGAGTCAAATTCCCCAAG
	Reverse	TGTATTAGTCAATGTTCTCCAGAGAC
TH01	Forward	GTGATTCCCATTGGCCTGTTC
	Reverse	ATTCCTGTGGGCTGAAAAGCTC
D3S1358	Forward	ACTGCAGTCCAATCTGGGT
	Reverse	ATGAAATCAACAGAGGCTTGC
PentaD	Forward	GAAGGTCGAAGCTGAAGTG
	Reverse	ATTAGAATTCTTTAATCTGGACACAAG
CSF1PO	Forward	CCGGAGGTAAAGGTGTCTTAAAGT
	Reverse	ATTTCCTGTGTCAGACCCTGTT
D16S539	Forward	GGGGGTCTAAGAGCTTGTAAAAAG
	Reverse	GTTTGTGTGTGCATCTGTAAGCATGTATC
D7S820	Forward	ATGTTGGTCAGGCTGACTATG
	Reverse	GATTCCACATTTATCCTCATTGAC
D13S317	Forward	ATTACAGAAGTCTGGGATGTGGAGGA
	Reverse	GGCAGCCCAAAAAGACAGA
D5S818	Forward	GGTGATTTTCCTCTTTGGTATCC
	Reverse	AGCCACAGTTTACAACATTTGTATCT

Figure 2.1: PowerPlex® 16 and PowerPlex® 16 BIO primer sequences. Forward and reverse primer sequences of PowerPlex® 16 System primer mixes are described here [Krenke, 2002].

While primer sequences and amplification parameters are conserved between the PowerPlex® typing systems designed for the FMBIO® II and the ABI PRISM® 3100-*Avant*, kit design for the two systems does differ. For the purpose of these studies, the FMBIO® II platform was employed to accommodate samples amplified with either the PowerPlex® 2.1 or the PowerPlex® 16 BIO Identification System. The ABI PRISM® 3100-*Avant* and 3130-series capillary electrophoresis platforms accommodate samples amplified using PowerPlex® 16 Identification System. While sensitivity studies for the PowerPlex® 2.1 are not presented in this work, results may be considered comparable to those observed with the PowerPlex® 16 BIO system

[Steadman, 2002b]. Because the PowerPlex® 2.1 kit was later used for STR typing of some samples in this study, the kit description is included alongside the PowerPlex® 16 system information. Table 2.1 describes the locus-specific information for the amplification systems analyzed on the FMBIO® II platform; Table 2.2 describes PowerPlex® 16 system information where different from PowerPlex® 16 BIO.

Locus	Repeat Sequence 5'-3'	Chromosome Location	Size Range of Allelic Ladder (bp)	Repeat Numbers of Allelic Ladder Components	Fluorescent Label PowerPlex® 16 BIO System	Fluorescent Label PowerPlex® 2.1 System
FGA	TTTC	4q28	322-444	*16-30, 31.2, 43.2, 44.2, 45.2, 46.2	RRX	TMR
TPOX	AATG	2p23-2pter	262-290	6-13	RRX	TMR
D8S1179	TCTA	8q	203-247	7-18	RRX	TMR
vWA	TCTA	12p12-pter	123-171	10-22	RRX	TMR
Amelo-genin	N/A	Xp22.1-22.3 and Y	106 (X), 112 (Y)	X,Y	RRX	
PentaE	AAAGA	15q	379-474	5-24	FL	FL
D18S51	AGAA	18q21.3	290-366	8-10, 10.2, 11-13, 13.2, 14-27	FL	FL
D21S11	TCTA	21q11-21q21	203-259	24, 24.2 ,25, 25.2, 26-28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2 ,34, 34.2, 35, 35.2, 36-38	FL	FL
TH01	AATG	11p15.5	156-195	4-9, 9.3, 10-11, 13.3	FL	FL
D3S1358	TCTA	3p	115-147	12-20	FL	FL
PentaD	AAAGA	21q	376-449	2.2, 3.2, 5, 7-17	JOE	
CSF1PO	AGAT	5q33.3-34	321-357	6-15	JOE	
D16S539	GATA	16q24-qter	264-304	5, 8-15	JOE	
D7S820	GATA	7q11.21-22	215-247	6-14	JOE	
D13S317	TATC	13q22-q31	176-208	7-15	JOE	
D5S818	AGAT	5q23.3-32	119-155	7-16	JOE	

Table 2.1: PowerPlex® 2.1 and PowerPlex® 16 BIO specifications. Chromosomal locations reported in accordance with the DNA Commission of the International Society for Forensic Haemogenetics (ISFH) [Bar, *et. al.*, 1997]. FL=fluorescein, TMR=carboxy-tetramethylrhodamine, RRX= rhodamine red™-X, JOE=6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein. *Allele 16 is omitted from the PowerPlex® 2.1 allelic ladder [Promega 2001; Promega, 2008b-d].

Locus	Repeat Numbers of Allelic Ladder Components	Fluorescent Label PowerPlex® 16 System
FGA	16-18, 18.2, 19, 19.2, 20, 20.2, 21, 21.2, 22, 22.2, 23, 23.2, 24, 24.2, 25, 25.2, 26-30, 31.2, 43.2, 44.2, 45.2, 46.2	TMR
TPOX	6-13	TMR
D8S1179	7-18	TMR
vWA	10-22	TMR
Amelogenin	X,Y	TMR
PentaE	5-24	FL
D18S51	8-10, 10.2, 11-13, 13.2, 14-27	FL
D21S11	24, 24.2 ,25, 25.2, 26-28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2 ,34, 34.2, 35, 35.2, 36-38	FL
TH01	4-9, 9.3, 10-11, 13.3	FL
D3S1358	12-20	FL
PentaD	2.2, 3.2, 5, 7-17	JOE
CSF1PO	6-15	JOE
D16S539	5, 8-15	JOE
D7S820	6-14	JOE
D13S317	7-15	JOE
D5S818	7-16	JOE

Table 2.2: PowerPlex® 16 specifications. Ladder and fluorophore information for the PowerPlex® 16 differs from the PowerPlex® 16 BIO Identification System (described in Table 1.2) and is separately described in this table. FL=fluorescein, TMR=carboxy-tetramethylrhodamine, JOE=6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein [Promega, 2008c].

With primer sequences conserved between the two systems and kit differences being well understood, data generated on these two common fluorescent detection system platforms could be analyzed and evaluated.

The capillary detection systems have become the method of choice because they are highly amenable to automation. While the gel platform systems are highly robust, popularity has waned due to the amount of analyst time necessary to prepare and load vertical polyacrylamide gels. Because this platform is less commonly recognized, a diagram and explanation of the internal mechanics of the FMBIO® II unit are presented in Figure 2.2.

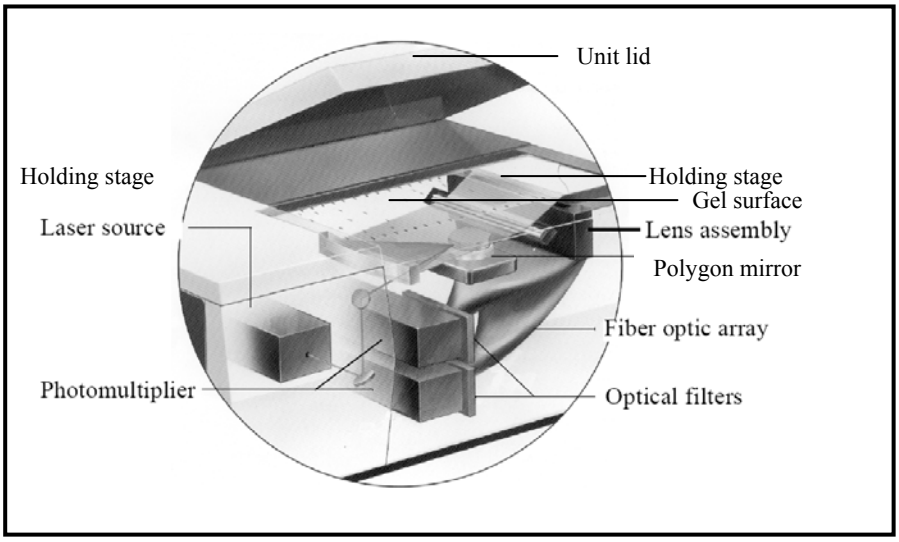


Figure 2.2: Interior view of the FMBIO® II scanning unit. Glass plates sandwiching the polyacrylamide gel contain dye-labeled DNA fragments that have been electrophoretically separated. The plates are suspended in the unit by the stage at the top and bottom of the gel. A laser source emits light onto a series of reflective surfaces and is ultimately directed onto the sample by a polygon mirror rotating at high speed. The laser light is diffused linearly across the surface of the glass plates and scans the platform from below as the optics move along the horizontal plane of the unit. Upon excitation by the laser, fluorophores attached to DNA fragments in the gel emit light; the signal is then collected by a lens, sent to a fiber optic array, and passed through fluorophore-specific filters (wavelengths are specific to multi-plex kit fluorophores). The targeted fluorescence is then photomultiplied, converted to digital signal and communicated to the computer collection software [Hitachi, 1999].

The preparation of amplification reactions are identical regardless of platform used, however kits are specific, since they employ different fluorophores. Capillary reaction products are loaded into 96 well plates and sampling is automated as the instrument inserts the capillary into the sample well. Gel platform reactions are loaded into comb-formed wells of the vertical gel. The gel is cast between low fluorescent glass plates which can be placed on the instrument directly for scanning. As described in Chapter 1, data output for the capillary system is an electropherogram, while the gel platform produces a band pattern image. Both systems employ platform specific collection and analysis software. But ultimately bands or peaks detected are designated as alleles and the resulting genotype data can then be interpreted and compared, regardless of platform origin.

2.1.3 Performance Verification Overview

Developmental validation studies involving the PowerPlex® 1.1/2.1, PowerPlex® 16, and PowerPlex® 16 BIO systems were previously published [Levedakou, 2002; Micka, 1999; Krenke *et. al*., 2002] and internal validation studies of both the PowerPlex® 2.1 and PowerPlex® 16 BIO studies were already established in the host lab upon commencement of this work [Steadman, 2002b]. Therefore, the main focus for this aspect of the project was the execution of a PowerPlex® 16 performance verification on capillary instrumentation, to include pertinent sensitivity and profile interpretation comparisons between gel and capillary platforms.

Guidelines set forth by the FBI's Quality Assurance Standards [FBI, 2004] require that certain testing be conducted internally prior to implementation of new chemistry and detection platforms in a laboratory. Because the primer sequences of PowerPlex® 16 BIO and PowerPlex® 16 are the same, one may expect the chemistry to perform similarly. However, because the manner in which the detection occurs and the data format are so different between these systems, and Sedgwick County was one of the first "FMBIO® labs" to implement capillary technology, multiple studies were conducted. These studies included concordance (typing of known and collaborative samples), interpretation threshold and stutter cut-off determination, sensitivity, specificity, mixture, and precision. Validation concluded in the casework lab with analysis of non-probative casework, statistical evaluation spreadsheet macro design, and qualification exams for analysts; these experiments were specific to case working labs and, with limited application to this project, are not presented herein. Collectively, these studies serve as subsections of this chapter's goal, that is, to demonstrate performance verification and comparison of the capillary and gel platform.

2.1.4 Capillary Technology Progression and Implementation

Capillary technology was acquired by the host lab in 2004 at a juncture where the four capillary platform was equipped with a manual dual syringe polymer delivery system on the front end. The 3100-*Avant* was the first four capillary instrument released and was popular because the model allows for upgrade to a 16 capillary instrument for

labs predicting a need for future throughput increase. Several improvements were made to the 3100-*Avant* that marked the next generation release of capillary instrumentation, the 3130-series. The 3130 (4 capillary) and 3130*xl* (16 capillary) instruments are equipped with the automated syringe polymer delivery system which eliminates manual execution of syringe removal, cleaning, filling, or bubble removal. Sedgwick County acquired a 3130 in 2006 and completed the required cross-over and internal validation studies for verification of this instrument in order to achieve redundancy in laboratory analysis capabilities. And finally, in 2007, the lab purchased the 3100-*Avant* system upgrade and converted the *Avant* to a 3130. This instrument is denoted as the 3130*u* as an indication of the upgrade.

2.2 Performance Verification Materials and Methods

Data from previously conducted gel-based performance verifications will be presented for comparison purposes in this chapter, and will also be presented in future chapters since early experiments were carried out using the FMBIO® with PowerPlex® chemistries. Therefore, the methods for standard extraction, quantification, amplification, electrophoresis, and data analysis for the applicable platforms will be included here and may be referenced when used in subsequent applications.

2.2.1 DNA Extraction and Quantification

Body fluid stains underwent standard organic extraction by treatment with 400 µL stain extraction buffer working solution to which 10 µL of 20 mg/mL proteinase K had been added [Laber, 1992]. Extraction was carried out at 56^{o} C for at least two hours. Substrates were removed from the extraction buffer, suspended in microcentrifuge tube recovery baskets, and centrifuged over their respective extraction buffers for 5 minutes at 15,000 RPM. Recovery baskets and cuttings were discarded and supernatents were extracted once with 500 µL phenol:chloroform:isoamyl alcohol (25:24:1). Following a 5 minute centrifugation at 15,000 RPM, the aqueous phases were removed for either Microcon® purification or ethanol precipitation. For Microcon® purification, aqueous phases were placed into Microcon® (50 or 100) concentrators and concentrated into TE or nuclease-free water

to a final volume of 10-20 μL [Millipore, 2000]. For ethanol precipitation, 1.0 mL cold 100% ethanol was added to the aqueous phase and placed in a -20° C freezer for at least 30 minutes. Samples were then centrifuged at 15,000 RPM for approximately 15 minutes, after which the alcohol was decanted. Pellets were then washed with1.0 mL 70% (v/v) ethanol and centrifuged at 15,000 RPM for 5 minutes. The ethanol wash was decanted; the pellets were allowed to dry and were then resolubilized at 56° C for approximately 30 minutes in TE. Some assays herein employed commercially obtained K562 or 9947A DNA supplied with PowerPlex® 16 typing kits and these cell line extracts were also used as positive amplification controls.

2.2.2 DNA Quantification-Yield Gel

Agarose yield gels were used for estimation of overall DNA quantity and quality present in any given extract. Agarose was added to TAE buffer at a concentration of 1-2% (w/v). The mixture was heated and poured into a 6 x 8.3 cm gel bed equipped with well-forming combs. Quantification standards were prepared at concentrations of 100, 50, 25, 10, and 5 ng/μL. Standards were mixed with 2 μL bromophenol blue loading solution [Promega, 1999] such that DNA could be added to the wells in the following quantities: 200, 100, 50, 25, 10, and 5 ng. Prior to sample loading, the desired volume of sample extract (usually 1 μL) was mixed with 2 μL of bromophenol blue loading solution. Electrophoresis was carried out under TAE buffer at 200 V for approximately 8 minutes. Staining was carried out in ethidium bromide staining solution consisting of 250 μg ethidium bromide dissolved in 200 mL TAE buffer [Ausubel, 1996]. Detection of DNA fragments was then achieved using the FMBIO® II Fluorescent Scanner with the standard scanning parameters listed in Table 2.3 [Hitachi, 1999; Steadman, 2002a]. The reading sensitivity and focal point were modified only in instances where non-standard gel depth or signal intensities required accommodation [Hitachi, 1999]. Intensities of the extracts were compared to the standard series to estimate the amount of DNA present in samples.

	Hitachi FMBIO® II Fluorescent Scanner
Material Type	Agarose gel
Resolution: Horizontal Vertical	150 dpi 150 dpi
Orientation	Flip Horizontal
Rate	NA
Repeat	150 times
Gray Level Correction Type	Range
Cutoff Threshold: Low (background) High (signal)	 1% 1%
Active Channel	3 (TMR)
Reading Sensitivity	1ch 100% (605nm - unused) 2ch 0% (505nm - fluorescein) 3ch 100% (585nm – TMR) 4ch 0% (650nm – unused)
Focusing Point	2.00mm

Table 2.3: FMBIO® II standard scanning parameters for agarose product gels. These parameters allow for optimal scanning and image preparation by defining the nature of the matrix (agarose), number of laser passes per scan and image resolution (repeat and resolution), the orientation you wish to view the image, signal to noise ratio (cutoff threshold), channels/filters used for the scan and respective reading sensitivities, and the focusing point for the detection [Hitachi, 1999].

2.2.3 DNA Quantification-Slot Blot

Human-specific DNA quantification was achieved with QuantiBlot® Human DNA Quantitation Kit [Applied Biosystems, 2000b; Life Technologies, undated]. Biodyne® B membranes were soaked in a pre-wetting solution while DNA samples were added to a spotting solution. A human DNA standard series was prepared from a stock solution supplied with the kit to achieve Standards A through G at concentrations described in Table 2.4. Calibrators supplied with the kit were also loaded in 5 μL quantities to observe 3.5 ng and 0.5 ng DNA slots.

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
Calibrator 1	0.7	3.5
Calibrator 2	0.1	0.5

Table 2.4: Dilution series for slot blot analysis. A serial dilution series prepared from a 2 ng/μL stock solution (A) for human DNA quantification. A volume of 5 μL of each standard was blotted to achieve slot quantities indicated in the last column of the table. Calibrators 1 and 2 concentrations are indicated as received with the kit.

Samples were then transferred to the membrane using the Convertible® Filtration Manifold System, equipped with a 48-slot top plate (each 7.5x0.75 mm). With samples bound, the membranes were placed into 100 mL of pre-warmed hybridization solution to which 5 mL hydrogen peroxide had been added. Pre-hybridization was carried out in a rotating water bath at 50° C for 15 minutes.

During the pre-hybridization period, a hybridization solution was prepared by adding 20 μL biotin-labeled primate-specific probe (D17Z1) to 30 mL of pre-warmed hybridization solution. Following decantation of the pre-hybridization solution the probe solution was added to the membrane and rotated in a water bath at 50° C for 20 minutes. Membranes were then rinsed briefly with wash solution, and treated with a solution comprised of 30 mL wash solution and 180 μL horseradish peroxidase-streptavidin conjugate and returned to the heated water bath for 10 minutes. Following two 1 minute rinses, each in 100 mL wash solution, a final 100 mL room temperature wash was carried out for 15 minutes. The membranes were rinsed at room temperature in 100 mL citrate buffer while a developing solution was prepared by mixing 750 μL Chromogen:TMB solution and 3 μL 30% (w/v) sodium hydroxide to 30 μL citrate buffer. The developing solution was then added to the membranes, which were rotated at room temperature for 30-45 minutes [Steadman, 2002a].

2.2.4 Multiplex PCR Amplification

Multiplex amplifications were set up according to the manufacturer's protocol [Promega, 2001; Promega, 2008-b-d]. A reagent cocktail was prepared from 4.2 µL nuclease-free water, 2.5 µL Gold ST*R 10X buffer, 2.5 µL primer mix (PowerPlex® 2.1, PowerPlex® 16 BIO, or PowerPlex® 16) and 0.8 µL Ampli*Taq* Gold® DNA polymerase. A volume of 10 µL of this master mix was added to each reaction tube. Template DNA was then added (0.5-1.5 ng DNA), and the reaction volume was brought to 25 µL with nuclease free water. In low quantity applications, where the template DNA must be consumed, a volume of 15 µL of DNA extract was added directly to the master mix. A positive amplification control (PAC) was prepared by addition of 1.0 µL 9947A DNA (diluted to 0.5 ng/µL in TE) and 14 µL nuclease-free water to the respective reaction tube. A negative amplification control (NAC) was prepared by addition of 15 µL nuclease-free water and no template DNA to the respective reaction tube. The 25 µL reactions then underwent 32 cycles of PCR, regardless of the typing kit used. For some research project assays, half volume reactions were used (where indicated) and reaction component volumes reduced by 50%. However, all validation experiments were full volume. For example a reaction would consist of 2.1µL nuclease-free water, 1.25 µL Gold ST*R 10X buffer, 1.25 µL primer mix and 0.4 µL Ampli*Taq* Gold® DNA polymerase; a volume of 5 µL of this master mix would be added to each reaction tube and a dilution of template DNA added (0.25-0.75 ng DNA) such that the reaction volume was brought to 12.5 µL. Target template amounts were also decreased and low quantity extracts requiring consumption would be added at a 7.5 µL volume. Although half-volume reactions were not validated for casework applications in Sedgwick County, they have been extensively described and are generally accepted for forensic applications [Castella, *et. al*. 2006; Taylor, *et. al*., 2005; Spathis and Lum, 2008]. When used, appropriate positive controls were employed and described.

Cycling parameters for the PowerPlex® 16 BIO System using either the GeneAmp 2400 or GeneAmp 9700 are those recommended by the manufacturers [Promega 2001; Promega, 2008b-d] and are described in Table 2.5.

GeneAmp 2400 Protocol	GeneAmp 9700 Protocol
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	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

Table 2.5: Cycling parameters for amplification using the PowerPlex® 16 BIO kit. Parameters for both the 2400 and 9700 instruments are described.

Amplification of the PowerPlex® 2.1 System is achieved using the GeneAmp 2400. Cycling parameters are those recommended by the manufacturer and are described in Table 2.6.

GeneAmp 2400 Protocol
95° C for 11 minutes 94° C for 5 minutes, then: (Using default ramp times) 94° C for 1 minute 60° C for 1 minute 70° C for 1.5 minutes For **10 cycles**, then: 90° C for 1 minute 60° C for 1 minute 70° C for 1.5 minutes For **22 cycles**, then: 60° C for 30 minutes 4° C for 4 minutes

Table 2.6: Cycling parameters for amplification using the PowerPlex® 2.1 kit. Amplifications were carried out on the 2400 instrument as described.

2.2.5 Post-Amplification Product Quantification

In some instances, agarose yield gels were used for estimation of product resulting from PCR amplification. Agarose was added to 1X TBE buffer at a concentration of 3% (w/v). The mixture was then heated to solubilize the agarose, and poured into a 6 x 8.3 cm gel bed equipped with well-forming combs. The positive amplification control (PAC) served as the quantification standard. Prior to sample loading, 2 µL of each amplified sample was mixed with 2 µL of bromophenol blue loading solution. Electrophoresis was carried out under TBE buffer at 200 V for approximately 10 minutes [Ausubel, 1996]. Detection of DNA fragments was then achieved using the FMBIO® II Fluorescent Scanner as described in section 2.2.2. Post-amplification product quantifications were widely used for flat-bed applications, but were abandoned upon implementation of capillary detection due to the ease in which injection parameters are modified and reinjections executed.

2.2.6 Multiplex Fragment Analysis with the FMBIO® II

For each gel, 50 mL of 6% (v/v) acrylamide solution was filtered and degassed just prior to gel preparation. To 50 mL of the 6% acrylamide solution, 333 µL 10% APS and 33.3 µL TEMED was added. The solution was mixed gently and poured between 43 cm low-fluorescent plates using 0.4 mm spacers and a 28 well comb. The gel was allowed to polymerize 30-60 minutes and pre-warmed at 65 Watts using 1X TBE until the gel temperature reached 48-52^{o} C [Promega, 2001; Promega 2008d].

A loading cocktail was prepared by combining and mixing the bromophenol blue loading solution and the Internal Lane Standard 600 appropriate for the amplification system used in a 3:1 ratio. For each sample, a quantity of 2 µL amplified product was combined with 4 µL loading cocktail. For each ladder lane, 4 µL prepared loading cocktail and 2 µL of the allelic ladder mix were combined. For the matrix, 2 µL of matrix and 2 µL bromophenol blue loading solution were combined. Samples, ladder, and matrix tubes were then heated to 95^{o} C for 5 minutes and snap cooled on ice. Urea was flushed from the wells and 3 µL of each sample, ladder, or matrix were loaded per well. Electrophoresis was carried out at 50-55 Watts, maintaining a gel temperature of 48-52^{o} C [Promega, 2001; Promega 2008d].

Detection was accomplished using the Hitachi FMBIO® II Fluorescent Scanner using the parameters described in Table 2.7 [Hitachi, 1999].

	Hitachi FMBIO® II Fluorescent Scanner
Material Type	Acrylamide gel
Resolution: Horizontal Vertical	150 dpi 150 dpi
Rate	NA
Repeat	256 times
Gray Level Correction Type	Range
Cutoff Threshold: Low (background) High (signal)	 50% 1%
Reading Sensitivity	1ch 100% 2ch 100% 3ch 100% 4ch 100%
Focusing Point	0.1 mm

Table 2.7: FMBIO® II standard scanning parameters for 6% acrylamide analytical gels. These parameters allow for optimal scanning and image preparation by defining the nature of the matrix (agarose), number of laser passes per scan and image resolution (repeat and resolution), the orientation you wish to view the image, signal to noise ratio (cutoff threshold), channels/filters used for the scan and respective reading sensitivities, and the focusing point for the detection specific to the gel matrix being scanned [Hitachi, 1999].

Gel analysis was performed using the FMBIO® analysis software version 8.0 and allelic designations made using STaR® Call software version 3.0 [Hitachi, 1999].

2.2.7 Multiplex fragment analysis with the 3100-*Avant*

A loading cocktail was prepared by combining and mixing the formamide and the Internal Lane Standard 600 in a 9:1 ratio. For each sample, a quantity of 1 μL amplified product was combined with 10 μL loading cocktail. For each ladder, a quantity of 1 μL allelic ladder mix was combined with 10 μL loading cocktail. Sample preparation is performed on a 96 well plate, which is then heated to 95° C for 5 minutes, centrifuged, placed in the plate assembly, and loaded onto the autosampler. Samples were injected using the parameters described in Figure 2.3, and data collected using Data Collection Software version 2.0 [Applied Biosystems, 2003].

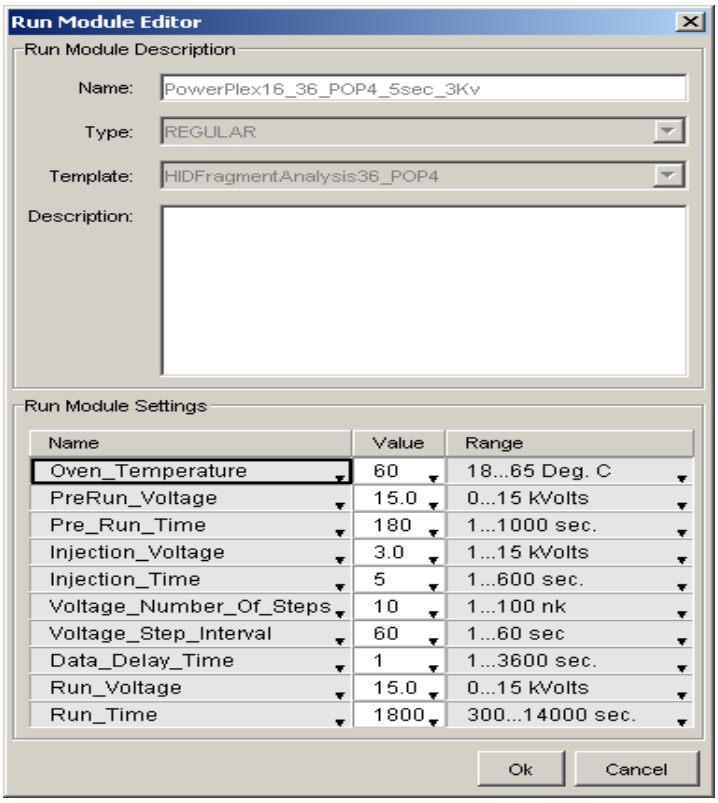


Figure 2.3: Run module parameters for PowerPlex® 16 fragment analysis on the ABI PRISM® 3100-*Avant*. Samples are subjected to 3kV injections; a range of modules are created for various injection durations, ranging from 1-11 seconds.

Performance of the 3130 series instruments in the host lab included operation of an upgraded collection software (version 3.0) [Applied Biosystems, 2005b]. This version has a slightly different run module option window, the settings of which are described in Figure 2.4.

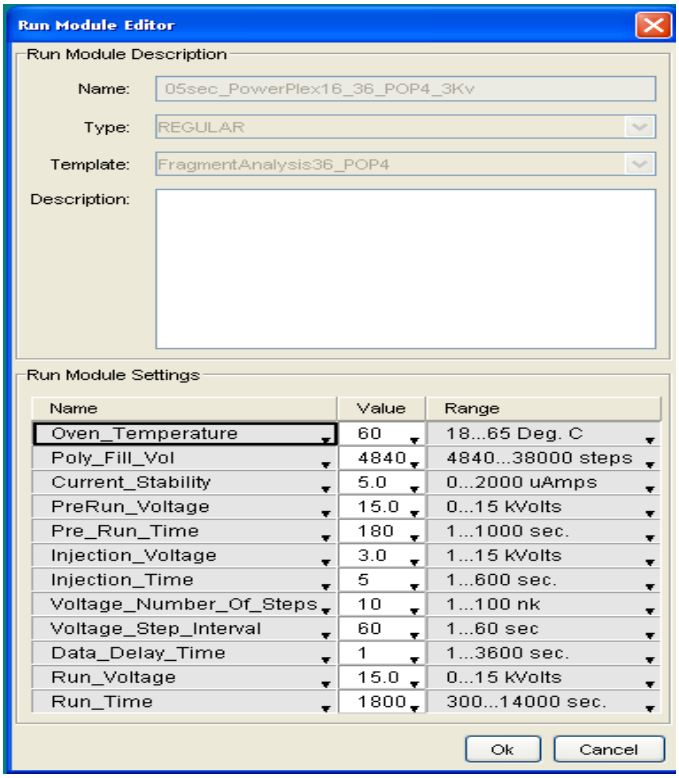


Figure 2.4: Run module parameters for PowerPlex® 16 fragment analysis on the ABI 3130 series instruments. Samples are subjected to 3kV injections; a range of modules are created for various injection durations, ranging from 1-11 seconds. The upgraded collection software for the 3130 series allows for modification of polymer fill volume and current stability within each module where previous software did not (Figure 2.3).

Data analysis was performed using GeneMapper™ ID Software version 3.1 (Applied Biosystems, 2003). Analysis method default settings were used with modifications to the Peak Detector parameters described in Figure 2.5. Five algorithms dictate the manner in which the software analyzes raw data files. For peak detection, selection of the advanced mode allows for manual selection of range, smoothing/baseline, size calling method, and peak detection parameters. The range dictates which portion of the collected data is analyzed for each sample. This is set at a point after the primer peak, and usually extends beyond the longest locus allele bin or past the longest internal lane standard peak. The smoothing and baseline options were set at light, which are recommended to provide the best results for most data, heavy or no smoothing may be selected if peaks are too narrow or too broad (respectively). The baseline value controls the baseliner, which computes the baseline for the electopherogram. Values too high will result in elevated baseline and shorter peaks, but if it is too low, the peaks may not be baseline resolved or "grounded." The size calling method selected is Local Southern, which sizes the fragments by using a reciprocal relationship between fragment length and mobility and is the most commonly employed method for STR profile analysis. Finally, peak amplitude threshold is the RFU value required for a peak to be called (given allelic assignment values); this is set at 100 based on in-house validation studies [Steadman, 2005b]. For some studies, where baseline is low and template is known to be highly purified, alternative threshold values may be applied.

.

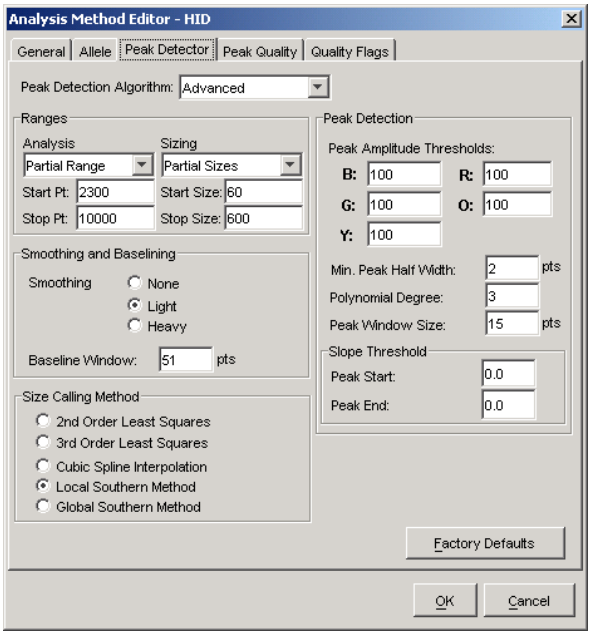


Figure 2.5: Peak detection parameters used for analysis of profiles with GeneMapper™ ID. Changes from default settings are reflected in the Analysis/Sizing and Peak Amplitude Threshold settings. These changes reflect optimized analysis range for PowerPlex® 16 chemistry and the amplitude thresholds determined from internal validation.

2.3 Performance Verification Results and Discussion

2.3.1 Concordance Studies

The objective of typing known samples for validation studies is to ensure that a single DNA profile is produced regardless of body origin (buccal, blood, semen, etc.) when collected from the same individual. The studies also provide verification that the PowerPlex® 16/3100-*Avant* platform produces profiles concordant with those obtained using previous platforms and, on a broad sense, those produced by the forensic community at large regardless of platform or amplification chemistry.

For blood samples, sixteen samples from four proficiency tests and dried samples from four volunteers were typed. For seminal fluid, samples from two volunteers were typed. For buccal samples, oral swabs from four volunteers were typed. For hairs, two samples were typed. In addition to this, ten purified DNA samples obtained from the National Institute of Standards and Technology (NIST) were commercially obtained as standard reference material (SRM2391b) and typed. Volunteer sources of blood and buccal samples were four individuals (two male and

two female). The samples of semen were obtained from the two males and hair samples were from the two females. Sampling from the same individuals serves as a mechanism for a built-in reproducibility study.

Allele calls for various body fluid specimens from the same individual were concordant with each other for all four volunteers. Furthermore, profiles were concordant with those previously obtained from PowerPlex® 16 BIO when detected using the FMBIO® II. Of interest, one of the male volunteers presumably expresses a reverse primer binding site mutation at the D5S818 locus which results in a heterozygotic profile with imbalanced peak heights (allelic imbalance at 30-50%) and this imbalance was also verified to be present in the PowerPlex® 16/3100-*Avant* electropherogram. This was discovered during the validation of the PowerPlex® 16 BIO system when the sample previously typed using the PowerPlex® 1.1 system yielded a different profile at D5S818 using the PowerPlex® 16 BIO system. The sample types as a homozygous 11 using the PowerPlex® 1.1 system, usually exhibiting a band consistent with stutter in the 10 position. Amplified product from the PowerPlex® 16 and PowerPlex® 16 BIO systems yields a heterozygous profile (10,11), where the 10 is approximately 30-50% the intensity of the 11.

This discrepancy results because the Promega D5S818 reverse primer falls on a polymorphic base and is likely caused by primer annealing differences that may occur when different cycling parameters are employed [Steadman, 2002a]. Although the primer sequence is the same in both systems, the reaction components, fluorophores, and cycling parameters are different. While the frequency of this mutation is not reported, the host lab had previously encountered a similar situation where a concordance sample provided from another lab was found to exhibit homozygosity at the D5S818 locus when typed with the PowerPlex® 1.1 system that yielded a heterozygous type when amplified with Applied Biosystems primers. Interestingly, when the inter-laboratory concordance sample was typed using the PowerPlex® 16 BIO system, a heterozygous profile was obtained at the D5S818 locus, with one allele ~43% the intensity of the other. While sequence analysis was not performed on either of these two samples, they were presumed to carry the mutation and it was concluded

that the discrepancy was likely due to the polymorphic base within the annealing site of the Promega primer. Regardless the cause, these considerations are important for interpretation of profile quality based on heterozygote ratios as this demonstrates one of many reasons imbalance can result at a locus.

Concordance studies and typing of knowns conducted for validation of the gel platform were otherwise unremarkable; all specimens performed as expected when compared to the PowerPlex® 1.1/2.1 dual amp results or other concordance samples.

During the internal validation, known samples were analyzed several different ways so that stutter and threshold parameters could be determined, and then once these values are set, the profiles are re-analyzed using the optimal/validated settings. The data from knowns is also useful for providing insight as to any artifacts a system may produce. For example, one artifact that was commonly observed during the typing of known reference samples (as well as others run for this study) was an n-10 peak at vWA. On some samples, this locus also had a tendency to produce split peaks. If these artifacts were documented throughout the course of the validation, appropriate accommodations were made when drafting interpretation guidelines so that they could be disregarded for comparison purposes. These vWA artifacts were later more fully described by McLaren *et. al*. and in 2008 the chemistry was modified by Promega to correct for the post-amplification secondary structure formations causing these peaks [McLaren *et. al*., 2008]. For the purposes of the studies herein, the n-10 artifacts, which present as off ladder peaks in GeneMapper™ ID electrpherograms, are amended to read "artifact" instead of the default "OL" designation.

2.3.2 Interpretation Threshold Determination

Analytical and/or interpretation threshold values can be established in many ways. Certainly, observations of numerous profiles can provide insight for an expected level of noise in the profile electropherogram. However, in an effort to arrive at a meaningful analytical threshold value a statistical approach was taken for this study.

The results of the ten standard reference material extracts obtained from NIST were analyzed down to 25 RFU so that data could be obtained from noise and background peaks. Peaks resulting from true alleles or predictable artifacts (stutter and pull-up) were not used for this study. RFU values were compiled for 236 noise peaks present within the ten profiles. A mean and standard deviation was calculated for these values.

Using Z values for confidence limits, it was determined that a threshold of 70 RFU would eliminate 94.63% of extraneous peaks using the following calculation and values obtained from the noise peak data:

$Z=(x-m)/s$

where:
x is the RFU cut-off value (70)
m is the average value of noise peaks (41.02)
s represents the standard deviation of noise peak values (18.03)
therefore:

$Z=(70-41.02)/18.03=1.61$

The percent of extraneous peaks eliminated was determined by the proportions of a normal curve which lie beyond this Z value (determined by reference to a standard Z table using the 1.61 value). Using this approach, it was determined that a 100 RFU value would eliminate 99.95% of extraneous peaks. Table 2.8 describes results obtained for a variety of threshold values using this calculation.

Z value	Extraneous Peaks Eliminated (%)	Threshold (RFU)
1.05	85.31	60
1.61	94.63	70
1.88	96.99	75
2.72	99.67	90
3.27	99.95	100
3.59	99.98	106

Table 2.8: Z-value calculations for various threshold settings. This table describes the percentage of extraneous peaks expected to be eliminated at various threshold values as determined by Z values.

Another approach to determining threshold value is to simply calculate the mean and standard deviation of noise peak intensity and apply a setting that is three standard deviations in excess of the mean. From this data, that value would be 95.11 RFU.

While an interpretation threshold of 70 RFU was considered, it was noted upon analysis of mixture samples that some artifacts exceeded this value making interpretation more complicated. This was weighed against the amount of probative information that might be overlooked when a 100 RFU threshold was applied (loss of sensitivity), and since this was found to represent a relatively insignificant amount of data loss, the interpretational threshold was set at 100 RFU. Although 100 RFU is generally applied to electropherograms from forensic specimens, lower thresholds may be suitable for highly purified samples with low background; profiles analyzed at lower levels should be interpreted with caution.

Several considerations should be discussed when setting threshold values and how these values should be applied to determine if a peak was a true allele or baseline artifact. For example, the value was determined from pristine samples which would be expected to produce very little background noise. Higher background samples would inflate the average and if analyzed alongside pristine samples, significantly increase the standard deviation. Therefore, higher baseline may be observed in sample extracts containing high background "contaminants" and such profiles may warrant more conservative interpretation. Also, once heterozygote ratios are understood, it should be recognized that heterozygote peaks near the threshold value could have sisters below the threshold. Observation of this is evident in sensitivity studies and further addressed by exploration of extended injection durations (discussed later).

No comparable threshold study exists for the gel platform, as detection of a band was dependent upon the morphology and intensity of the band signal on the image. The gel analysis software does have automated band calling, however placement of bands is determined by the user, not by intensity-based analytical software settings. This was a major disadvantage to the gel platform, since no empirical or numeric detection

threshold can be discerned. Because of this, band calling was subject to interpretation, which may be affected by bleed-through, over subtraction, or even the experience of the analyst. Not only does threshold determination offer an empirical analytical limit and reduces discussion surrounding subjective allele determinations, it streamlines profile interpretation and the technical review process.

2.3.3 Stutter Cut-off Determination

Stutter bands, described as artifacts inherent to STR-PCR due to *Taq* polymerase slippage during amplification in Chapter 1, are usually evident as peaks one repeat smaller than the true/parent allele. Some loci exhibit more stutter than others, and the longer the repeat unit, the less stutter is observed. Because the presence of stutter can complicate mixture interpretation, establishing stutter cut-off values for each locus provides a useful tool for analyzing STR profiles.

Data generated from known reference blood samples and SRM extracts were used for this study. These profiles were analyzed down to 15 RFU and stutter peaks were identified across the profiles. For tetranucleotide repeat loci, these were n-4 bands; for Penta E and Penta D, these were n-5 bands. Percent stutter was calculated by dividing the RFU value of the stutter peak by that of the RFU value of the parent allele. Stutter values were used for this study regardless of whether the parent peak represented a heterozygote or homozygote peak and regardless of heterozygous peak pattern since this is the manner in which values will be applied. Stutter values were compiled for each locus by combining data from the various samples. Once compiled, the mean (x) percentage and standard deviation (σ) of the percentages for each locus were determined. For each locus, stutter cut-off values were then set at $x+3\sigma$ (rounded up to the nearest whole integer).

These values were compared to those included in the PowerPlex® 16 published developmental validation, which represents the collaborative validation of the system by numerous laboratories [Krenke, *et. al*., 2002]. Figure 2.6 and Table 2.9 summarize in-house data and comparison to the published values.

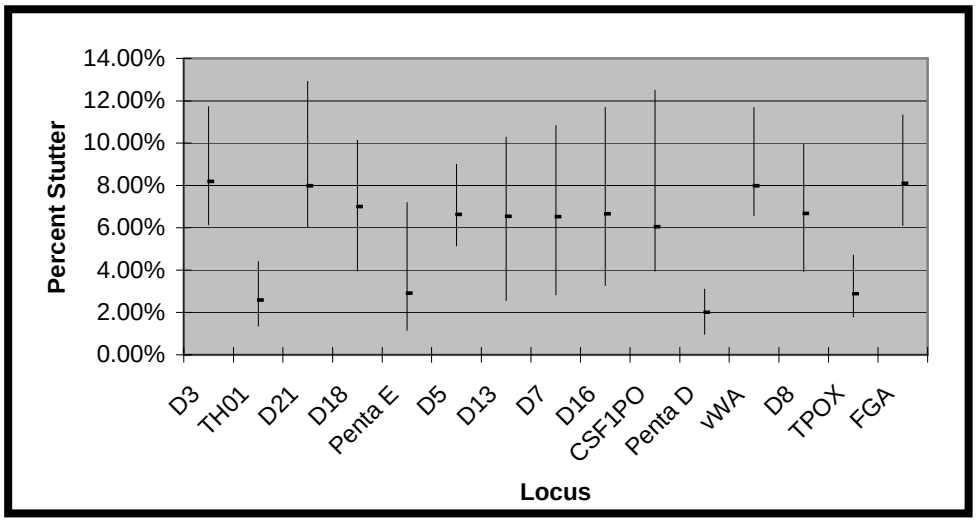


Figure 2.6: Summary of in-house stutter range observations. The graph illustrates the average percent stutter value for each locus (-), with minimum observed and maximum observed stutter values indicated (|).

Locus	Data Points	Mean	SD	In-house Cut-Off	Published Mean	FMBIO® cut-offs
FGA	*n=23*	8.1	2.0	15	7.4	9
TPOX	*n=19*	2.9	1.0	6	2.0	8
D8S1179	*n=21*	6.7	1.7	12	6.0	8
vWA	*n=18*	8.0	1.4	13	6.6	14
Penta E	*n=18*	3.0	1.5	8	2.5	2
D18S51	*n=25*	7.0	1.6	12	6.7	9
D21S11	*n=22*	8.0	1.7	14	8.7	10
TH01	*n=14*	2.6	0.9	6	1.8	5
D3S1358	*n=21*	8.2	2.1	15	7.8	10
Penta D	*n=8*	2.0	0.8	5	1.0	2
CSF1PO	*n=20*	6.0	2.0	13	4.5	11
D16S539	*n=18*	6.7	2.0	13	5.4	12
D7S820	*n=19*	6.5	1.8	12	4.0	9
D13S317	*n=17*	6.5	2.1	13	5.3	9
D5S818	*n=15*	6.6	1.0	10	5.5	11

Table 2.9: Stutter comparisons. Reported are the following for the PowerPlex® 16 – 3100-*Avant* platform: mean stutter percentage values for each locus, the number of data points incorporated into the mean, standard deviation, and the percent stutter values determined by $x+3\sigma$ after rounding up to the nearest whole integer. Also presented are PowerPlex® 16 published mean values [Krenke, *et. al*., 2002] and cut-off values previously applied for the PowerPlex® 16 BIO – FMBIO® II platform; these were determined by in-house validation using $x+3\sigma$ for each locus [Steadman, 2002b].

The results described here indicate that there is a great deal of stutter variation between loci. In-house and published data indicate that the loci with the highest average stutter percentages were vWA, FGA, D21S11, and D3S1358. Both D3S1358 and vWA represent loci with short amplicons, however D21S11 and FGA are moderate and long amplicon loci (respectively). Although the purpose of this study was not to elucidate the mechanism by which a locus falls susceptible to stutter, it is interesting to note that both D21S11 and FGA have a high degree of observed microvariant alleles. While the average observed stutter percentages may reflect trends in stutter propensity, cut-off ranges vary greatly because of standard deviation differences that occurred for each locus. For example, while CSF1PO exhibited a relatively low mean percentage, a great deal of variation was observed at the locus, resulting in an overall high cut off value (13%). Therefore, while the D5S818 mean percentage value was 6.6% (greater than CSF1PO at 6%), the standard deviation was lower at the D5S818 locus, resulting in a cut-off lower than that for CSF1PO (10% at D5S818 versus 13% at CSF1PO).

The published values were fairly consistent with trends observed in-house, and while it has already been explained why these values alone have limitations when determining appropriate cut-offs, it is useful to show that in-house observations are similar to that obtained by other users on some level. When compared to those values used for the gel platform, it appears that the values are lower overall for the capillary system, but that the trends from locus to locus are similar. Similarities are probably due to the fact the systems are designed with the same primers, and the elevated values for the gel platform are probably a function of higher standard deviation caused by variation in optical density for bands due to manual band and range placement when using the imaging software.

Most importantly, one can use the stutter data figure and table together to verify that determined cut-offs exceed the stutter percentage of the maximum observed stutter value at each locus, which indicates that the cut-offs are sufficient for proper filtering of slippage artifacts.

2.3.4 Specificity Studies

Although the PCR primers included in the PowerPlex® 16 chemistry are designed in accordance with human DNA sequences, non-specific priming and amplification may occur in non-human species. Levels of cross-priming should be evaluated against common domestic animals as well as upper primates. Therefore, DNA extracted from chimpanzee, orangutan, cat, dog, rat, rabbit, mouse, deer, dove, and cow were analyzed with this platform. An estimated 1.0 ng of target template from each species was provided in the PCR reaction, and was determined crudely by agarose gel and/or slot blot (upper primates can be quantified via blot).

DNA samples from rat, deer, mouse (non-primate mammals), and dove (bird) resulted in no peaks above 70 RFU. Three of the non-primate mammals, cat, rabbit, and cow exhibited single off-ladder peaks between 70 and 100 RFU. The off ladders appeared at vWA for cat and at D5S818 for rabbit and cow. A substantial peak shorter than Amelogenin X was generated from dog. Because upper primate samples had been run

for the PowerPlex® 16 BIO validation, results from the chimpanzee and orangutan were not surprising; these are summarized in Table 2.10.

Locus	Chimpanzee	Orangutan
D3S1358	15	15.2, weak 16,17
TH01	6,>10	4
D21S11	25.2	
D18S51	13.X, 14.X	
Penta E	10,11	5
D5S818	<7	9.X, weak 9
D13S317	12	9.X,13
D7S820	9.X	7,<7
D16S539	11.X	11.X,13.X
CSF1PO	9,11.X	
Penta D	3.X,4.X	<2.2
Amelogenin	X	X
vWA	12,13	
D8S1179	11,14	
TPOX	9, 10.X	
FGA	19.X, 20.X	31.2, 40.x

Table 2.10: Summary of upper primate profiles obtained from the PowerPlex® 16/3100-*Avant* platform. The table indicates peaks detected above 100 RFU for the upper primates. Bands indicated as weak were noted between 70 and 100 RFU. Where < or > is indicated, these peaks fell shorter or longer than bins for these loci. Documentation of these peaks by locus is for descriptive purposes only; verification that the peaks were generated from primers specific to the designated locus was not pursued.

These specificity studies indicate that conserved sequences between humans and upper primates can result in detection of peaks upon amplification of non-human template. However, more common domestic animals did not result in a substantial number of peaks, and when peaks were generated, they did not bin as alleles. Documentation of these off-ladder peaks are interesting; however, combined human and upper primate mixture samples are unlikely to be encountered in a forensic specimen. Because most non-human DNA extracts are not detected by human quantification methods, it is unlikely that a non-human blood sample would be

processed beyond the quantification without some concern for sample quantity visually as compared to data obtained from a quantitative method. Nevertheless, specificity limitations are all important aspect of the performance validation and the results herein indicate that the PowerPlex® 16 typing kit is sufficiently specific for human forensic DNA casework and research.

While not all of the above mentioned species were tested using the PowerPlex® 16 BIO – FMBIO® II platform (mouse, deer, and dove were not included), the upper primates examined for this performance verification were previously typed by the lab on the gel platform and yielded similar results. The PowerPlex® 16 BIO platform validation studies did not produce the band near Amelogenin from domestic dog, however a similar artifact was documented for the PowerPlex® 1.1 + Amelogenin chemistries [Steadman, 2002b]. Primer sequences between the 16-plex systems are the same, so this could be because different canines were used for the various validation studies. These results are expected due to primer conservation between the 16-plex systems and indicate that canines may express a conserved sequence within the Amelogenin priming sites.

2.3.5 Mixture Analysis

Since forensic samples often contain DNA from multiple sources, an understanding of detectable contribution levels for major and minor contributors to a mixture should be pursued during the course of performance verification.

It is acknowledged that forensic mixtures are often comprised of more than two individuals, however, the purpose of this study is to observe the dynamic range of minor contributions that can or cannot be detected in the presence of equal or greater amounts of additional contributors and to verify the system's capability to detect allele intensities in a manner consistent with the predicted ratios. This study sets an example for limitations of mixture resolution and demonstrates the complex nature of differentiating between true minor alleles and system artifacts. The behavior of these samples will offer some insight, although limited, for three or more contributor mixture interpretations, should this project present the need for such applications.

The mixture samples used for this study were supplied by Promega; these were previously obtained for the purposes of a collaborative study involving multiple laboratories and had been previously analyzed using the PowerPlex® 16 BIO system. The DNA concentration, as determined by Promega, was 0.4 ng/μL; a volume of 1 μL was used in each amplification. The concentrations were not further verified since this standard amount had been analyzed with the PowerPlex® 16 BIO system and consistency in reaction template quantity would allow for a more direct comparison of platform mixture detection capability.

The Male:Female ratios amplified were as follows: 1:0, 19:1, 9:1, 4:1, 2:1, 1:1, 1:2, 1:4, 1:9, 1:19, and 0:1. This corresponds to 100%, 95%, 90%, 80%, 66.7%, and 50% contributorship analysis for each donor.

The profiles were analyzed at 100 RFU, with in-house stutter cut-offs. Given the multitude of detected alleles, the results are best presented in summary. Minor components of most loci were detected at mixture ratios of 1:9 (M:F and F:M). Minor components were detectable, although partial, when the minor component comprised as little as 5% of the mixture. For these pristine samples, the most robust loci seemed to be D8S1179, Penta E, and D18S51 for detection of minor components, but were not necessarily the most robust loci (based on intensity) within the profiles overall. Other loci where minor contributor was detected at 5% were FGA, vWA, TH01, Penta D, D16S539, D13S317, and Amelogenin. While amplification of minor contributor occurred at these loci, several important considerations should be taken when interpreting this data. First, these mixture studies were not carried out in replicate, nor were they repeated using different donors when preparing the mixture ratios. The ability of a locus to resolve minor contributors is a function of primer binding efficiency, stochastic effects taking place in early rounds of amplification, fluorophore detection and separation parameters, and the composite profile of mixed alleles being detected at the locus. So while amplification and detection limitations maintain a certain expected level of stability, the composition of the mixture alleles also affects mixture resolution. For example, in a 4:1 male to female mixture, if the

male is homozygous at a locus and the female heterozygous, sharing an allele with the male, the sister of the shared allele may fall below the detection threshold if the profile is optimized for the detection of the shared allele. Since the composition of mixtures over 16 loci become complicated and could be researched at length, the exercise demonstrates the dynamics of mixture detection and loss of allele detection across a wide range of contributor levels. Certainly one could make quite an interesting study of the dynamics of mixture ratio detection if each locus was studied for multiple mixtures of different paired sources amplified in replicate, the purpose of this study is to verify the performance of the system in-house. This verification was apparent based on the allele intensity differences observed from these mixture ratio samples.

Mixture studies from the PowerPlex® 16 BIO validation yielded similar results with minor components detectable, although partially, at 1:19 ratio. Most minor contributions were detected at the 1:9 ratio, however the trends in the PowerPlex® 16 BIO system seemed to be more fluorophore or detection based. The three channels appeared to exhibit different sensitivity levels with respect to minor component detected, with the JOE dye-labeled loci being least robust and allowing full detection of minor contributors only at the 1:4 ratio. The RRX and fluorescein channels were stronger, with full detection at 1:9 and partial detection at 1:19.

2.3.6 Precision Studies

Forensic DNA analysis requires the use of commercial internal lane standards of known size for the determination of DNA fragment sizes generated from samples. The applicability of the PowerPlex® 16 system for typing depends on reproducible base pair size designations, especially when GeneMapper™ ID software is employed for allelic determinations. The objective of this study was to explore the precision and reproducibility of amplicon length determinations and encompassed three different studies. The first study consisted of an inter-run precision analysis of allelic ladder band base pair size determinations and summarized data from twelve ladders run on seven different occasions. The second study is an intra-run precision analysis of allelic ladder band base pair size determinations and was further divided into two

mini studies. The first mini study investigates size determination of ladders from seven different injections and wells during one run; the second mini study explores size determination of ladders from two different wells (G10 and H10) injected six times each using different injection durations. The third and final study consists of an inter-capillary and inter-injection study, which allows the evaluation of differences between capillaries in the same array and differences between injections made by the same capillary.

2.3.6.1 Inter-run Precision Analysis

For the first study, inter-run ladder precision was assessed by importing all ladders into a single GeneMapper™ ID project and selecting one ladder as the allelic ladder, while leaving the others to be sized as samples. RFU cut-off ranges were modified, up to a 500 RFU threshold, to limit characterization of insignificant peaks. Size information tables were then exported to Excel so that sizing columns could be aligned. The mean base pair size and standard deviation was calculated for each ladder peak. The average of the standard deviations was calculated for each locus and 3σ was then determined per locus, these values are presented in Table 2.11.

LOCUS	3σ 3100-*Avant*	3σ FMBIO® II
D3S1358	0.597	0.31
TH01	0.462	0.35
D21S11	0.552	0.59
D18S51	0.480	1.72
Penta E	0.480	0.94
D5S818	0.603	0.23
D13S317	0.462	0.29
D7S820	0.396	0.42
D16S539	0.291	0.55
CSF1PO	0.396	0.91
Penta D	0.426	2.15
Amelogenin	0.096	NA
vWA	0.111	0.30
D8S1179	0.123	0.34
TPOX	0.147	0.52
FGA	0.159	0.92

Table 2.11: Precision data from inter-run ladder analysis. The table reports three times the average of the standard deviation values observed for alleles at each locus; data was compiled from 12 ladders from 7 separate runs. Also reported are the most comparable calculations obtained from inter-gel PowerPlex® 16 BIO studies; data was compiled from 33 ladders run on 8 gels.

2.3.6.2 Intra-run Precision Analysis

For the second study (intra-run ladder studies), seven ladders from different plate wells injected within the same run were added to a single GeneMapper™ ID project (project 1). Another project was created containing twelve ladders within a run, generated from 1, 3, 5, 7, 9, and 11 second injections of two ladders from different wells on the plate (project 2). For each project, one ladder was designated as such and the others were sized as samples. As for the inter-plate analysis, the threshold was increased to minimize characterization of insignificant peaks. Size tables were exported to Excel so that columns could be aligned. The average of the standard deviations was calculated for each locus and 3σ was then determined per locus as previously described, these values are presented in Table 2.12.

LOCUS	3σ PROJECT 1	3σ PROJECT 2	3σ FMBIO® II
D3S1358	0.090	0.096	0.16
TH01	0.081	0.102	0.18
D21S11	0.117	0.087	0.29
D18S51	0.105	0.105	0.45
Penta E	0.102	0.126	0.77
D5S818	0.093	0.096	0.12
D13S317	0.090	0.090	0.17
D7S820	0.102	0.087	0.24
D16S539	0.096	0.105	0.35
CSF1PO	0.117	0.102	0.42
Penta D	0.126	0.120	0.54
Amelogenin	0.075	0.108	NA
vWA	0.078	0.090	0.18
D8S1179	0.105	0.078	0.23
TPOX	0.102	0.096	0.30
FGA	0.111	0.108	0.65

Table 2.12: Precision data from intra-run ladder analysis. The table reports three times the average of the standard deviation values observed for alleles at each locus. Project 1 represents analysis of seven ladders injected from different wells of the sample plate/run. Project 2 represents analysis results of ladders injected for various durations. Also reported are the comparable calculations obtained from intra-gel PowerPlex® 16 BIO studies; values derived from 11 ladders from one gel (NA=not analyzed).

Collectively, the precision studies indicate that a high degree of precision is exhibited by this system, which is demonstrated by the small standard deviation between allele calls, even when samples from different plate runs were sized against each other. The data is presented in the form of three times the average of the standard deviation observed per locus because this is intended to provide some insight for a 99.7% confidence interval that would result for an allele call at a locus [Johnson and Bhattacharyya, 1996]. This value is important to consider because the GeneMapper™ ID software employs binning windows when designating discrete alleles. Therefore, the expected fluctuation between designations was investigated to make sure that the range falls within the window used by the software for allele designation (±0.5 base pairs) [Applied Biosystems, 2003]. The sizing conducted between plate runs (inter-plate studies) did not all fall within this range, this indicates the importance of including ladders on each plate run. For the gel platform, a general trend existed where short fragment size determinations exhibited more precision than long fragment

sizes, and this was related to the detection platform since small differences in band placement at the top of the gel constituted a greater base-pair variance than placement differences at the bottom of the gel. The data from the gel platform validation illustrates this since the precision decreases as amplicon length increases for each channel; vWA has greater precision than FGA; D5S1358 has greater precision that Penta D; D3S1358 has greater precision than Penta E, and the loci between the shortest and longest progress according to amplicon length within each channel. The exception to this trend for the inter-gel studies was D18S51; this was likely due to the fact that the triplicate band patterns at this locus often had to be manually placed by the user, resulting in more variation than would occur during automated band placement. This same trend was not observed on the capillary platform. Loci that exceeded the range were D3S1358, D21S11, and D5S818 and these loci do not represent those with long amplicon products.

Inter-run differences may be due to differences in polymer viscosity, room temperature, capillary age, or a variety of factors that change over time between runs. Certainly, migration occurs differently when amplicons are separated with fresh polymer than occurs when separated with polymer that has been on the instrument for several days. Since these studies were all conducted using the same kit, the observed differences are not due to ILS lot-to-lot variation, and can be attributed to environmental and detection factors that one should expect throughout the course of thesis or casework analysis.

When implementing a new platform, it is of interest if the new technology performs as well or better than that previously used by the lab. While the main advantage to capillary detection, compared to gel platforms, is automation of the typing process, system performance aspects should also be recognized. Since the platforms exhibited very different trends between loci, a parallel cannot be drawn between the systems. However, while a gross representation of overall platform precision, it was noted that the average of the inter-run 3σ values reported across all loci for the 3100-*Avant* was 0.379, while comparable studies using the gel platform (inter-gel) was nearly twice as great (0.703).

More applicable to practice is the precision achieved within a run since samples are sized by ladders from the same run plate; this is encompassed by the second part of the precision testing (intra-plate). All of the estimated intervals from the intra-plate studies fell well within the ±0.5 base pairs range, indicating the sizing can be expected to occur at a level of precision satisfactory for allele designation. Very little difference occurred between the different projects, when values are compared locus to locus. And while there is no amplicon size trend exhibited by the capillary detection system similar to that so clearly present on the gel platform, the intra-run table illustrates that precision range estimates from the capillary platform are much tighter than that achieved within a gel. For each locus, the estimates seem to indicate that the capillary detection system out-performs the gel platform with respect to sizing precision.

2.3.6.3 Capillary and Injection Variance

The third precision study was related to differences between capillaries and injections of like samples and actually consisted of three studies: intra-array capillary precision, inter-injection capillary precision, and reinjection precision. These were conducted using twelve "identical" sample wells that were prepared by mixing an amplified sample with internal lane standard/formamide in a cocktail prior to aliquotting the mixture into the plate wells.

For *intra-array capillary precision*, three injections were performed to inject the twelve samples. Peak height values were analyzed for each injection; the mean, standard deviation, and ratio of the deviation to the mean (allelic standard deviation divided by mean allele RFU) was calculated for data from capillary 1-4 of injection 1; capillary 1-4 of injection 2, and capillary 1-4 of injection 3. See Table 2.13 for a summary of these calculations. For *inter-injection capillary precision,* the same peak height data was used, but analyzed from the perspective of differences between the capillaries during a single injection. For this study, the mean, standard deviation, and ratio of the deviation to the mean (allelic standard deviation divided by mean allele RFU) was calculated for data from capillary 1 for three injections, capillary 2 for

three injections, capillary 3 for three injections, and capillary 4 for three injections. The data is compiled in Table 2.14a/b. Finally, the *reinjection precision* was conducted where capillaries reinjected sample from the same well three times and differences in peak height values observed between these injections were considered. For this study, the mean, standard deviation, and ratio of the deviation to the mean (allelic standard deviation divided by mean allele RFU) was calculated for data from a sample injected three times using capillary 1, a sample injected three times using capillary 2, a sample injected three times using capillary 3, and a sample injected three times using capillary 4. Reinjection data is presented in Table 2.15a/b. This further standardizes capillary performance because even though the wells were prepared from the same cocktail, the contents may not truly be "identical" due to pipetting error. Furthermore, it reveals whether sample depletion results from reinjecting a sample multiple times.

Locus	Allele	Inj 1 χ	Inj 1 σ	Inj 1 σ:χ	Inj 2 χ	Inj 2 σ	Inj 2 σ:χ	Inj 3 χ	Inj 3 σ	Inj 3 σ:χ
D3S1358	14	766.25	68.59	0.090	772.00	49.48	0.064	762.50	99.99	0.131
D3S1358	15	888.25	74.60	0.084	898.00	55.86	0.062	874.50	105.77	0.121
TH01	8	650.00	67.31	0.104	657.75	39.85	0.061	655.00	77.65	0.119
TH01	9.3	675.25	52.53	0.078	688.50	39.75	0.058	683.00	84.09	0.123
D21S11	30	2649.75	229.78	0.087	2701.50	180.83	0.067	2690.00	342.78	0.127
D18S51	15	1690.50	166.92	0.099	1711.00	126.33	0.074	1742.50	228.83	0.131
D18S51	19	1733.75	171.48	0.099	1767.25	130.58	0.074	1779.25	229.85	0.129
Penta_E	12	1765.75	207.07	0.117	1817.00	138.57	0.076	1826.75	250.34	0.137
Penta_E	13	2332.00	249.42	0.107	2396.00	187.42	0.078	2402.50	313.81	0.131
D5S818	11	2258.00	197.12	0.087	2299.00	175.98	0.077	2252.00	294.28	0.131
D13S317	11	1812.75	169.24	0.093	1843.00	118.65	0.064	1814.75	233.90	0.129
D7S820	10	904.75	93.23	0.103	924.75	72.85	0.079	907.75	123.10	0.136
D7S820	11	674.50	61.13	0.091	686.25	50.61	0.074	677.75	89.80	0.133
D16S539	11	1115.00	113.13	0.101	1137.50	94.88	0.083	1132.00	148.97	0.132
D16S539	12	905.00	91.71	0.101	921.00	70.60	0.077	918.50	125.17	0.136
CSF1PO	10	1372.25	146.08	0.106	1394.25	122.86	0.088	1404.25	193.03	0.137
CSF1PO	12	1667.25	183.61	0.110	1697.25	142.04	0.084	1703.25	239.25	0.140
Penta_D	12	2986.75	324.25	0.109	3051.50	280.70	0.092	3040.25	455.02	0.150
AMEL	X	1438.50	166.05	0.115	1456.25	142.71	0.098	1486.25	203.58	0.137
vWA	17	980.75	117.94	0.120	1004.50	98.21	0.098	1033.50	134.63	0.130
vWA	18	1175.50	138.36	0.118	1214.75	129.59	0.107	1247.00	162.91	0.131
D8S1179	13	2685.25	294.04	0.110	2739.00	261.46	0.095	2828.50	397.56	0.141
TPOX	8	1816.25	214.11	0.118	1859.25	186.29	0.100	1921.50	253.79	0.132
FGA	23	2105.50	248.98	0.118	2146.50	209.80	0.098	2244.50	312.62	0.139
FGA	24	1986.50	232.81	0.117	2040.00	215.54	0.106	2112.00	303.91	0.144

Table 2.13: Intra-array capillary precision results. The study investigates differences between capillaries during an injection. Therefore, these results represent data obtained from each of the four capillaries over three injections (twelve samples). The average allele peak heights (RFU) obtained, standard deviation, and σ/x from capillary 1 through 4 are reported in the table for each of the three injections.

Locus	Allele	Cap 1 χ	Cap 1 σ	Cap 1 σ:χ	Cap 2 χ	Cap 2 σ	Cap 2 σ:χ
D3S1358	14	751.67	38.70	0.051	680.67	24.79	0.036
D3S1358	15	868.67	46.61	0.054	797.00	41.39	0.052
TH01	8	642.00	25.94	0.040	582.00	34.12	0.059
TH01	9.3	670.00	21.17	0.032	610.00	29.55	0.048
D21S11	30	2622.33	102.83	0.039	2392.33	93.15	0.039
D18S51	15	1694.67	54.60	0.032	1511.67	59.37	0.039
D18S51	19	1737.33	66.65	0.038	1557.67	65.29	0.042
Penta_E	12	1788.67	71.12	0.040	1572.67	105.00	0.067
Penta_E	13	2373.67	100.23	0.042	2082.00	106.59	0.051
D5S818	11	2195.67	119.23	0.054	2016.67	71.81	0.036
D13S317	11	1784.00	78.26	0.044	1625.33	92.57	0.057
D7S820	10	891.33	50.52	0.057	804.00	41.87	0.052
D7S820	11	667.33	28.88	0.043	602.33	25.70	0.043
D16S539	11	1115.00	50.39	0.045	990.00	39.66	0.040
D16S539	12	902.67	39.55	0.044	804.33	38.89	0.048
CSF1PO	10	1381.67	61.60	0.045	1211.67	51.33	0.042
CSF1PO	12	1679.67	80.31	0.048	1467.00	67.45	0.046
Penta_D	12	3008.67	136.54	0.045	2631.67	136.29	0.052
AMEL	X	1262.67	23.97	0.019	1468.67	48.42	0.033
vWA	17	874.67	10.97	0.013	1011.67	46.61	0.046
vWA	18	1053.67	15.18	0.014	1215.33	72.07	0.059
D8S1179	13	2375.33	36.47	0.015	2778.00	126.35	0.045
TPOX	8	1611.00	8.54	0.005	1875.67	95.20	0.051
FGA	23	1853.67	11.24	0.006	2178.33	104.21	0.048
FGA	24	1750.00	13.53	0.008	2065.00	110.04	0.046

Table 2.14a: Inter-injection capillary precision results. The study investigated differences between injections from different wells performed by the same capillary. The results represent average, standard deviation, and σ/*x* for allelic peak heights (RFU) obtained for capillary 1 and 2.

Locus	Allele	Cap 3 χ	Cap 3 σ	Cap 3 σ:χ	Cap 4 χ	Cap 4 σ	Cap 4 σ:χ
D3S1358	14	785.00	9.54	0.012	905.50	16.34	0.018
D3S1358	15	905.00	22.11	0.024	1048.75	33.95	0.032
TH01	8	669.33	21.13	0.032	776.25	21.09	0.027
TH01	9.3	703.00	19.08	0.027	812.75	27.01	0.033
D21S11	30	2727.67	47.23	0.017	3209.75	64.72	0.020
D18S51	15	1731.33	71.59	0.041	2073.75	35.61	0.017
D18S51	19	1771.00	59.57	0.034	2151.75	57.76	0.027
Penta_E	12	1816.33	56.19	0.031	2219.50	44.59	0.020
Penta_E	13	2385.00	73.78	0.031	2892.00	58.55	0.020
D5S818	11	2340.33	34.70	0.015	2706.75	72.03	0.027
D13S317	11	1856.67	55.08	0.030	2195.00	66.34	0.030
D7S820	10	927.67	27.01	0.029	1107.50	34.90	0.032
D7S820	11	688.33	12.42	0.018	825.75	25.24	0.031
D16S539	11	1138.00	31.51	0.028	1381.50	40.90	0.030
D16S539	12	923.33	31.72	0.034	1114.00	28.80	0.026
CSF1PO	10	1397.00	52.00	0.037	1702.25	37.88	0.022
CSF1PO	12	1703.33	60.53	0.036	2077.75	55.58	0.027
Penta_D	12	3004.00	115.31	0.038	3751.75	103.24	0.028
AMEL	X	1672.00	60.02	0.036	1546.50	32.89	0.021
vWA	17	1151.67	39.00	0.034	1071.50	17.14	0.016
vWA	18	1391.00	42.30	0.030	1290.50	18.52	0.014
D8S1179	13	3133.33	157.13	0.050	2949.50	64.88	0.022
TPOX	8	2132.67	83.97	0.039	2011.00	49.65	0.025
FGA	23	2470.33	125.99	0.051	2349.00	41.20	0.018
FGA	24	2349.00	116.81	0.050	2209.50	55.16	0.025

Table 2.14b: Inter-injection capillary precision results. The study investigated differences between injections from different wells performed by the same capillary. The results represent average, standard deviation, and σ/*x* for allelic peak heights (RFU) obtained for capillary 3 and 4.

Locus	Allele	Inj 1 χ	Inj 1 σ	Inj 1 σ:χ	Inj 2 χ	Inj 2 σ	Inj 2 σ:χ
D3S1358	14	763.50	41.13	0.054	696.00	25.07	0.036
D3S1358	15	890.25	54.01	0.061	812.50	38.93	0.048
TH01	8	663.25	37.62	0.057	603.75	22.69	0.038
TH01	9.3	686.25	31.12	0.045	626.75	28.04	0.045
D21S11	30	2711.00	153.23	0.057	2465.50	88.68	0.036
D18S51	15	1749.75	87.72	0.050	1576.75	52.77	0.033
D18S51	19	1814.50	110.00	0.061	1623.50	53.05	0.033
Penta_E	12	1848.25	105.09	0.057	1637.25	46.61	0.028
Penta_E	13	2451.00	135.15	0.055	2182.75	74.04	0.034
D5S818	11	2253.25	145.88	0.065	2071.00	77.37	0.037
D13S317	11	1830.00	103.73	0.057	1677.75	77.98	0.046
D7S820	10	914.75	63.24	0.069	832.00	32.53	0.039
D7S820	11	691.25	41.71	0.060	627.75	30.65	0.049
D16S539	11	1156.50	73.47	0.064	1033.75	45.22	0.044
D16S539	12	936.75	56.69	0.061	843.00	42.92	0.051
CSF1PO	10	1423.50	81.79	0.057	1261.75	39.63	0.031
CSF1PO	12	1738.25	106.97	0.062	1540.25	58.55	0.038
Penta_D	12	3127.50	201.73	0.065	2714.00	108.46	0.040
AMEL	X	1298.25	46.77	0.036	1513.75	29.94	0.020
vWA	17	901.75	29.38	0.033	1052.50	15.70	0.015
vWA	18	1081.00	30.99	0.029	1266.50	15.07	0.012
D8S1179	13	2459.00	89.07	0.036	2906.75	67.62	0.023
TPOX	8	1676.50	58.75	0.035	1974.50	37.62	0.019
FGA	23	1936.25	67.84	0.035	2292.50	37.28	0.016
FGA	24	1829.00	68.68	0.038	2171.25	46.42	0.021

Table 2.15a: Reinjection precision results. The study investigated differences between injections from any single capillary injecting repeatedly from the same well. The results reflect the average, standard deviation, and σ/x for allelic peak heights (RFU) obtained for capillary 1 and 2 reinjections.

Locus	Allele	Inj 3 χ	Inj 3 σ	Inj 3 σ:χ	Inj 4 χ	Inj 4 σ	Inj 4 σ:χ
D3S1358	14	810.50	18.38	0.023	905.50	16.34	0.018
D3S1358	15	939.25	20.79	0.022	1048.75	33.95	0.032
TH01	8	694.25	22.50	0.032	776.25	21.09	0.027
TH01	9.3	721.50	16.90	0.023	812.75	27.01	0.033
D21S11	30	2751.75	130.38	0.047	3209.75	64.72	0.020
D18S51	15	1762.25	112.83	0.064	2073.75	35.61	0.017
D18S51	19	1785.25	132.13	0.074	2151.75	57.76	0.027
Penta_E	12	1801.50	207.16	0.115	2219.50	44.59	0.020
Penta_E	13	2355.50	303.06	0.129	2892.00	58.55	0.020
D5S818	11	2428.75	45.07	0.019	2706.75	72.03	0.027
D13S317	11	1895.00	85.90	0.045	2195.00	66.34	0.030
D7S820	10	937.50	49.68	0.053	1107.50	34.90	0.032
D7S820	11	696.25	37.36	0.054	825.75	25.24	0.031
D16S539	11	1147.50	73.34	0.064	1381.50	40.90	0.030
D16S539	12	933.25	56.16	0.060	1114.00	28.80	0.026
CSF1PO	10	1396.00	109.37	0.078	1702.25	37.88	0.022
CSF1PO	12	1705.75	131.74	0.077	2077.75	55.58	0.027
Penta_D	12	2958.25	370.97	0.125	3751.75	103.24	0.028
AMEL	X	1745.00	37.71	0.022	1546.50	32.89	0.021
vWA	17	1188.75	25.43	0.021	1071.50	17.14	0.016
vWA	18	1434.00	35.21	0.025	1290.50	18.52	0.014
D8S1179	13	3232.50	182.85	0.057	2949.50	64.88	0.022
TPOX	8	2203.50	123.67	0.056	2011.00	49.65	0.025
FGA	23	2513.75	215.04	0.086	2349.00	41.20	0.018
FGA	24	2384.25	212.19	0.089	2209.50	55.16	0.025

Table 2.15b: Reinjection precision results. The study investigated differences between injections from any single capillary injecting repeatedly from the same well. The results reflect the average, standard deviation, and σ/x for allelic peak heights (RFU) obtained for capillary 3 and 4 reinjections.

This third study (encompassing data sets summarized in Tables 2.13-2.15) provides information regarding variance that one may expect between samplings of like amplified products due to capillary performance. The standard deviation for the comparison between capillaries over three injections ranged from 39.75 to 455.02 base pairs, representing average peak heights of 688.50 to 3040.25 (respectively). The standard deviation to average peak height ratios for this range was 5.8% and 15%. The sample with the greatest standard deviation also reflected the highest standard deviation to average peak height ratio, and the sizing for this allele was lowest in capillary 2 at 2556 RFU and highest in capillary 4 at 3626 RFU (raw data not shown), the lower value is approximately 70% that of the larger. This represents the broadest observed ratio, and it should be noted that the raw intensity data clearly indicate that capillary 2 gave consistently lower peak heights and capillary 4 gave consistently higher peak height values, with capillaries 1 and 3 falling somewhere in

RFUbetween (raw data not shown). This indicates that not only does some variation occur between capillaries, and it suggests the differences may be inherent to the array or spatial settings used during detection of signal. Because multiple arrays were not used to conduct this study, this data alone is not sufficient for differentiating between these two possibilities. However, routine trouble-shooting data was provided to the array manufacturer after completion of this performance verification which documented substantial differences in capillary performance within an array. The study presented evidence that the peak pattern resulting from the spatial run for a newly installed array may be indicative of detection intensity differences expected between capillaries in the array and that these differences can be as much as 50-70% (data not shown).

Variation between injections from different wells by the same capillary indicated that less variation occurred than data comparisons made between different capillaries. This is based on the fact that the range for standard deviation was smaller as were the values constituting the least and greatest standard deviation (15.07 to 370.97). These were obtained from alleles with average peak heights of 1266.55 and 2958.25, therefore the standard deviation to peak height ratios were 1.2% and 12.5%. Interestingly, the allele with the greatest variation is the same as that with the greatest variation between capillaries, and represents a homozygote 12 at Penta D. While this is a long amplicon, the exact cause of this variation was not pursued. However it is noted that this locus did exhibit greater variation than the many others in this system during inter and intra plate studies. The other important thing to note regarding this trend is that often times locus-specific variance is a function of secondary structures formed, even though samples are injected from formamide and heated prior to injection. While this is one possible explanation for the observations here, documentation of this specific to Penta D has not been reported.

Finally, the study for reinjections resulted in a third range of variance. The standard deviations ranged from 8.54 to 157.13 for average peak heights of 1611.00 to 3133.33 (with standard deviation to peak height ratios of 0.5% to 5%). This shows that a high degree of precision is obtained upon reinjection and also indicates that for duplicate

samplings, optimal reproducibility will more likely be achieved if the same prepared well and capillary are used for the reinjection. The raw data (not shown) indicates that there was no trend toward sample "depletion" when a sample is injected up to three times, as the overall profile intensities did not increase or decrease progressively between injections.

2.3.6.4 Precision Overview

In summary, the capillary chemistry and detection instrumentation seem to exhibit a level of precision equal to or better than that of the gel-based chemistry and platform, and data explored through several minor studies allowed for an understanding of differences that occur between capillaries of the same array, between injections made of same samples prepared in different wells, and also variation that can occur when same sample preparations undergo multiple injections.

2.3.7 Sensitivity Studies

One key element for obtaining an optimal PCR profile is incorporating the appropriate amount of template DNA into the PCR reaction, especially in the multiplex reactions requiring balance between many primer sets. Excessive template causes imbalance between loci, peak saturation, and saturation related artifacts. Insufficient amounts of template in the reaction cause allele drop out due to stochastic effects and preferential amplification. A sensitivity study was conducted for the performance verification of the 3100-*Avant*, and that data will be presented first, with the primary goal of determining the least amount of template required for obtaining full profiles and optimized template and injection parameters. The second goal is to assess increased injection durations for profile enhancement. Additionally, crossover studies were conducted throughout the span of this thesis, allowing for the compilation of sensitivity data for the 3100-*Avant,* the 3130, and the 3130*u*. Discussion of some of this data offers a broad understanding of sensitivity of this platform throughout instrument upgrades and advancements in DNA quantity determination (progression from blots to real-time PCR). Therefore, these additional studies will be included and compared to sensitivity achieved by the gel platform and will also be used for an ancillary study which assesses heterozygote balance between sister alleles.

For the initial sensitivity study, DNA was quantified by slot blot and the following target template amounts amplified: 2.0 ng, 1.0 ng, 0.75 ng, 0.5 ng, 0.1 ng, 0.05 ng, and 0.025 ng. Following amplification, these samples were each injected for 1, 3, 5, 7, 9, and 11 second durations.

2.3.7.1 Visual Analysis

Because one important aspect of profile evaluation involves overall appearance, intensity, presence/absence of artifacts, examples of sensitivity study profiles are first presented. The FMBIO® II data is illustrated by gel imaging and is represented in Figure 2.7, while data obtained from the ABI PRISM® 3100-*Avant* consists of electropherograms and is represented in Figure 2.8 (a-d). The channel or pane containing data from FGA, TPOX, D8S1179, vWA, and Amelogenin were selected for presentation here. For the FMBIO® II, this constitutes loci tagged with Rhodamine Red™-X; for the ABI PRISM® 3100-*Avant*, the loci are tagged with carboxy-tetramethylrhodamine.

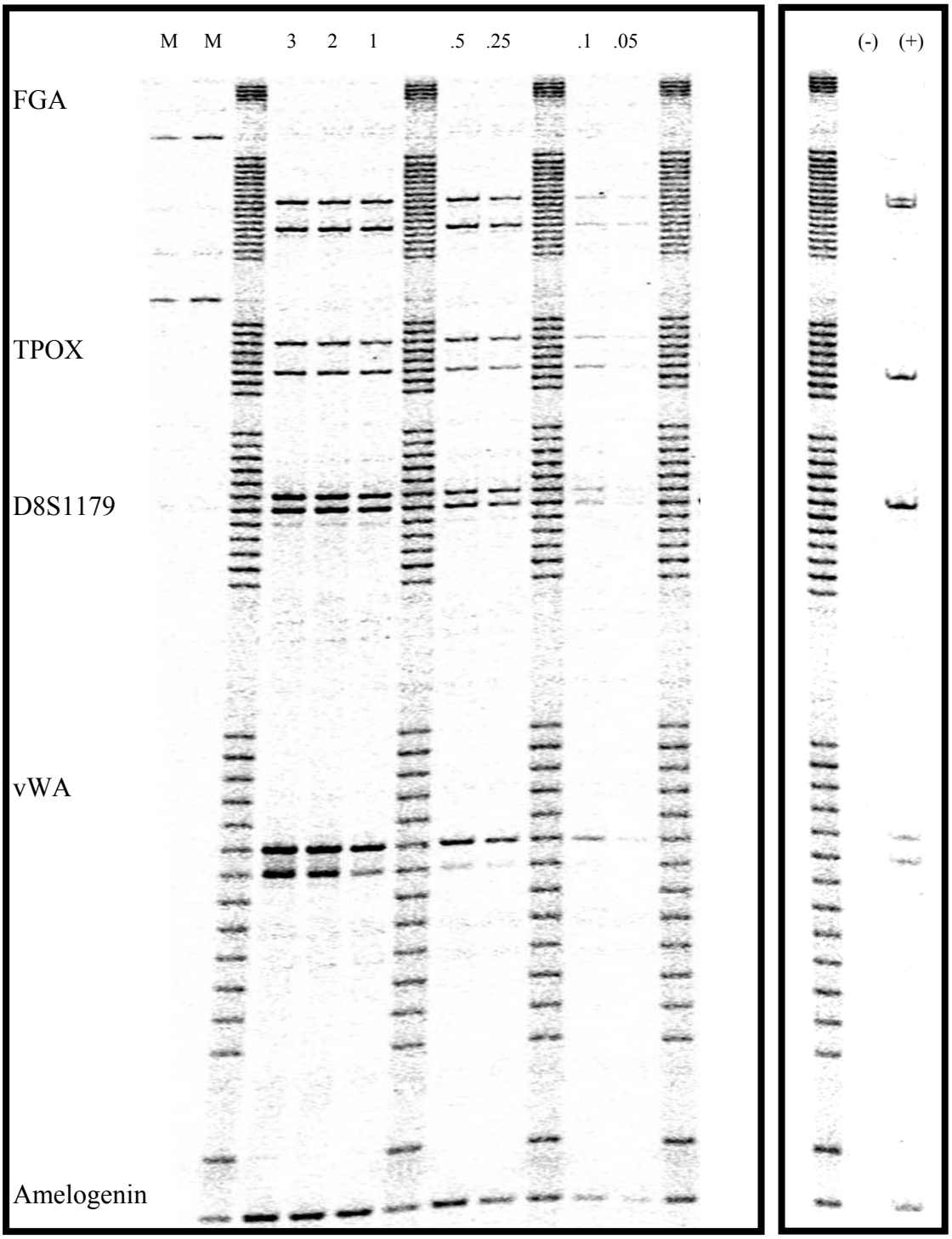
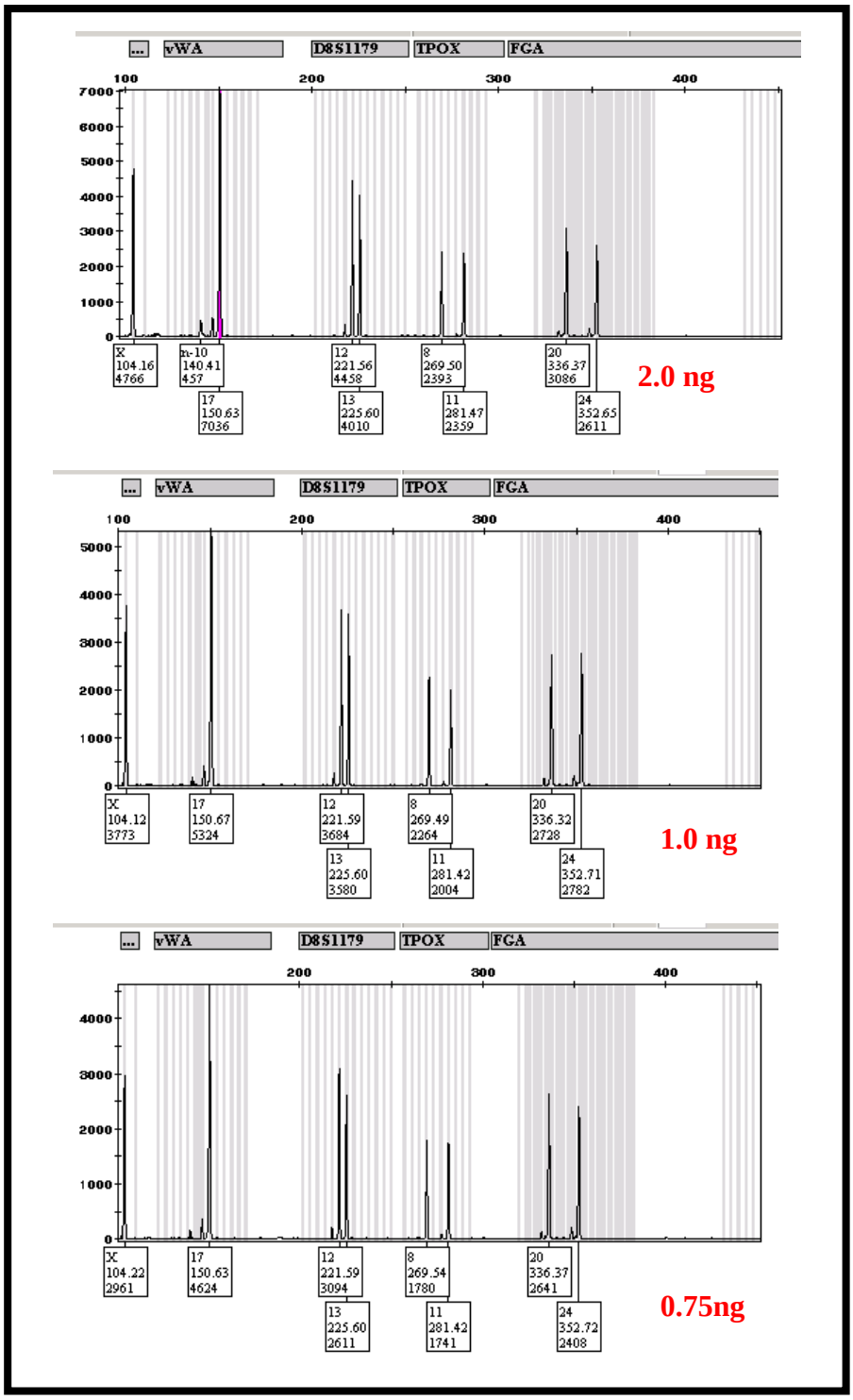


Figure 2.7: Sensitivity of the PowerPlex® 16 BIO/FMBIO® II systems. Rhodamine Red™-X loci are illustrated, with locus designations indicated next to the corresponding ladders. Template quantities amplified (ng) are indicated for each well. M=color separation matrix, (-)=negative amplification control, (+)=positive amplification control (9947A).



2.8a: Sensitivity of the PowerPlex® 16/ABI PRISM® 3100-*Avant* systems. Carboxy-tetramethylrhodamine labeled loci are illustrated, with locus designations indicated above corresponding bins. Template quantities amplified (ng) are indicated in red below each electropherogram, the profiles resulted from 5 second injections. Each peak label indicates allelic designation, base pair size, and peak height.

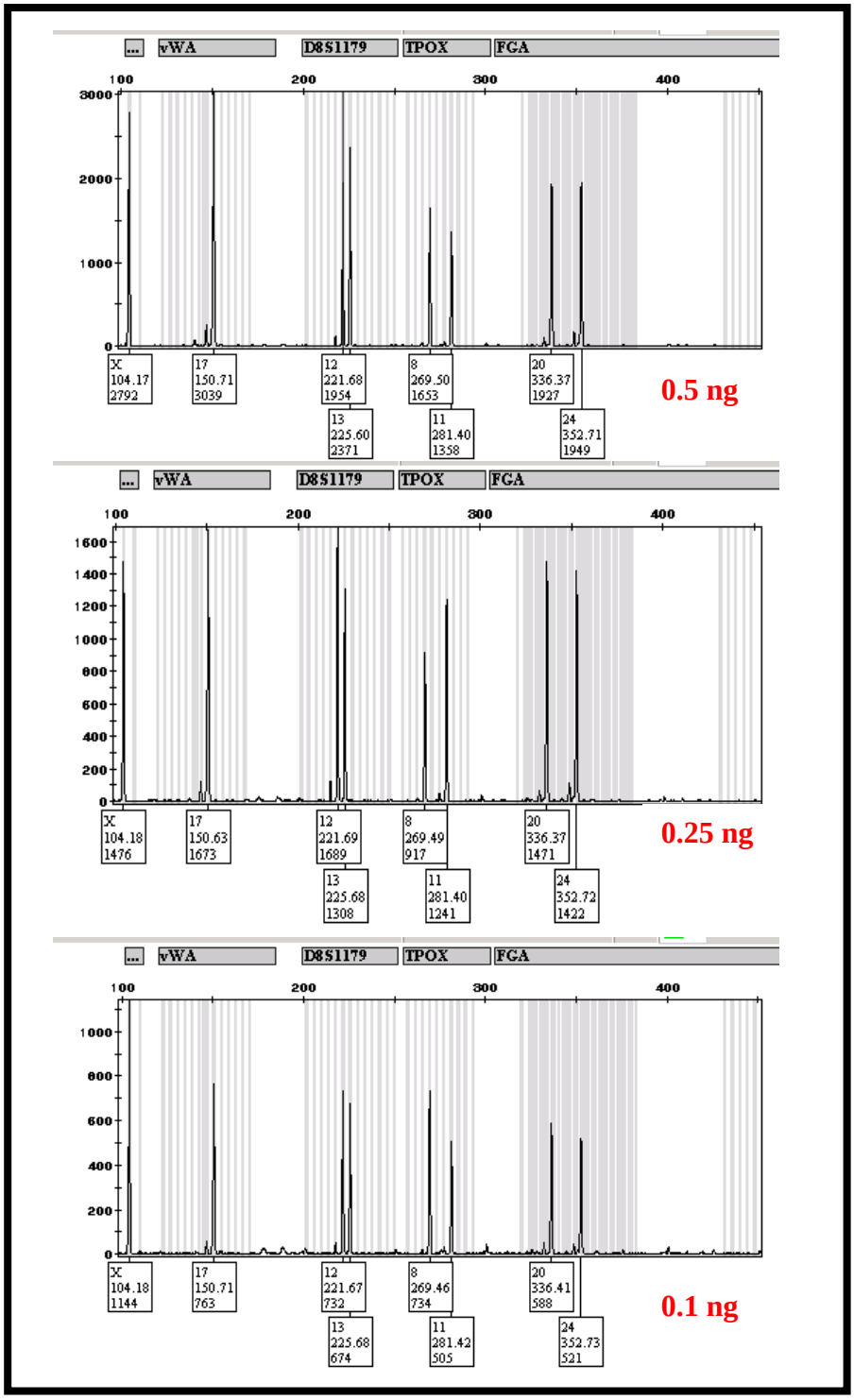


Figure 2.8b: Sensitivity of the PowerPlex® 16/ABI PRISM® 3100-*Avant* systems. Carboxy-tetramethylrhodamine labeled loci are illustrated, with locus designations indicated above corresponding bins. Template quantities amplified (ng) are indicated in red below each electropherogram, the profiles resulted from 5 second injections. Each peak label indicates allelic designation, base pair size, and peak height.

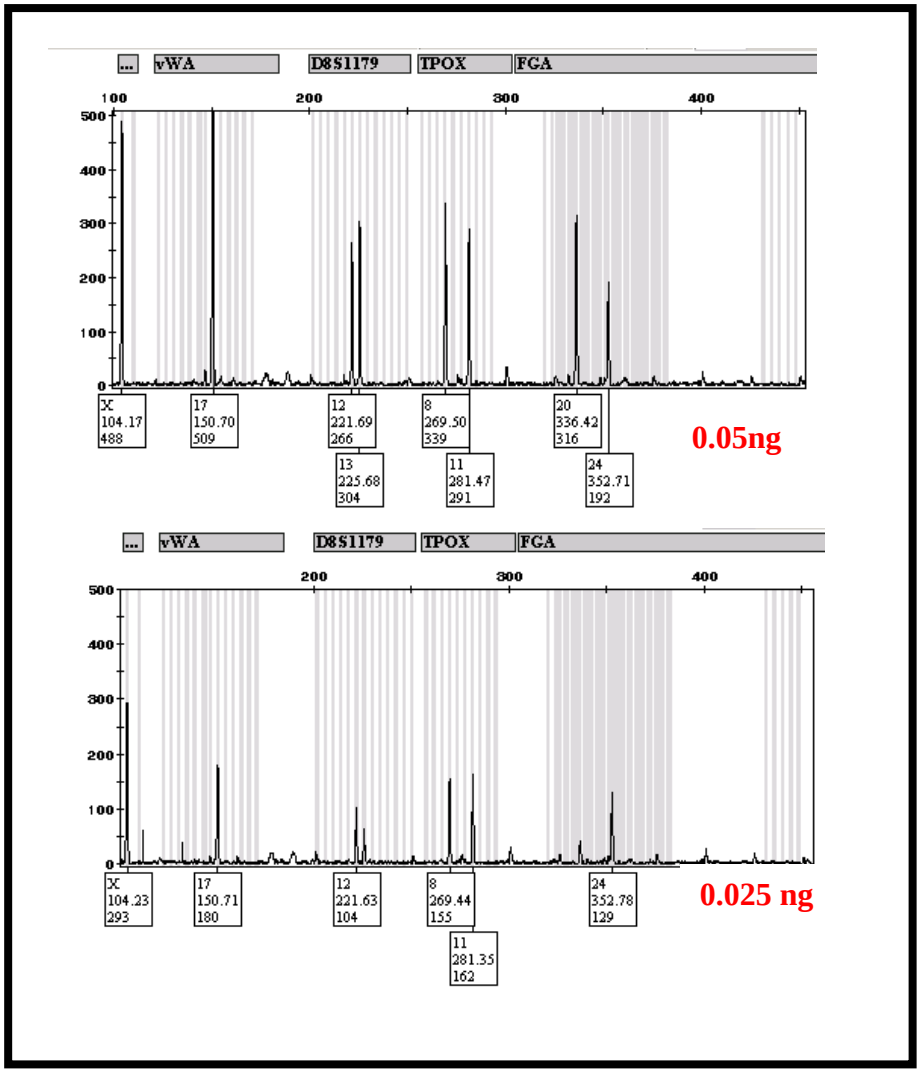


Figure 2.8c: Sensitivity of the PowerPlex® 16/ABI PRISM® 3100-*Avant* systems. Carboxy-tetramethylrhodamine labeled loci are illustrated, with locus designations indicated above corresponding bins. Template quantities amplified (ng) are indicated in red below each electropherogram, the profiles resulted from 5 second injections. Each peak label indicates allelic designation, base pair size, and peak height.

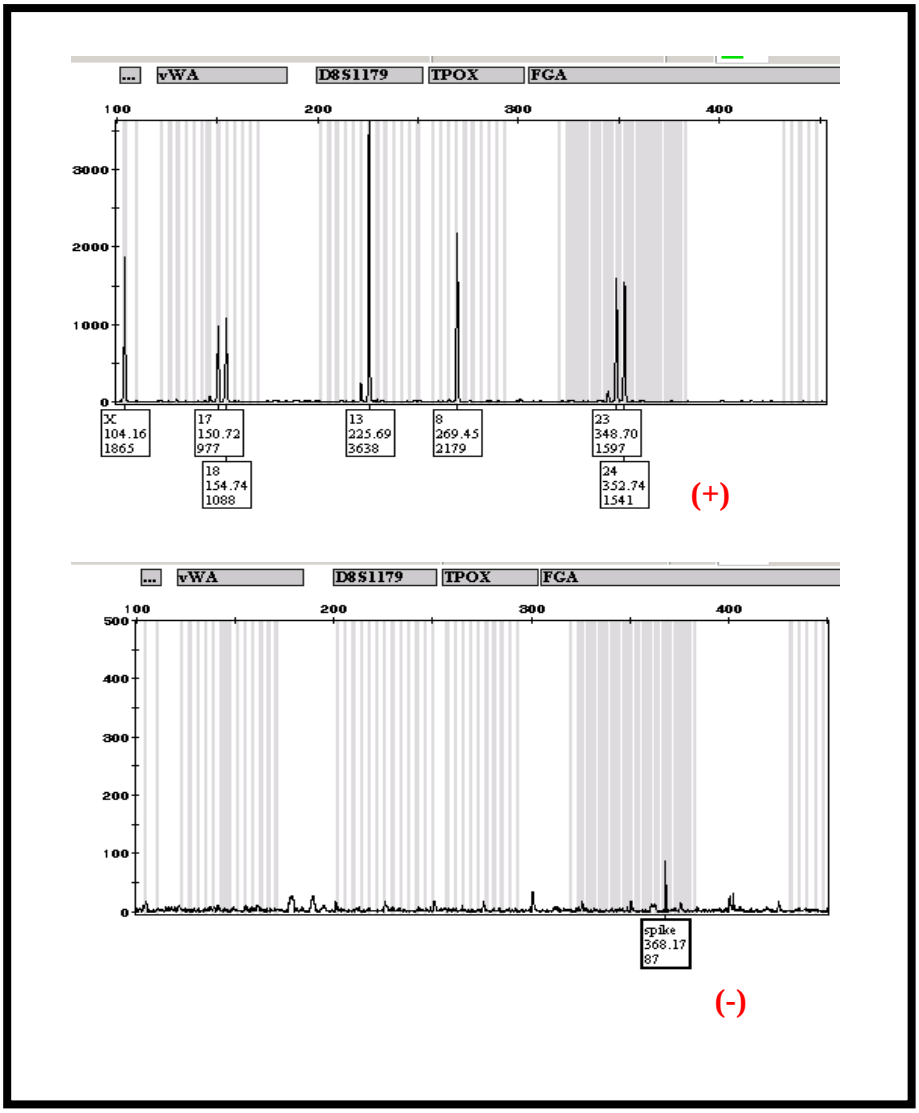


Figure 2.8d: Sensitivity of the PowerPlex® 16/ABI PRISM® 3100-*Avant* systems. Carboxy-tetramethylrhodamine labeled loci are illustrated, with locus designations indicated above corresponding bins. Positive and negative amplification controls are indicated in red below each electropherogram, the profiles resulted from 5 second injections. Each peak label indicates allelic designation, base pair size, and peak height.

The gel data generated by the PowerPlex® 16 BIO/FMBIO® II system can also be viewed such that each band is represented by a peak within the lane trace. The lane trace data from lanes representing 1, 0.5, and 0.25 ng of template for Rhodamine Red™-X loci from the PowerPlex® 16 BIO/FMBIO® II gel were analyzed and recorded in Figure 2.9.

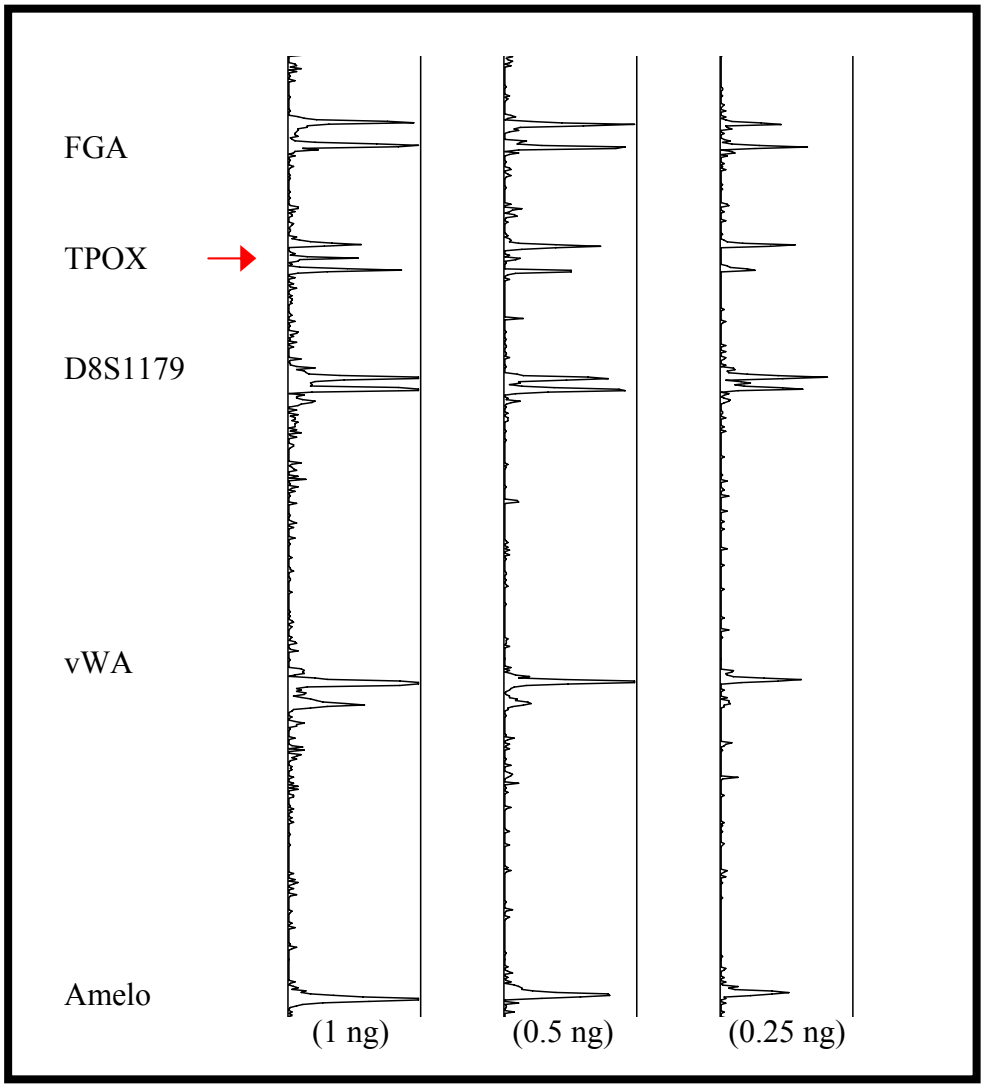


Figure 2.9: Lane trace view for PowerPlex® 16 BIO/ FMBIO® II data. Lanes where amplified products from template quantities of 1, 0.5, and 0.25 ng were analyzed are illustrated to provide an overview of optical density imbalance between and within loci for the PowerPlex® 16 BIO/ FMBIO® II data. The red arrow indicates a non-band artifact (debris on plate) that appears in this gel image's region of the lane.

Overall profile quality of the various template quantities amplified using the two systems can be observed visually at these analysis parameters, both the PowerPlex® 16 BIO/FMBIO® II data and the PowerPlex® 16/ABI PRISM® 3100-*Avant* data revealed that little saturation occurred at any loci where 0.5 ng template DNA was amplified. However, peak saturations started to occur at or greater than 1 ng template DNA at a number of loci in both systems, as reflected by signal intensity on the gel image (Figure 2.7), peak saturation on the lane trace (Figure 2.9) and peak heights exceeding 3000 RFU on electropherograms (Figures 2.8). At template quantities of 2 ng or greater, saturation was evident at most loci; saturation resulted in significantly elevated stutter at the vWA locus, where stutter band intensities began approaching that of true allele intensities on the gel image (Figure 2.7). Capillary data revealed that an n-10 artifact at vWA is detectable when template quantities of 0.5 ng or greater were amplified (Figure 2.8a/b), although the artifact would likely be far less predominant if a heterozygotic type was being observed at this locus.

2.3.7.2 Mathematical Analysis

In addition to visual evaluation of profile quality, mathematical analysis of peak height values was performed. When the amplification is optimized, sister allelic products, arising from amplification of the same locus of the homologous chromosomal pair, are expected to amplify at an equal rate to each other. The PowerPlex® 16 BIO/FMBIO® II system describes band intensity by optical density units, which is somewhat analogous to peak heights reported in relative fluorescent units (RFU) on capillary instruments. This results in equal band intensities or peak heights for sister alleles. Evaluation of relative imbalance between sister alleles was qualitatively performed with a heterozygote ratio calculation. This consisted of dividing one allele's OD or RFU value by the other allele's OD or RFU value at each locus; the smaller OD value served as the numerator and the larger OD value served as the denominator in the calculation. No calculation was performed in the event of allele drop-out (unobserved on the gel or below 100 RFU on the electropherogram) or homozygous type. For the capillary performance verification, the average of these heterozygote ratios was calculated across each profile/injection in an effort to achieve an overall imbalance level for each template quantity/injection duration combination.

Additionally, levels of imbalance occurring between (rather than within) loci was also investigated by calculating the average of heterozygote ratios for each dye within the profiles. These calculations were performed in an effort to generate a mathematical basis for selection of optimal template quantity and injection duration for standard samples processing. This information, along with general observations of profile quality and intensity were used to determine a standard target template quantity of 0.5 ng to be used with 5 second injection durations for most samples. These parameters were generally applied throughout the course of these studies (i.e. typing of knowns) as the various aspects of performance verification did not occur in isolation.

Results of these calculations are presented in Appendix 3 and can be approached from many directions. First, it was verified that the intensity of peak heights increased as injection durations increased for each template quantity. One exception to this trend was noted for the 0.25 template quantity when the 9 second injection was compared to the 11 second injection. Some alleles had slightly lower RFU values in the 11 second injection. For example, TPOX intensities were 1511/2077 for the 9 second injection but were 1539/2038 for the 11 second injection. Since the other injection durations performed predictably for this template quantity, this may be due to a spurious injection event (isolated poor injection).

Another point of interest was whether the heterozygote ratios could be used to draw any conclusions regarding optimal amplification and injection parameters. Predictably, heterozygote ratios were fairly stable for each template quantity regardless of injection duration. Since the ratio is a function of the amplification, this would be fully expected. However, ratios did differ slightly according to template quantity. The ranges of heterozygote ratios observed for each template amount are compiled in Table 2.16. Because the 0.25 target amount did not perform in a predictable manner (ratio was less than that observed for the 0.1 template quantity range), injections of 0.75, 0.5, 0.25, and 0.1 ng template quantities using 3, 5, 7, 9, and 11 second durations were repeated (1 second injections were not performed on the verification run). The raw data is presented in Appendix 3, and the repeated analysis generated the same trend for the 0.25 template sample. This indicates that

the discrepancy is not due to detection and is inherent to the sample dilution or amplification.

Template Quantity (ng)	Heterozygote Ratio Range (%) Initial Run	Heterozygote Ratio Range (%) Repeat Run
2.0	84.6-85.6	
1.0	84.6-85.6	
0.75	86.7-87.5	83.6-84.1
0.5	87.6-87.8	86.9-87.5
0.25	73.6-76.4	68.3-69.2
0.1	78.6-81.0*	78.3-80.6
0.05	72.7-76.1*	
0.025	61.5-64.7*	

Table 2.16: Heterozygote ratio ranges observed during sensitivity study. The table describes the amount of template used for the sensitivity series and reports the range of observed heterozygote ratios observed during various injection durations (1-11 seconds). *Indicates that some short injection duration ratios could not be considered; due to drop out the mean ratio values were erroneously inflated because profiles were obtained under low template/low injection duration conditions.

Another apparent discrepancy is that the 2.0 ng template quantity seemed to achieve better overall balance. However, this was likely due to peak saturation within many of the profiles, contributing to false impression that peaks are of similar intensity. In fact, many of the profiles were so saturated and contained so many artifacts that interpretation was difficult. This is one reason why overall profile quality was important to consider alongside the mathematical values.

While the sensitivity series could be repeated to further resolve the 0.25 ng imbalance progression discrepancy, it was determined that this would not be necessary for the laboratory's verification given that it was unlikely this template quantity would be selected as the optimal target. This was based on the fact that the 0.5 ng template quantity resulted in desirable peak height ranges across the profile; this was true for a range of injection durations. While the heterozygote imbalance did not perform in the expected manner for the 0.25 ng template, the overall profile intensity for a given

injection duration, did seem to fall between intensities achieved by the 0.5 ng-0.1 ng quantities at equal injection durations. While this does make the heterozygote ratio dilemma even more interesting, it indicates that the template quantity may have been in line with the dilution series, and that for some reason the PCR process was inefficient in early rounds of amplification for this particular sample. Repeating the study may have resolved the dilemma, and perhaps determined if it was dilution series dependent or PCR dependent (this would require repeating the series amplification/detection twice), but was unlikely to affect the decision regarding optimal template/injection parameters for the validation. Therefore, in the interest of very limited resources, the study was not repeated.

One last consideration for standard protocol optimization is that the data suggest a high degree of balance is achieved between panels at the 0.5 ng template quantity. This conclusion is based on the fact that the standard deviation: mean peak height ratio for the three colors is fairly stable between colors at this template amount. For example, Appendix 3 reports that the ratios are 17-18% at the 5ng/5 second sample regardless of panel. However, for the 0.75 ng/5 second injection the range was broader (8-24%). The broader range indicates greater standard deviation between panels, therefore optimal balance would be expected when this range is tight between fluorophore panels. And although the 0.25 ng results may be erroneous, the range between panels for this quantity were even greater and also generally higher, indicating more difference between panels and more difference between loci within a panel. Therefore, the selected target template quantity also seems to achieve satisfactory balance between fluorophore panels.

2.3.7.3 Minimum Template & Injection Duration

With a five second injection duration set as the standard for this instrument, some determination could then be made regarding the minimum amount of template required to achieve a complete STR profile for this source. A full profile was obtained at 0.1 ng template, and one allele dropped out at the 0.05 ng level. At 0.025 ng, eleven alleles fell below 100 RFU across 8 loci, constituting a loss of

approximately half the genetic information. Based on this, one may expect to achieve a full profile at template quantities between 50 and 100 pg input DNA.

In reviewing data obtained during sensitivity studies conducted with the gel platform, it was confirmed that the minimum amount of target required to produce a profile was similar, but that a full profile was achieved at 100 pg of DNA using the FMBIO® II. Drop out was evident at template levels lower than this. Although this may appear to indicate the gel platform was more sensitive, several considerations should be made. First of all, the same samples were not tested on both platforms; different dilution series were prepared for each and were based on different quantifications to determine the stock DNA concentration. These studies took place at very different time points in the laboratory, and even though they were conducted by the same individual, allele calling is a far less automated process using gel analysis software. Also, the minimum required target amount determined for the capillary verification was based on a 5 second ratio, and it is fair to say that if the same amount of individual bias be placed on this system as was commonly applied to the analysis of gels, the same minimum quantities would have been determined for these two platforms. Moreover, throughout the course of multiple performance verifications over time, there were studies on the capillary platform where 0.025 ng of DNA did result in a full profile (data not included); this is not unexpected given that each instrument has slightly different sensitivity, laser strength and that sensitivity dilution series cannot be exactly reproduced or stored in a stable manner over the course of several years. Therefore the apparent difference between the gel and 3100-*Avant* sensitivity study results may not be considered substantially different or beyond normal variation one might observe when similar studies are carried out at different time points. Because the studies were carried out at different times, using different quantification system to characterize of stock solutions, this abbreviated study merely illustrates that the host lab achieves results similar to published data [Tomsey, *et. al*. 2001]. The data comparison, while not exhaustive, indicates that the instruments operate within a similar sensitivity range, that sensitivities are comparable and likely suitable for thesis research purposes. It also provides a general understanding of template quantity required to achieve a full profile under standard STR typing conditions.

Another issue explored using data from Appendix 3 was the use of increased injection durations to enhance low-level profiles. The studies herein have shown that additional true alleles can be detected above the threshold and raw data from the electropherograms did not indicate an increase in detection of noise or artifacts when low level samples were injected longer. A good example of the usefulness of a longer duration is the example discussed above, where one allele fell below threshold when 50 pg of DNA were amplified and injected using a 5 second duration. When the sample was injected for durations of 7 seconds or greater, the peak was above 100 RFU and was about 75% that of the sister allele. There were not detrimental effects from the longer duration with respect to raised baseline. Alternatively, profiles with overall peak heights 1500-2500 RFU should not be injected for "enhancement" purposes because saturation will occur and artifacts will be present above the threshold. Therefore, while the studies show a practical advantage to the use of extended injection durations, they also show that this practice should be used on low level template samples that are fairly pristine in nature. Furthermore, interpretations should be cautious and heterozygote imbalances considered when determining if the amplification process was subject to strenuous stochastic effects and the PCR reliability more fully assessed.

2.3.7.4 Multi-Instrument Heterozygote Ratio Analysis

Because the exact same heterozygote study was not performed repeatedly when validating the gel platform, a comparison between expected levels of heterozygosity cannot be directly extracted from existing data for comparison to capillary system ratios. Nevertheless it is important to note that the ratios were evaluated for the gel platform validation and, although not reflective of replicate sensitivity studies, results were compiled in the format presented in Table 2.17.

Template Quantity (ng)	Mean profile heterozygote ratio (%)
0.05	46.1
0.1	74.9
0.25	78.9
0.5	79.5
1	83.3
2.0	90.1
3	89.1

Table 2.17. Heterozygote ratios from the FMBIO® II sensitivity study data. The OD values of sister alleles were compared to determine heterozygote ratios. The mean of heterozygote ratios for all loci with a profile was calculated for each template level and these average values are reported here [Steadman 2002b].

As shown in the table, there seems to be a correlation between the percentage of imbalance loci and the amount of template DNA amplified, as the amount of template DNA moves away from 0.5-1.0 ng, the number of imbalanced loci increases. These results suggested 0.5-1.0 ng of template DNA gave the most optimal and balanced heterozygous genotypes; however, this was also the basis for determining the sensitivity study conducted using the 3100-*Avant* did not perform to expectation with respect to the 0.25 ng template amplification. Although the 3 ng template sample exhibited imbalance at only one locus, this profile is saturated which makes sister peaks approach the same OD value since the instrument can no longer detect true density when saturation occurs.

Therefore, because the studies contained in this thesis depend upon evaluation of profile quality for low-level samples, additional data sets were consulted to further ascertain whether the heterozygote ratios could be used to evaluate profile quality at low template quantities. The data was obtained from sensitivity studies conducted within the host lab throughout the course of this research. Data sets include the sensitivity study presented for the 3100-*Avant* (data from initial run but not the confirmation run), sensitivity data from the 3130 validation, and sensitivity data from the 3130*u* crossover study. The overall heterozygote ratios were determined for profiles at four template levels when optimal injection durations were used, this was

determined through injections at various durations using the same calculations presented for the 3100-*Avant*. Due to the volume of data this encompasses, the raw intensity data is not included here. A summary of results from each sensitivity series is presented in Table 2.18, as are the mean ratios for each template quantity when all sets are collectively considered.

Template Quantity (ng)	3100-*Avant* 8/2004*	3130 1/2006	3130u 3/2008	Mean % Ratio 2004-2008 Studies
0.1	79.6	67.2	79.0	75.3
0.25	75.8	81.6	86.6	81.3
0.5	87.6	82.0	86.5	85.4
1.0	84.6	90.8	84.4	86.6

Table 2.18: Combined sensitivity data for 0.1-1.0 ng template amplifications on three capillary detection instruments over time. This table reports the mean percent heterozygote ratios for profiles amplified at the specified template quantities and injected for standard durations (as determined for each instrument through performance verification in-house). The far right column indicates the mean of the ratios for each template quantity when data from all three instruments are considered together (averaged). *Data from this series is reflected in Appendix 3, other raw data not included

The data here indicate that at the chosen optimal template quantity, using standard injection and analysis parameters, single source profiles would be expected to exhibit heterozygote ratios around 85%. The data also illustrates that additional replicate sensitivity series, when collectively considered, do exhibit a trend similar to that expected based on gel-platform performance. This exercise indicates that generalizations to reaction performance should be made from replicate samplings since individual sets may contain spurious events. While replicate series studied on each instrument are specifically useful, these studies taken at various time points during which studies in this thesis were carried out provide an applicable general understanding of overall laboratory sensitivity and heterozygote ratio analysis, regardless of template, quantification method, or instrument model employed.

Since the manner in which data was compiled differed between gel and capillary platforms, one last examination was conducted to compare platform performance. For this study, archived data sets from known single source reference samples, processed with the intent of amplifying 0.5 ng template using standard injection parameters, were analyzed for heterozygotic balance. Mean profile ratios were calculated by determining the average of ratios observed for each heterozygous locus within the profile. For the capillary instruments, 20 profiles were examined and resulted in an average peak ratio of 84.8% (standard deviation 11.5%). For the FMBIO® instrument, 20 profiles were examined and resulted in a mean peak ratio of 85.5% (standard deviation 11.7%). While mixture interpretation is not the focus of this project, standard deviation of ratios within the profiles may be useful in determining a lower boundary for imbalance tolerance within a profile, a lab may consider a lower boundary of x-3σ for mixture interpretation purposes. Using either platform, this would allow a 50% imbalance between true sister alleles. In practice, this would accommodate most true heterozygotes, but each platform did have a single instance where one heterozygote ratio was below 50%. This occurred at 43.6% on the capillary platform (D8S1179) and at 38.2% on the gel platform (also at D81179). It is unknown whether this is due to amplification or template sequence but these two events did not arise as a function of typing a common DNA source.

2.3.7.5 Sensitivity Overview

The sensitivity studies performed provide a strong foundation for profile interpretation of low level samples for future studies related to this project. Results from these analyses indicate that the gel and capillary platforms perform at similar sensitivity levels and that similar heterozygote ratios can be expected from either system when optimal template quantities are provided for the reaction and profiles analyzed using standard injection and analysis parameters.

In summary, profiles generated on either platform were evaluated based on the total number of alleles detected for a range of template quantities within each system after suitable analysis parameters were defined. Results indicate that both systems are capable of producing adequate genotype information in the 50-100 pg template range

and that when amplified products were detected from template quantities below 50 pg, the profiles were more likely to exhibit a great deal of drop-out and/or imbalance of signal between and within loci. While overall data quality between loci could be observed visually, it was also useful to examine profile quality using quantitative measures.

In an effort to apply heterozygote ratio analysis as a means for confirming target template quantity, heterozygote ratios were averaged for each template quantity analyzed on the FMBIO® II and the 3100-*Avant*. While replicate studies had not been conducted using the gel platform (prior to the commencement of this thesis), three different sensitivity series were analyzed using capillary instrumentation; these were conducted on different instruments over the course of four years. Although analysis was carried out for multiple injection durations for sensitivity samples run on the capillary instruments, only data from "optimized" parameters could be directly compared to the gel data. In both platforms, data supports a 0.5 ng target template and, while exceptions do occur, heterozygote balance generally worsens as template quantity decreases in the PCR reaction. Furthermore, when optimized template quantities and analysis parameters were used to type single source samples on either platform, expected levels of heterozygote balance were virtually identical, as was standard deviation between allelic pair ratios.

2.4 Performance Verification Summary

Studies comparing the performance of the PowerPlex® 16 BIO/ FMBIO® II and the PowerPlex® 16/ABI PRISM® 3100-*Avant* systems were designed to explore and compare sensitivity limitations of two common STR typing systems and establish the capability of current technology to type LCN exhibits. The full range of performance verification exercises carried out on the 3100-*Avant* explore system performance and are contrasted to the gel platform previously validated in the lab.

Because this project involves LCN analysis, the sensitivity studies and comparisons offer the most significance. A comparison of the gel and capillary platforms was achieved by designing a sensitivity study to assess the minimal template limitations of

two the 16-plex STR typing systems commonly used in forensic applications. The objectives of the study were to determine the optimal amount of template DNA required for STR-PCR multiplex typing and the least amount of template DNA required for detection of a complete profile. These efforts served to demonstrate the capability of current technology available for typing LCN exhibits and propose mathematical evaluation of heterozygote balance as a meaningful tool for assessing profile quality.

The performance verification presented here is foundationally important to data analysis carried out throughout this project, especially since multiple platforms were used to analyze data. The in-house studies indicate that host laboratory instrumentation performs comparably regardless of platform. Furthermore, results of in-house validation studies were similar to those obtained during developmental validation, based on comparison to published validation data and expectations declared by the manufacturer [Krenke, 2002].

3.0
Whole Genome Amplification

3.1 Whole Genome Amplification (WGA) Theory

The primary objective of whole genome amplification is to increase the amount of total DNA in a reaction non-specifically so that the result is simply greater amounts of DNA that is representative of the starting template. For forensic applications, the technique would have to be successful with starting template quantities equal to or less than that from which types are achieved by conventional STR typing processes.

It is well established from data generated in Chapter 2 that multiplex PCR kits commonly used to type casework samples will often yield DNA profiles from low copy number templates in quantities as low as 25-100 pg. However, situations still arise where there is too little DNA to obtain STR DNA profiles. Therefore, it is desirable to develop methods of improving DNA extraction or by replicate means to avoid problems associated with stochastic effects of the multiplex reaction. The basis of an accurate typing process is providing adequate template for the STR PCR reaction so that allelic drop-out can be avoided and a balanced representation of true allelic contribution can be obtained for end-point interpretation. This chapter explores increasing template availability by whole genome amplification (WGA), which has been reported as useful for generating large fragment copies of high molecular weight genomic DNA. The technique has been used to generate large quantities of DNA for sequencing applications and proven to result in sequence information identical to that obtained from samples that had not undergone WGA [Nara, *et. al*., 2009]. A commercial kit designed for whole genome amplification, GenomiPhi™, was tested for the capability to increase template quantity prior to multi-locus STR analysis. Prior to describing the specific aspects of the commercial kit, research and development of this technology will be discussed.

3.1.1 Classical PCR Techniques

Early WGA reactions were primer extension PCR (PEP) methods described in the 1990's which made use of *Taq* polymerase and random primers. The reactions were carried out using 15 base random oligonucleotides with low stringency annealing parameters to achieve repeated primer extensions resulting in copy fragments approximately 3 kb in length [Zhang, *et. al*., 1992]. These initial attempts were

successful in achieving copies of a majority of the sequences in the haploid genome, with an estimated 78% coverage expected 95% of the time. Sequences that were generally difficult to copy using this method were highly repetitive areas, and the process was not efficient on extremely small samples. The potential implications for medical diagnostics were immediately recognized and improved methods (improved-PEP) were reportedly successful on unfixed tumor cell clusters, as well as frozen, formalin fixed, paraffin-embedded, or stained (immunocytochemically) microdissected samples requiring mutational analyses [Dietmaier *et. al*., 1999]. The PEP methods were successfully optimized for preimplantation genetic diagnosis of inherited disorders [Sermon *et. al*., 1996; Hughes *et. al*., 2005].

Another general DNA amplification method made use of partially degenerate primers rather than 15-oligo random primers and is termed degenerate oligonucleotide-primed PCR (DOP-PCR). These primer mixtures can be prepared to target known semi-conserved sequences interspersed in more predictable distances throughout the genome. The mixtures are comprised of oligonucleotide mixtures with slight variations to accommodate sequence variation between species. Developed about the same time as PEP methods, the method employed such oligonucleotides and a low initial annealing temperature to accomplish priming from 10^6 sites within a genome. This was followed by a large number of cycles with a higher annealing temperature to accomplish evenly dispersed copies of the genome that achieved amplification of virtually all species [Telenius *et. al*., 1992]. Some groups reported products up to 10 kb in length, but problems with fidelity and coverage were still prevalent with less than 1 ng of template DNA using DOP-PCR [Kittler, *et. al*., 2002]. Recently, the method has been combined with real-time amplification technology such that the accumulation of product can be monitored and reaction stopped when the desired level of end-point product is achieved [Feher *et. al*., 2006].

3.1.2 Shearing Techniques

WGA methods which rely on chemical cleavage and endonuclease digestion are ligation-mediated PCR (LMP) and T7-based linear amplification of DNA (TLAD). LMP was initially used for developing probes that could be used for mapping

applications [Ludecke *et. al*., 1989]. However, the idea of fragmenting the DNA and using linkers and primers to amplify it was adapted for WGA by Tanabe, who first described successful PCR of randomly sheared genomic DNA (PRSG) based on successful downstream amplification of evenly dispersed genes, amplification of microsatellites, and comparative genomic hybridization (CGH) analysis [Tanabe *et. al*., 2003; Pirker, *et. al*., 2004]. The technique was reported successful on low quantity or single cell samples by Klein, who assessed the amplification was unbiased via CGH, undetectable loss of heterozygosity, and the ability to detect mutations [Klein *et. al*., 1999]. An overview of the method is illustrated in Figure 3.1.

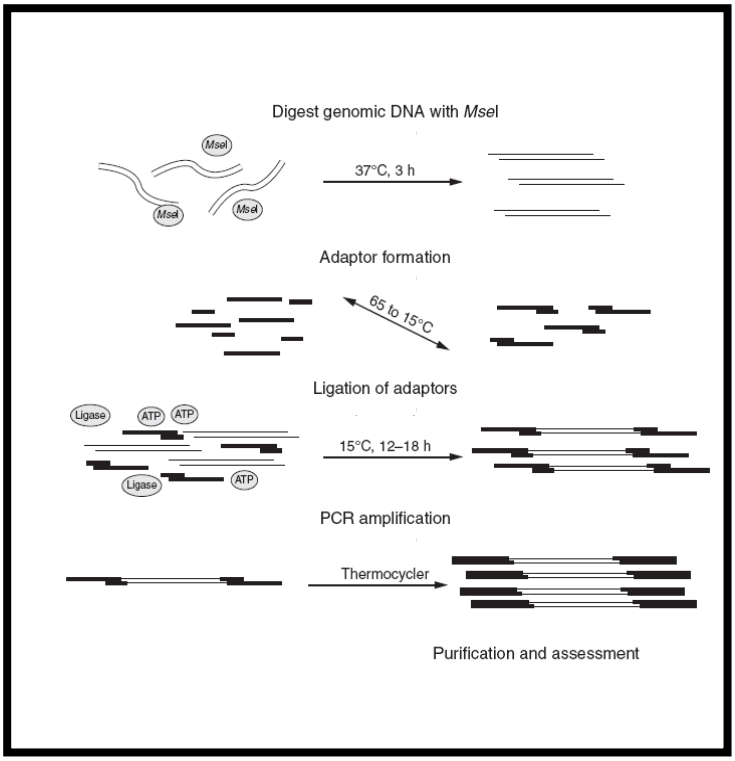


Figure 3.1: Ligation-mediated PCR. This amplification is facilitated by the ligation of a conserved sequence to the 3' ends of restriction-digested template DNA with the assistance of a linker. Depending on the restriction enzyme or shearing method, the linkers may be staggered on one end or both and are prepared by stepwise annealing; this is followed by ligation to template ends. The PCR is then achieved based on primer recognition of the ligated sequence [Arneson, *et. al*., 2005].

Reports of successful ligation-mediated whole genome analysis was the basis for coining the process called single cell comparative genomic hybridization, or "SCOMP," claiming advantages over DOP-PCR for fixed tissue analysis by use of a single primer and more stringent annealing conditions [Stoecklein *et. al*., 2002].

Nonetheless, the main limitation of the LMP method proved to be the efficiency of the ligation reaction and this shortcoming brought forth yet another alternate extension/recognition method. TLAD is a method that uses restriction endonuclease digestion followed by a terminal transferase to add poly-T tails to the DNA fragments. This is followed by annealing with an adapter that has a 5' T7 promoter sequence attached to a 3' polyA sequence, and after second strand synthesis, the sample is reverse transcribed [Liu, *et. al.*, 2005]. This produces fragments of DNA complimentary to the original fragment sequences that can be used to probe and whole-genome amplify future templates. The advantage of this method is reduced sequence bias, but it is not commonly used due to the complicated and lengthy protocol [Hughes, *et. al*., 2005], which is illustrated in Figure 3.2.

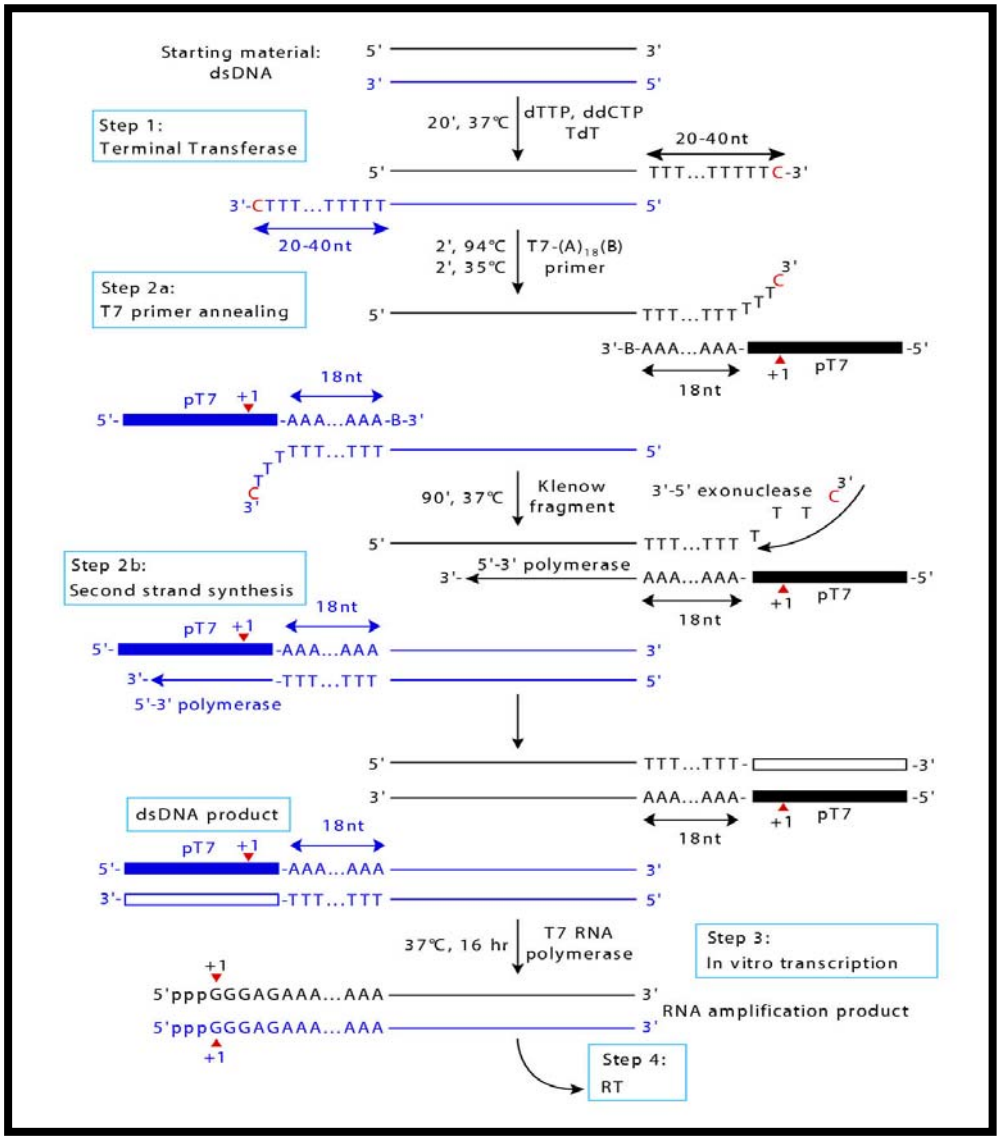


Figure 3.2: T7-based linear amplification of DNA. From Liu, *et. al.*, 2005, who described the steps of the process as follows: "Step 1: Double stranded DNA starting material (shown with one strand in black and one strand in blue) is tailed on the 3' end of each strand to generate a 20–40 bp polyT tail with a terminal dideoxycytidine base. Step 2a: A T7-$(A)_{18}$B anchored primer adaptor is annealed to the polyT tail of each template strand. Step 2b: During second strand synthesis Klenow fragment of DNA Polymerase I removes the excess bases from the tail overhang via its 3'-5' exonuclease activity, and extends from the primer to produce the second strand. This results in two double stranded DNAs identical to the original template, except that each has a T7 promoter at a different end. Step 3: The product of second strand synthesis is used as template in an in vitro transcription reaction. Step 4: To generate DNA probes for microarray analysis, amplified RNA is reverse transcribed."

3.1.3 Isothermal WGA

The largest breakthrough in whole genome amplification has been the isothermal methods which are based on random hexamer priming followed by Phi29 (ϕ29) DNA polymerase assembly of the complimentary strands. The activity of this enzyme was elucidated throughout the 1980s and catalyzes polymerization in a highly processive manner, coupled with excellent strand displacement capabilities [Blanco and Salas, 1984; Blanco *et. al*., 1989]. The reaction occurs at constant temperature and has been shown effective for synthesis of greater than 10 kb fragments of DNA [Dean *et. al*., 2002]. Unlike *Taq* polymerase that is used in STR PCR applications, the greater processivity allows ϕ29 polymerase to assemble lengthy strands of DNA before dissociating from the template. Furthermore, the strand displacement capability negates the need for cycling the reaction temperature to make single-stranded template available in the reaction.

Proofreading activity is among the most important enzyme attributes for achieving fidelity [Eckert and Kunkel, 1991], and the ϕ29 DNA polymerase has a 3'-5' exonuclease activity giving it an error rate of only 1 in 10^6–10^7, approximately 100 times better than *Taq* DNA polymerase [Esteban *et. al*., 1993; Paez *et. al*., 2004]. The enzyme exhibits a high level of fidelity; therefore products are often used for sequencing and SNP analysis [Faruqi *et. al*., 2001; Rook, *et. al*., 2004]. The method is illustrated in Figure 3.3 and is reportedly better than previously described methods of WGA for downstream genotyping applications [Bergen, *et. al*., 2005], probably due to reduced sequence bias within the system [Lizardi, *et. al*., 1998].

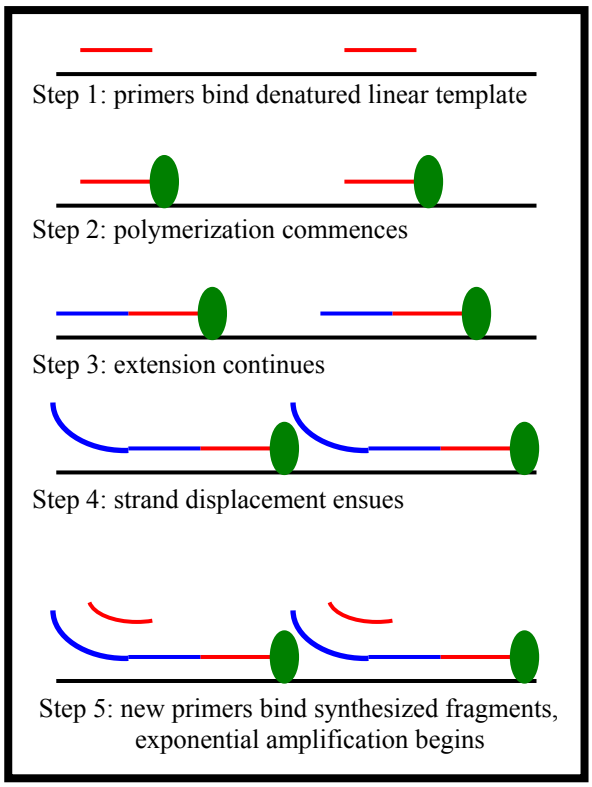


Figure 3.3: Illustration of the GenomiPhi™ Kit amplification process. During the whole genome amplification process, random hexamer primers bind the template (step 1), ϕ29 enzyme initiates polymerization (step 2), and strand displacement allows for continued extension into downstream primer binding sites (steps 3 and 4). Ultimately, hexamer primers recognize newly synthesized fragments and the process is repeated resulting in exponential amplification of the linear genomic template (step 5) [Davis *et. al*., 2002].

3.1.4 Forensic Applications

The GenomiPhi™ Kit, marketed by GE Healthcare (formerly Amersham Biosciences), was developed to amplify linear genomic DNA exponentially by strand displacement amplification [Davis *et. al.*, 2002; Sriramen *et. al*., 2003]. The GenomiPhi™ system may be considered extended technology of the TempliPhi™ system that was developed for amplification of circular plasmid templates, a practice long used for sequencing applications [Amersham, 2003]. Both the TempliPhi™ and GenomiPhi™ systems employ ϕ29 polymerase to assemble lengthy strands of DNA. The GenomiPhi™ kit reportedly produces microgram quantities of DNA from nanogram amounts of template, producing fragment lengths in the range of 10,000 bases [Sriraman, 2003]. During GenomiPhi™ amplification method, template DNA is added to a sample buffer containing random hexamer primers. An initial heat denaturation

allows for strand separation of the linear genomic DNA and is followed by cooling to allow for hexamer primers to randomly anneal to the template. The remaining reaction components, consisting of ϕ29 polymerase, deoxynucleotide triphosphates, and a buffer optimized for linear DNA synthesis, are then added and extension occurs during an overnight incubation at 30^{o} C. During the extension process, the nucleotides are incorporated into high molecular weight fragments complimentary to the input DNA [Amersham, 2003].

WGA has entered all aspects of molecular research, including bacteriology and microbial genetics [Raghunathan *et. al*., 2005]. Multiple displacement amplification is frequently used in clinical research and medical diagnostics [Hellani, *et. al*., 2004; Handyside, *et. al*., 2005]. Advancements in all of the methods described herein have since made WGA a widely accepted and broadly applied technique in clinical diagnostics, especially in the area of preimplantation genetic diagnoses [Spits and Sermon, 2009]. Nevertheless, possible forensic uses were not well-described as of 2002. The GenomiPhi™ literature suggests that the system does not require highly purified DNA and that WGA with this kit can be carried out on cell lysates directly [Amersham, 2003]. Therefore, upon commencement of this project, shortly after the GenomiPhi™ kit was released, it was thought a strong candidate system for replicating forensic templates.

The primary purpose of this chapter is investigating WGA as a forensic application to determine if the technology could be useful for improving the ability to capture information from challenging specimens of biological material. Since current STR-PCR technology can generate quality STR profiles from 100 pg of template or less (as demonstrated in chapter 2), it was understood that WGA would have to exceed this sensitivity, provide a unique DNA capture/amplification method, or otherwise amplify degraded or inhibited template in special circumstances where general multiplex PCR fails. Initial studies explore the generation of WGA products from a range of template quantities and the ultimate usefulness of the resulting products as PCR-STR templates. After testing on purified DNA extracts, WGA on crude cell lysates followed by PCR-STR typing of cell lysate amplicons was also carried out.

These studies afforded the opportunity to explore possible advantages of WGA prior to direct STR-PCR multiplexing (supplementing template quality by using WGA prior to STR-PCR vs. direct STR-PCR amplification of purified product) as well as the possible advantage of direct WGA of lysed cell components and therefore bypassing the DNA purification/concentration step and direct amplifying DNA from lysis reactions.

3.2 WGA Methods and Materials

Whole genome assays were conducted using two procedures. The first attempts to amplify DNA that has been purified, concentrated, quantified, and then diluted to specified concentrations. The second was conducted on whole cell pellets. Cellular composition was based on estimated quantities of cells in blood volumes, along with considerations concerning the quantity of DNA present in a single cell. For cell lysate assays, whole genome amplification was carried out immediately after cell lysis, with no intermediate DNA purification/concentration step.

WGA experiments made use of the GenomiPhi™ amplification kit and were based on manufacturer's instructions [Amersham, 2003]. Components of the kit are sample buffer, reaction buffer, and enzyme mix. Control DNA (Lambda 10 ng/μL) is also supplied but was not used for these studies.

The experiments for each of the two studies (WGA of purified DNA and WGA of cell lysates) are described and presented systematically within the respective section for each. The preparation of template is first described (dilutions of extracted DNA for the purified DNA studies and dilutions of whole blood for lysate tests). Quantification of the WGA products by yield gel and slot blot are then presented (crude products and Microcon® treated post-WGA product are compared analyzed), followed by the results of STR typing of the WGA product.

3.2.1 Whole Genome Amplification of Purified DNA Extracts

For this study, DNA was extracted from human blood, Microcon® concentrated, and quantified using the QuantiBlot® Human DNA Quantitation Kit. Dilutions of the extracted DNA were prepared in TE and given identifiers as described in Table 3.1.

Sample Identification	Concentration (ng/μL)
1A	1.00
1B	1.00
1C	0.50
1D	0.25
1E	0.10
1F	0.05
1G	0 (TE added instead)

Table 3.1: Concentrations of purified DNA prepared for the GenomiPhi™ sensitivity study. Target concentrations were selected given that a quantity of 1 μL of sample would serve as template in WGA reactions.

Reactions were prepared according to the manufacturer's protocol using the kit components, which consisted of sample buffer, reaction buffer, and enzyme mix. The Lambda control DNA included in the kit was not used for these studies. Each reaction was prepared by placing 1 μL of each sample into 9 μL of sample buffer (included with amplification kit). Tubes were heated to 95° C for three minutes and snap-cooled on ice water; this denaturation step was omitted for sample 1B (containing 1 ng template) since some literature reported incubation at this temperature in the presence of some reaction components can severely damage the DNA template [Dean *et. al*., 2002]. A reaction pre-mix, consisting of 1 μL ϕ29 enzyme and 9 μL of reaction buffer (included with amplification kit) was added to each reaction tube while kept cold. Reaction tubes were placed at 30° C for 18 hours. Following amplification, the samples were heated to 65° C for 10 minutes and then stored at 4° C [Amersham, 2003]. A quantity of 1 μL of each crude amplified product was visualized on 1-2% agarose gels prior to and after Microcon® concentration/purification [Ausubel, 1996]. Microcon® retentates were then diluted and underwent slot blot quantification, multiplex amplification, and fragment analysis

using the FMBIO® II platform as described in Chapter 2. PowerPlex®16 BIO amplification included 0.5 μL of each sample 1A-1F, a volume of 1 μL of negative control sample 1G was amplified (as a conservative measure given quantification results indicated possible presence of human DNA). Appropriate controls accompanied the PowerPlex® 16 BIO amplifications and consisted of a positive amplification control (0.25 ng template from 9947A supplied with the typing kit), an internal positive extraction control (Microcon® -purified human DNA extracted from a blood stain), and a negative amplification control.

3.2.2 Whole Genome Amplification of Whole Cell Lysates

GenomiPhi™ amplification of cell lysate material was conducted using dilutions of human blood. Because the manufacturer's literature stated it possible to obtain reliable WGA products from the DNA of only 10 cells, blood dilutions were prepared to investigate this claim.

The estimated calculations were initially based on the expected amount of recoverable DNA to be 20-50 ng/μL for humans [Applied Biosystems, 2004]. It was accepted up front that the dilution range used was based on literature that reports expected yields of DNA from human blood, which does not necessarily reflect accurate quantities of DNA in blood volumes since this will vary based on a donor's white blood cell (WBC) count. Nevertheless, generally accepted ranges for normal humans would be 4,500-10,000 cells per microliter [Applied Biosystems, 2004; Bagby, 2007; GE Healthcare, 2007]. The amount of recoverable DNA in blood as reported above by Applied Biosystems can be explained with the following calculation:

Given that 1 bp = 618 g/mol, a single genome copy = approx. 3 x 10^9 bp, and that 1 mole = 6.02 x10^{23} molecules, the following math is used to calculate the amount of DNA in a single cell:

1 genome copy = (3 x 10^9 bp) x (618 g/mol/bp) = 1.85 x 10^{12} g/mol
= (1.85 x 10^{12} g/mol) x (1 mol/6.02 x 10^{23} molecules)
= (3.08 x 10^{12} g) x (1 pg/10^{12} g)
= 3.08 pg

Then amount (g) of DNA/cell = (3.07 pg/1 genome copy) x (2 genome copies/cell)
= approx. 6 pg [Collaborative Electronic Learning Center, 2003]

Therefore, if a donor has 7000 cells present in 1 μL of blood (a value between the reported 4,500 and 10,000), and 6 pg of DNA is present per cell (0.006 ng), then one would expect 42 ng of DNA per μL of blood at 100 % recovery (7000 cells x 0.006 ng/cell= 42 ng). This is within the range of recoverable DNA reported by Applied Biosystems, 2004.

Dilutions described in Table 3.2 (designated 2A-2M) were prepared and 10 μL of each sample was lyophilized in a microcentrifuge tube. The pellets were kept frozen until commencement of the lysis reactions. PBS was added to whole blood and dilutions were prepared. Sample 2A was prepared by mixing 100 μL whole blood with 100 μL PBS; this represents a 50% dilution. Dilutions were designed to achieve the estimated range of cell numbers per sample based on the expected amount of DNA present in standard whole blood volumes; this was based on lower boundary of range reported as 4,500-10,000 cells/μL. Since the total lysing/neutralization solution volume was 20 μL, and only 1 μL of this lysate was called for by the GenomiPhi™ reaction, the number of cells placed in the lysis reaction was twenty-fold the final WGA reaction target quantity (1/20 of the lysis solution is placed into the WGA reaction). Pellets 2D through 2H were selected for use in initial lysis experiments since 1/20 of the total lysed cells from the pellet would result in transfer of DNA from approximately 9-141 cells into the WGA reaction, representing a range slightly higher than the manufacturer's claim of success with 10 cells and equating to an approximate range of 54-846 pg DNA going into the WGA reactions (6 pg per cell).

Sample identifier	Blood component to which 100 µL PBS was added	*estimated number of cells/10 µL	Cells represented by 1/20 of lyophilized pellet
2A	100 µL whole blood	22500	1125
2B	100 µL 2A	11250	563
2C	100 µL 2B	5625	281
2D	100 µL 2C	2813	141
2E	100 µL 2D	1406	70
2F	100 µL 2E	703	35
2G	100 µL 2F	352	18
2H	100 µL 2G	176	9
2I	100 µL 2H	88	4
2J	100 µL 2I	44	2
2L	100 µL 2J	22	1
2M	100 µL 2K	11	0.5

Table 3.2: Serial dilution process for WGA of cell lysates. PBS was added to whole blood and dilutions were prepared. Sample 2A was prepared by mixing 100 µL whole blood with 100 µL PBS; this represents a 50% dilution. Dilutions were designed to achieve the estimated range of cell numbers per sample based on the expected amount of DNA present in standard whole blood volumes. *based on lower boundary of range reported as 4,500-10,000 cells/µL; therefore if 45,000 cells are expected in 10 µL of whole blood 22,500 cells would be present in the 50% dilution sample 2A. Samples/quantities in blue are those that were selected for experimental analysis.

To each of pellets 2D through 2H, 10 µL of alkaline lysis solution was added. Following 10 minutes on ice, 10 µL of neutralization solution was added. From this, 1 µL of each was placed into sample buffer (included in amplification kit), and the amplification was carried out as described for DNA extracts [Amersham, 2003]. A portion (5 µL) of crude amplified product was reserved, 1 µL of which was visualized on a 1-2% agarose gel as previously described. A quantity of 285 µL TE was then added to the remaining 15 µL of crude product. The dilutions were PCI-extracted (using 300 µL of PCI), Microcon®-concentrated and purified to a final volume of 20 µL in TE. Microcon® retentates then underwent slot blot quantification, multiplex amplification, and fragment analysis using the FMBIO® II platform as described in section 2.2.

3.3 WGA Results and Discussion

3.3.1 Whole Genome Amplification of Purified DNA Extracts

Samples 1A through 1G were amplified according to GenomiPhi™ WGA kit procedures as described in section 3.2.1. Following amplification, the products were visualized on a 1% agarose gel. Figure 3.4 represents the product gel obtained when 1 μL of each sample was analyzed per well.

All template quantities tested resulted in saturated gel signal, including the negative control. In an effort to discern whether WGA reaction products, rather than amplified DNA fragments, were causing the signal, Microcon® devices were used to purify the samples by removal of small DNA fragments. While 4 μL of each reaction product was reserved and stored frozen in the crude form; the remaining portion of each (~15 μL) underwent Microcon® purification and was then brought to a final volume of 20 μL in TE. Following purification, 1 μL of each sample was again visualized on a 1% agarose gel and shown in Figure 3.5.

The diluted products, noted in Figure 3.6, exhibit high molecular weight bands at the same migration point as the standards. However, appreciable quantities of smaller fragments are represented by the smears below noted bands at this dilution.

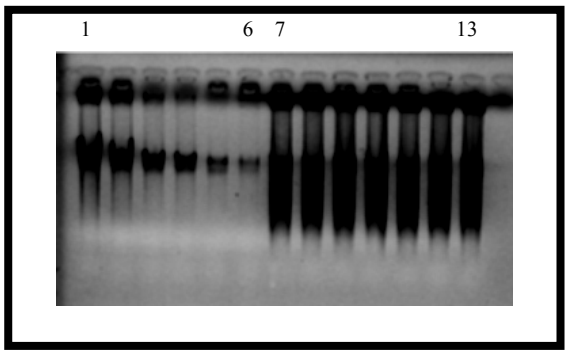


Figure 3.4: Product gel (1%) of crude WGA products. Lanes 1-6 (left to right) contain the quantification standard series in the amounts of 200 ng, 100 ng, 50 ng, 25 ng, 10 ng, and 5 ng of DNA. Lanes 7-13 contain 1 μL of 1A through 1G, respectively. 1A=1.00 ng, 1B=1.00 ng (not denatured); 1C=0.50 ng; 1D=0.25 ng; 1E=0.10 ng; 1F=0.05 ng; 1G=no template negative control.

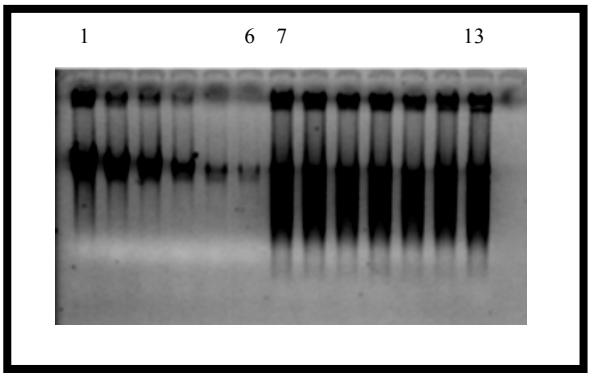


Figure 3.5: Product gel (1%) of Microcon®-purified WGA products. Lanes 1-6 (left to right) contain the quantification standard series in the amounts of 200 ng, 100 ng, 50 ng, 25 ng, 10 ng, and 5 ng of DNA. Lanes 7-13 contain 1 μL of each of the Microcon®-purified samples (each of 1A through 1G). 1A=1.00 ng, 1B=1.00 ng (not denatured); 1C=0.50 ng; 1D=0.25 ng; 1E=0.10 ng; 1F=0.05 ng; 1G=no template negative control.

By comparison of Figures 3.4 and 3.5, little difference was observed between the intensity of the Microcon® -purified products and the signal intensity of crude products quantified initially. Therefore, the additional purification process had little affect on product quality. Microcon®-purified samples were then diluted 1:49 so that products could be observed at a quantifiable level (diluted by a factor of 50 using nuclease free water), and a third product gel was run for these samples. The image of the agarose gel containing the diluted samples is Figure 3.6.

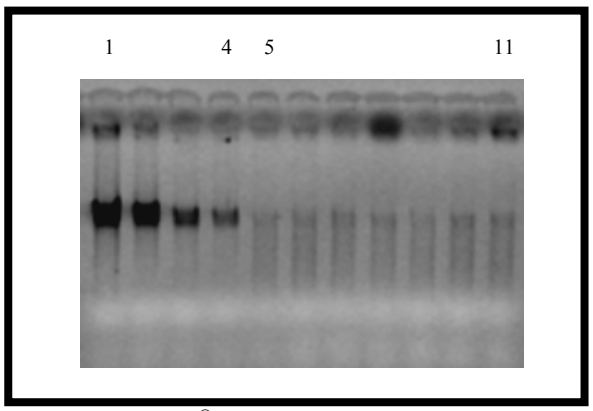


Figure 3.6: Product gel (1%) of Microcon®-purified and diluted WGA products. Lanes 1-4 (left to right) contain the quantification standard series in the amounts of 50 ng, 25 ng, 10 ng, and 5 ng of DNA. Lanes 5-11 each contain 1 μL volumes of Microcon® -purified samples following a 1:49 dilution in TE (1A through 1G, respectively). 1A=1.00 ng, 1B=1.00 ng (not denatured); 1C=0.50 ng; 1D=0.25 ng; 1E=0.10 ng; 1F=0.05 ng; 1G=no template negative control.

The Microcon® purified samples were then quantified using the QuantiBlot® Human DNA Quantitation Kit. Samples 1A through 1G were each analyzed without dilution, diluted 1:49, and diluted 1:399. Figure 3.7 represents the blotting pattern for the samples employed for this assay, while Figure 3.8 represents the resulting blot.

	1	2	3	4	5	6
A		10 ng std	1A (1:399)	1A (1:49)	1A (N/D)	Cal 1 (3.5 ng)
B		5 ng std	1B (1:399)	1B (1:49)	1B (N/D)	Cal 2 (0.5 ng)
C		2.5 ng std	1C (1:399)	1C (1:49)	1C (N/D)	
D		1.25 ng std	1D (1:399)	1D (1:49)	1D (N/D)	
E		0.63 ng std	1E (1:399)	1E (1:49)	1E (N/D)	
F		0.31 ng std	1F (1:399)	1F (1:49)	1F (N/D)	
G		0.15 ng std	1G (1:399)	1G (1:49)	1G (N/D)	
H						

Figure 3.7: Blot arrangement for analysis of Microcon®-purified products from WGA of purified DNA. Each well received 1 µL of samples 1A through 1G at the indicated dilutions. Standard and calibrator quantities are expressed in ng/well; a loading volume of 5 µL was used to achieve each of the indicated amounts. Std= standard; Cal=calibrators; N/D=no dilution. 1A=1.00 ng, 1B=1.00 ng (not denatured); 1C=0.50 ng; 1D=0.25 ng; 1E=0.10 ng; 1F=0.05 ng; 1G=no template negative control.

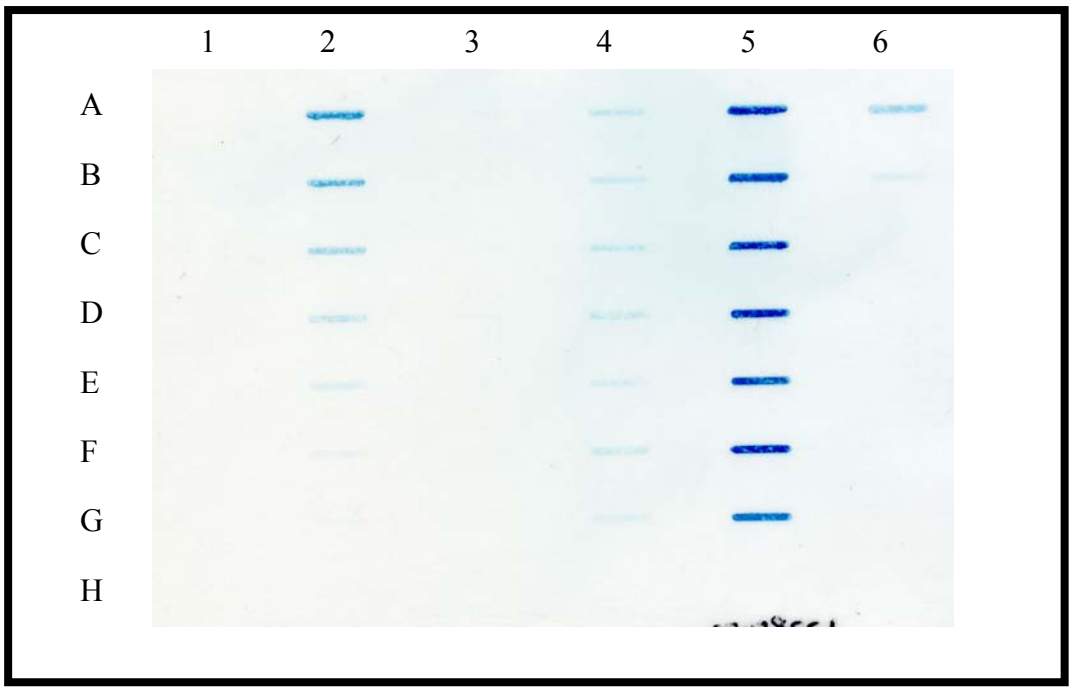


Figure 3.8: Colorimetric detection of Microcon®-purified products from WGA of purified DNA. Column 2 contains the standard series, column 3 contains the 1:399 dilutions of samples 1A through 1G; column 4 contains the 1:49 dilutions of 1A through 1G; and column 5 contains the undiluted 1A through 1G. 1A=1.00 ng, 1B=1.00 ng (not denatured); 1C=0.50 ng; 1D=0.25 ng; 1E=0.10 ng; 1F=0.05 ng; 1G=no template negative control. Calibrators representing 3.5 and 0.5 ng of DNA were loaded in 6A and 6B, respectively.

Human quantification of undiluted and diluted purified samples revealed that the 1:49 dilutions yielded slot blot signal within the range of the blot standards based on data obtained from Figure 3.8. Interestingly, the negative control yielded such signal as well, based on bands detected in wells 3G, 4G, and 5G. Initially, it was not known if this signal was due to contaminant human DNA that was amplified during the WGA process or if this represented non-specific binding of WGA reaction products. Since the WGA reaction primers consist of random hexamers, these can bind nylon and recognize probe sequence or otherwise form concatamers that ultimately result in probe recognition and colorimetric signal. However, further discussions with the manufacturer [Nelson, 2007] revealed that detection of product on the agarose gel could be due to amplification of non-human template (the enzyme is produced in microbial systems and trace levels of microbial DNA can be present in the kit components). While this does not fully explain the detection of human specific signal via the blot techniques, it is noted that the complex reactions contain random

hexamers which would be present on the blot and may serve to recognize probe sequences at levels high enough to result in signal.

Bands resulting from 1:49 dilutions of samples 1A through 1F were quantified at 1 ng. Based upon loading volume and sample dilution factor, each sample eluate was quantified at approximately 50 ng/μL of DNA. Therefore, the samples were diluted such that 0.25 ng of template could be introduced into the reduced-volume PowerPlex® 16 BIO typing reactions (0.5 μL of each sample). Appropriate controls accompanied the PowerPlex® 16 BIO amplifications and consisted of a positive amplification control (0.25 ng template from 9947A supplied with the typing kit), an internal positive extraction control (Microcon® -purified human DNA extracted from a blood stain), and a negative amplification control. In an effort to fully test reagent integrity, twice as much of the negative control was incorporated as template as compared to the volume amplified for any WGA sample (1 μL for the negative control; 0.5 μL for all WGA samples).

Following amplification with the PowerPlex® 16 BIO typing system, a product gel was used to assess product quantity for each sample so that loading volumes could be optimized for the polyacrylamide analytical gel (as described in section 2.2.2). Each well in Figure 3.9 contains 2 μL of amplified reaction volume (approximately 27% of each amplified sample).

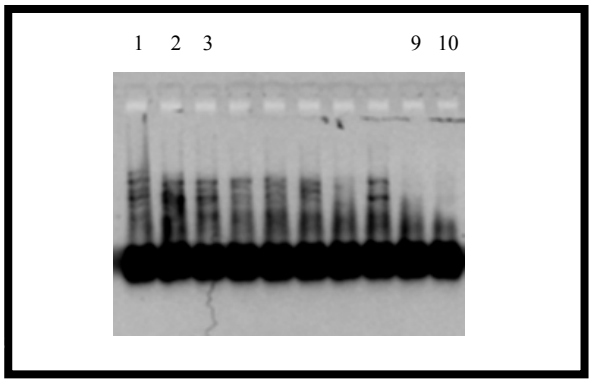


Figure 3.9: Post-amplification product gel (3%) of PowerPlex® 16 BIO amplified WGA products. Lane 1 contains the positive amplification control (9947A DNA), lane 2 contains an internal positive control (Microcon®-purified human DNA), lanes 3 through 9 respectively contain PowerPlex® 16 BIO amplified WGA products from samples 1A through 1G (1A=1.00 ng, 1B=1.00 ng (not denatured); 1C=0.50 ng; 1D=0.25 ng; 1E=0.10 ng; 1F=0.05 ng; 1G=no template negative control), and lane 10 is the negative amplification control.

Product was readily observed for all samples except the negative controls (WGA sample 1G and the negative amplification control). The samples were then separated using a 6% denaturing polyacrylamide gel and shown in Figures 3.10 through 3.12. These represent the analytical gel results of the PowerPlex® 16 BIO amplification. Since product was not readily observed for sample 1G, this sample was run at 1X and 2X concentrations. Likewise, less product was observed for sample 1E on the post-amplification product gel, therefore 1X and 2X concentrations of this sample were analyzed on the polyacrylamide gel.

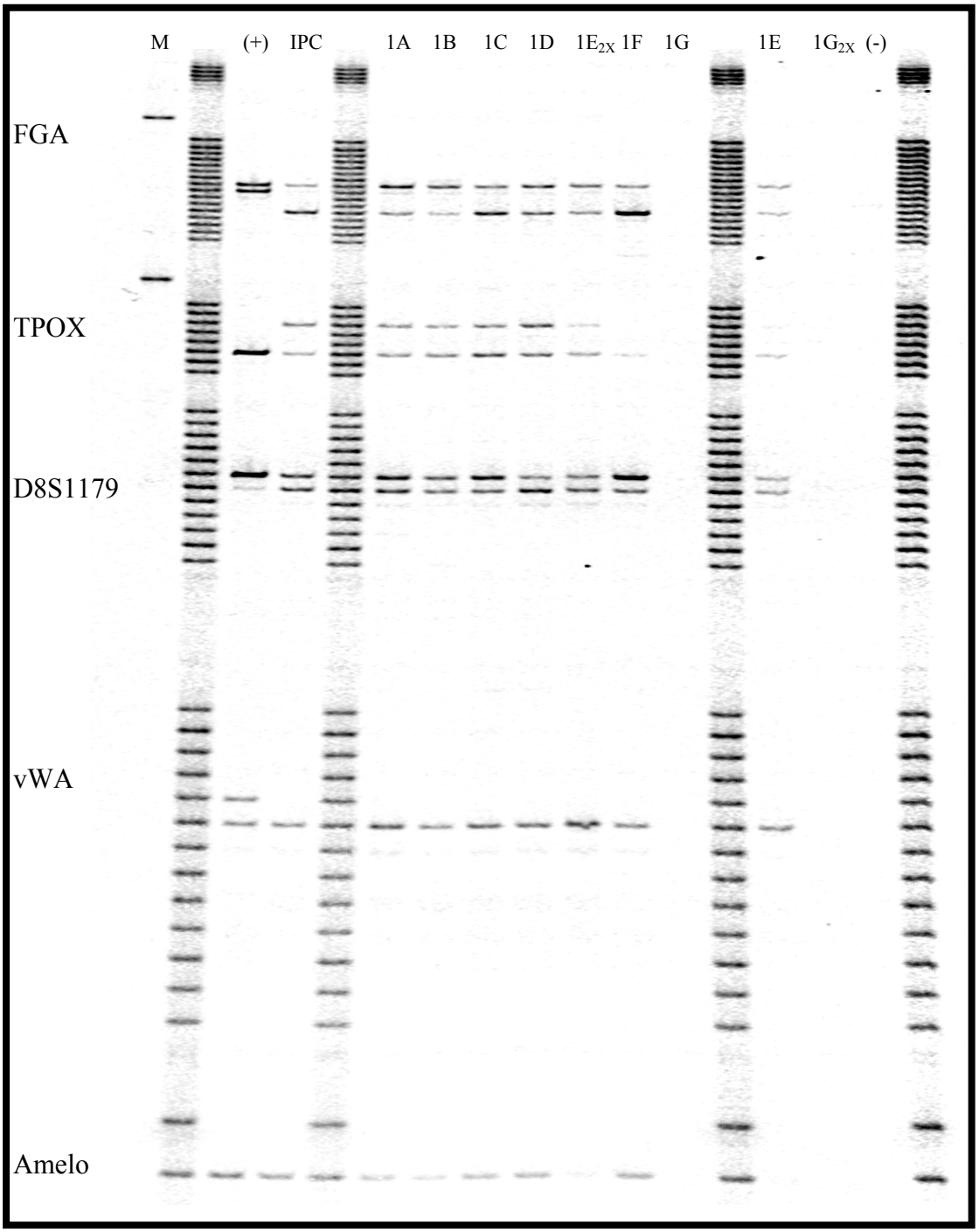


Figure 3.10: PowerPlex® 16 BIO amplified WGA products-Rhodamine Red™-X (RRX) loci. Image color separation was optimized for RRX-labeled products, with locus designations indicated next to the corresponding ladders. WGA reaction designations are indicated for each lane. All samples were analyzed at standard volume; however twice as much amplified product was also analyzed for samples 1E and 1G (as indicated by 2X). 1A=1.00 ng, 1B=1.00 ng (not denatured); 1C=0.50 ng; 1D=0.25 ng; 1E=0.10 ng; 1F=0.05 ng; 1G=no template negative control. M=color separation matrix, (-)=negative amplification control, (+)=positive amplification control (9947A), IPC=internal positive control (Microcon®-purified human DNA).

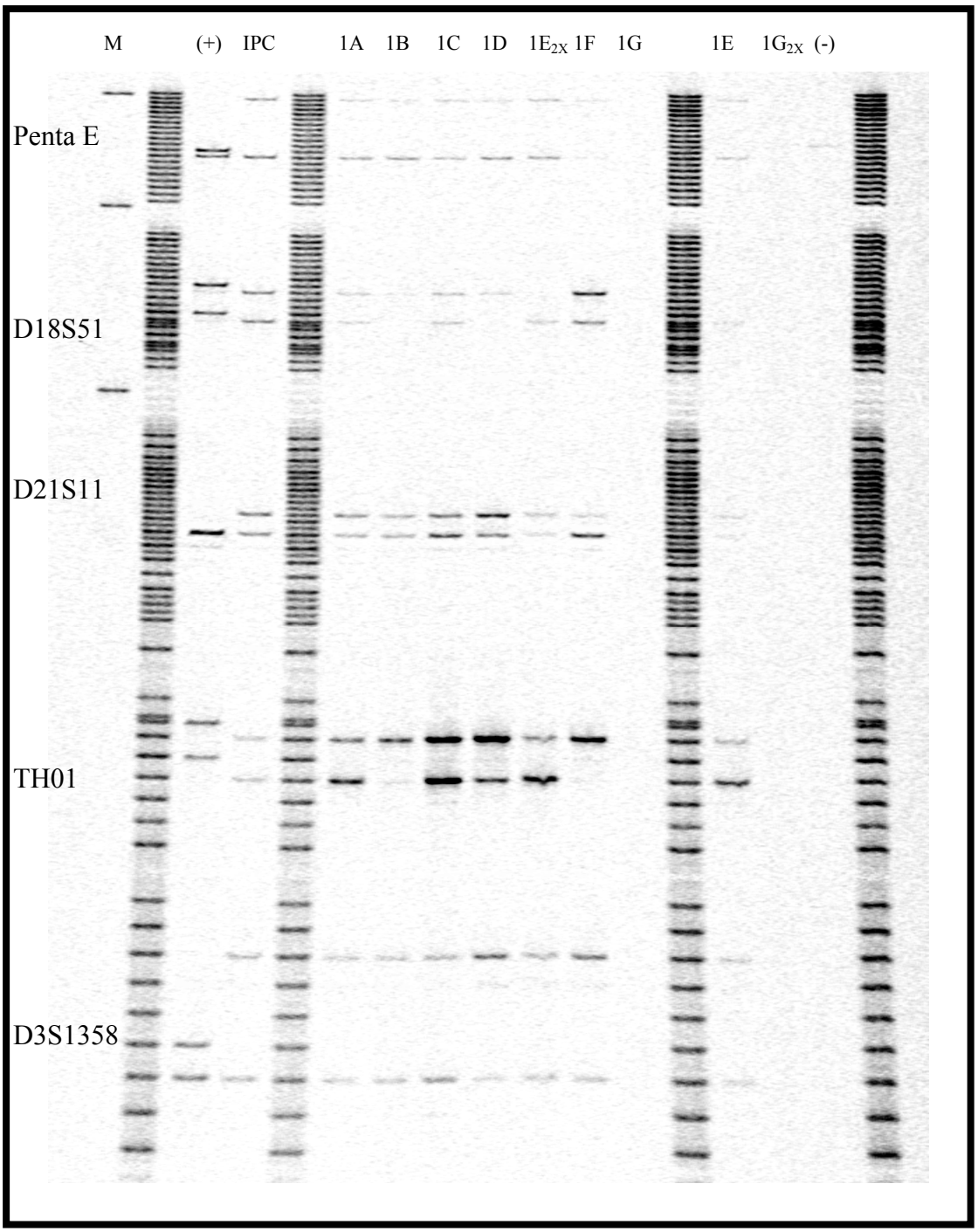


Figure 3.11: PowerPlex® 16 BIO amplified WGA products-fluorescein (FL) loci. Image color separation was optimized for FL-labeled products, with locus designations indicated next to the corresponding ladders. WGA reaction designations are indicated for each lane. All samples were analyzed at standard volume; however twice as much amplified product was also analyzed for samples 1E and 1G (as indicated by 2X). 1A=1.00 ng, 1B=1.00 ng (not denatured); 1C=0.50 ng; 1D=0.25 ng; 1E=0.10 ng; 1F=0.05 ng; 1G=no template negative control. M=color separation matrix, (-)=negative amplification control, (+)=positive amplification control (9947A), IPC=internal positive control (Microcon®-purified human DNA).

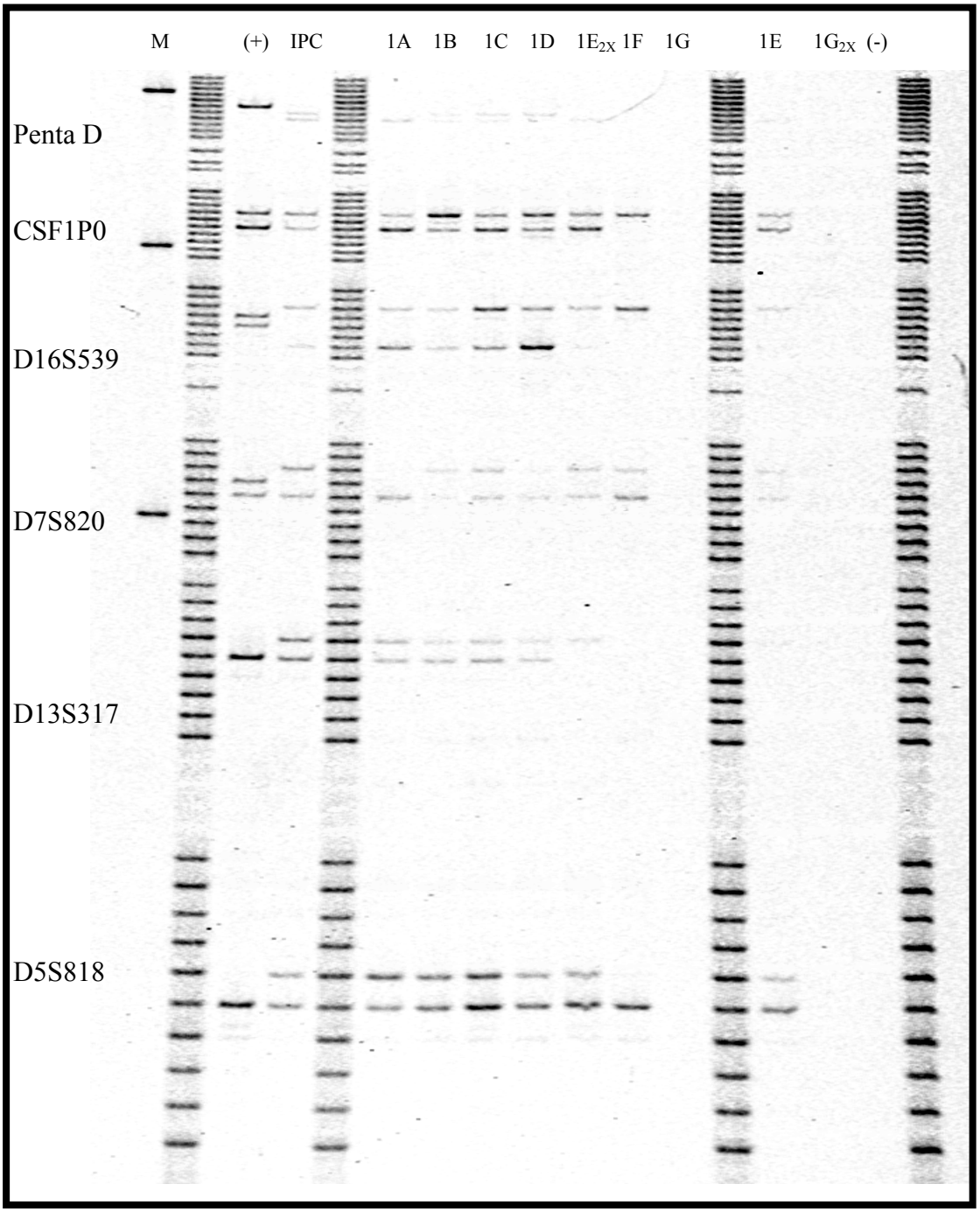


Figure 3.12: PowerPlex® 16 BIO amplified WGA products-6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE) loci. Image color separation was optimized for JOE-labeled products, with locus designations indicated next to the corresponding ladders. WGA reaction designations are indicated for each lane. All samples were analyzed at standard volume; however twice as much amplified product was also analyzed for samples 1E and 1G (as indicated by 2X). 1A=1.00 ng, 1B=1.00 ng (not denatured); 1C=0.50 ng; 1D=0.25 ng; 1E=0.10 ng; 1F=0.05 ng; 1G=no template negative control. M=color separation matrix, (-)=negative amplification control, (+)=positive amplification control (9947A), IPC=internal positive control (Microcon®-purified human DNA).

In an effort to better quantify the visually apparent imbalance of sister alleles, OD values were used to calculate percent ratios for the profiles detected. All loci's heterozygote ratios were then averaged for each profile and the overall profile averages are reported in Table 3.3. Profile quality was also evaluated by the amount of drop-out occurring within each profile. The number of expected, but undetected, alleles was determined for each resulting profile and is also reported in Table 3.3.

	PAC	IPC	1A	1B	1C	1D	1E	1F
Heterozygote Ratio	78.5	73.8	63.9	58.4	61.8	44.6	58.1	32.1
Undetected Alleles	0	0	2	1	0	3	4	9

Table 3.3: Heterozygote ratios and undetected allele quantities for PowerPlex® 16 BIO amplified WGA products. Percent heterozygote ratios reflect the average value of all loci ratios calculated within each profile. The number of true alleles expected to result from this template was determined by direct amplification with the PowerPlex® 16 BIO kit (IPC) and was compared to profiles resulting from WGA reaction products in order to determine the number of alleles that were undetected to reflect allelic drop-out occurring within each profile. Loci exhibiting allelic drop-out were not considered for the heterozygote ratio calculations. 1A=1.00 ng, 1B=1.00 ng (not denatured); 1C=0.50 ng; 1D=0.25 ng; 1E=0.10 ng; 1F=0.05 ng.

Surprisingly, the number of undetected alleles did not directly correspond with WGA template quantity. While one may have expected higher template quantities to result in better STR template post-WGA, this was not the case (Table 3.3). The 1.0 ng quantities both exhibited drop-out (1A and 1B), yet a full profile was detected at the 0.5 ng WGA template quantity (1C). Overall, the WGA reaction template samples did show an appreciable amount of allelic drop-out and such occurrences were especially evident in the sample where 0.05 ng of template was provided to the WGA reaction (1F) with the loss of nine alleles over seven loci.

Also from Table 3.3, there appeared to be little or no relationship between WGA template quantity and resulting profile quality. While both controls gave average heterozygote ratios in the range of 75%, WGA templates gave ratios from 32.1% (50 pg sample 1F) to 63.9 % (1 ng with denaturation sample 1A). However, the 100 pg WGA template (1E) resulted in less mean imbalance than did the 250 pg template

(1D), indicating that no trend was observed using the single amplification sampling method.

It was unclear whether the heat denaturation prior to WGA serves to increase product (and ultimately template) integrity. The 1 ng template samples, denatured (1A) and non-denatured (1B), yielded profiles of similar quality (Table 3.3). While less sister allelic imbalance occurred overall in the heat-denatured sample (1A), the non-denatured sample (1B) exhibited less allelic drop out. Duplicate samplings would likely be necessary to further investigate the usefulness of denaturation prior to WGA. However, further tests regarding the effects of omitting this step will not be performed as the performance of the denatured samples of less than 1 ng were no better than direct STR testing.

A primary consideration was if the QuantiBlot® results accurately reflected suitable template quantities for STR-PCR template amounts, or if observed profile shortcomings were a result of poor quantification prior to amplification. As expected, the WGA reaction negative control did not produce an STR profile, confirming that signal detected on the blot likely arose due to probe recognition of bound random hexamer primers and/or primer concatamers. Template quantities were targeted with caution and carefully considered when interpreting post-amp data since signal detected on the blot for the negative control may indicate system background. However, the overall intensity of the STR profiles for the WGA samples containing template (as determined by the post-amplification product gel and the analytical gel images) appeared satisfactory and this indicated that template amounts for the STR reactions were on target. While imbalance was noted within and between loci, trends exhibited by the WGA template reactions were not indicative of poor template quantification. Based on previously conducted validation studies with this typing system, addition of excess template generally results in “bottom-heavy” profile morphology, where short fragment loci are extremely robust and long fragment loci are weak or undetectable. To the other extreme, inadequate template quantities result in random drop-out events and overall weak signal. The profile from 1F is weak overall and does exhibit random dropout and it is possible that additional alleles may

be detected if more template was added to the STR reaction. However, higher WGA template quantities also resulted in profiles with significant imbalance between and within loci, indicating that stochastic effects associated with such imbalance were likely imparted during the WGA amplification as opposed to the PowerPlex® 16 BIO STR amplification.

Given the results of the WGA sensitivity study on purified DNA samples, it was determined the WGA process offers little advantage to typing low copy number samples. While the whole genome amplified DNA can be subsequently typed using current STR methods, the resulting profile quality is compromised. Moderate to severe allelic dropout is apparent and sister allelic imbalance is accentuated in reactions where template was generated using WGA. These studies indicate that the overall system sensitivity was not enhanced when WGA preceded conventional STR typing. In fact, profiles of similar quality occurred with direct STR amplification of 0.05 ng template (studied in Chapter 2) as occurred in the sample where WGA was used prior to STR typing using approximately ten times as much starting DNA.

3.3.2 Whole Genome Amplification of Whole Cell Lysates

While it was concluded that WGA of purified aqueous DNA samples offered no clear advantage for downstream STR typing applications, the possibility existed that WGA could be conducted in less stringent conditions than conventional multiplex STR amplifications. The manufacturer's protocol suggested that WGA could be carried out on sample cell lysates in an effort to amplify the DNA directly after cell lysis and prior to template concentration and purification. Therefore studies were designed to investigate the possible forensic applications for cell lysate WGA for template quantity enhancement prior to purification and conventional STR amplification. GenomiPhi™ amplification of cell lysate material was conducted using dilutions of human blood. Because the manufacturer's literature stated it possible to obtain reliable WGA products from the DNA of only 10 cells, blood samples were diluted and lyophilized to target cell numbers per sample to test this claim. These procedures

were described in section 3.2.2; samples 2D through 2H were targeted to estimate WGA on lysates of 141, 70, 35, 18, and 9 cells (respectively).

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Lysis reactions were initiated on samples 2D through 2H; following the recommended lysis procedure, amplification was carried out. Upon completion of the WGA, 5 μL of product volume (25%) was removed and reserved on ice (crude), while the remaining ~15 μL (75%) underwent concentration/purification (purified).

Purification/concentration was carried out on the remaining 75% of each sample and a portion of the 20 μL purified eluates were diluted 1:49 for visualization on a 1% agarose gel. Figure 3.13 represents the product gel obtained when 1 μL of each sample was analyzed.

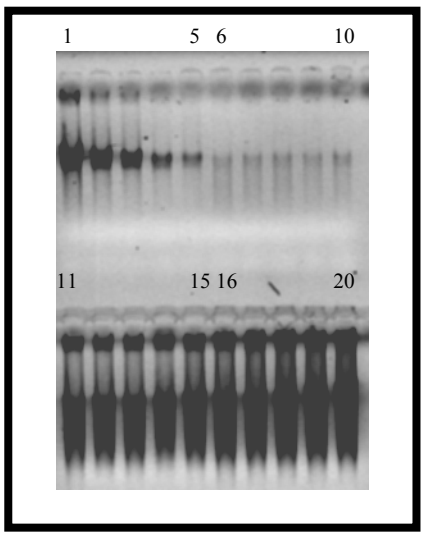

Figure 3.13: Product gel (1%) of lysis WGA products. Lanes 1-5 (left to right) contain the quantification standard series in the amounts of 100 ng, 50 ng, 25 ng, 10 ng, and 5 ng of DNA. Lanes 6-10 contain samples 2D through 2H following Microcon® purification and 1:49 dilution in TE. Lanes 11-15 contain the undiluted Microcon®-purified products of 2D through 2H; lanes 16-20 contain the crude WGA lysis products from 2D through 2H. 2D=~141 cells; 2E=~70 cells; 2F=~35 cells, 2G=~18 cells and 2H=~ 9 cells.

The yield gel revealed little difference between undiluted Microcon®-purified and undiluted crude products (wells 11-15 and 16-20 of Figure 3.13, respectively), both of which appeared saturated and similar to that observed following WGA of purified

DNA. The Microcon®-purified samples diluted 1:49 were then quantified using the QuantiBlot® Kit. Samples 2D through 2H were analyzed at the 1:49 dilutions. Figure 3.14 represents the blotting pattern for the samples employed for this assay, while Figure 3.15 represents the resulting blot.

	1	2	3	4	5	6
A		10 ng std	2D (1:49)			
B		5 ng std	2E (1:49)			
C		2.5 ng std	2F (1:49)			
D		1.25 ng std	2G (1:49)			
E		0.63 ng std	2H (1:49)			
F		0.31 ng std	Cal 1 (3.5 ng)			
G		0.15 ng std	Cal 2 (0.5 ng)			
H						

Figure 3.14: Blot arrangement for analysis of Microcon®-purified products from WGA of cell lysates. Each well received 1 μL of samples 2D through 2H at the 1:49 dilution. 2D=~141 cells; 2E=~70 cells; 2F=~35 cells, 2G=~18 cells and 2H=~ 9 cells. Standard and calibrator quantities are expressed in ng/well; a loading volume of 5 μL was used to achieve each of the indicated amounts. Std= standard; Cal=calibrators.

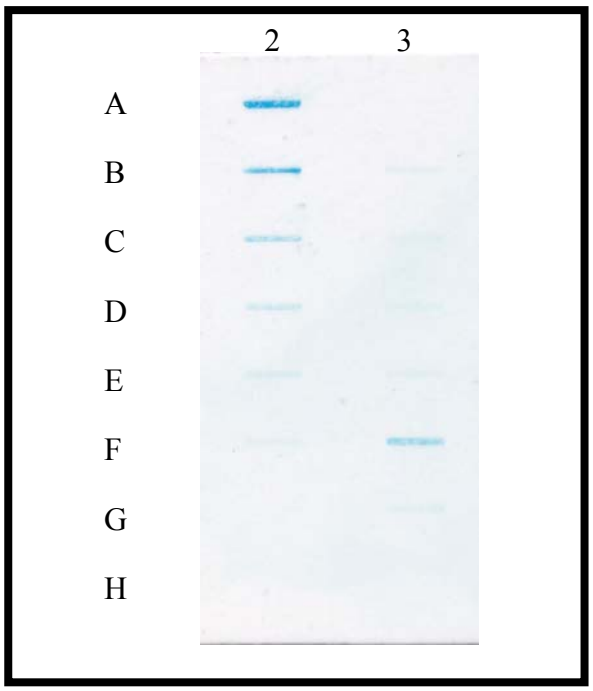

Figure 3.15: Colorimetric detection of Microcon®-purified products from WGA of cell lysates. Column 2 contains the standard series, wells 3A through 3E contain the 1:49 dilutions of samples 2D through 2H; calibrators representing 3.5 and 0.5 ng of DNA were loaded in wells 3F and 3G, respectively. For experimental samples, 2D=~141 cells; 2E=~70 cells; 2F=~35 cells, 2G=~18 cells and 2H=~ 9 cells.

Results from Figure 3.15 revealed little correlation between cell number in the lysis reaction and WGA product yield. A target template amount of 0.25-0.3 ng of template DNA per tube was used for the reduced-volume PowerPlex® 16 BIO typing reactions of the WGA products obtained from the lysates. Positive controls accompanied the PowerPlex® 16 BIO amplifications consistent with those previously described for the WGA sensitivity study and a post–amplification product gel was run as previously described. Each well in Figure 3.16 contains 2 μL of amplified reaction volume (approximately 27% of each amplified sample).

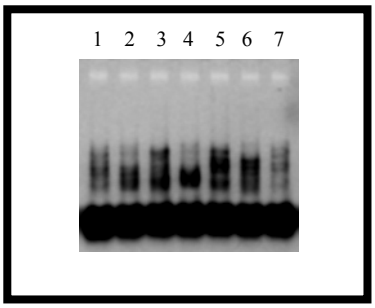

Figure 3.16: Post-amplification product gel (3%) of PowerPlex® 16 BIO amplified WGA cell lysis products. Lane 1 contains the internal positive control (Microcon®-purified human DNA), lanes 2 through 6 contain WGA cell lysis products from samples 2D through 2H (respectively), and lane 7 contains the positive amplification control (9947A DNA). For experimental samples, 2D=~141 cells; 2E=~70 cells; 2F=~35 cells, 2G=~18 cells and 2H=~ 9 cells.

The post-amplification product gel (Figure 3.16) indicated that, while product is present in most of the WGA cell lysis product reactions, the amplifications may have occurred in a poorly balanced manner, as many samples exhibited unevenly distributed signal compared to the positive controls. Unlike well 7 that contains a fairly even distribution of signal, others appear clustered or banded. No dilutions were performed to any of the samples prior to loading on the 6% polyacrylamide gel; Figures 3.17 through 3.19 represent the analytical gel results following the PowerPlex® 16 BIO amplification.

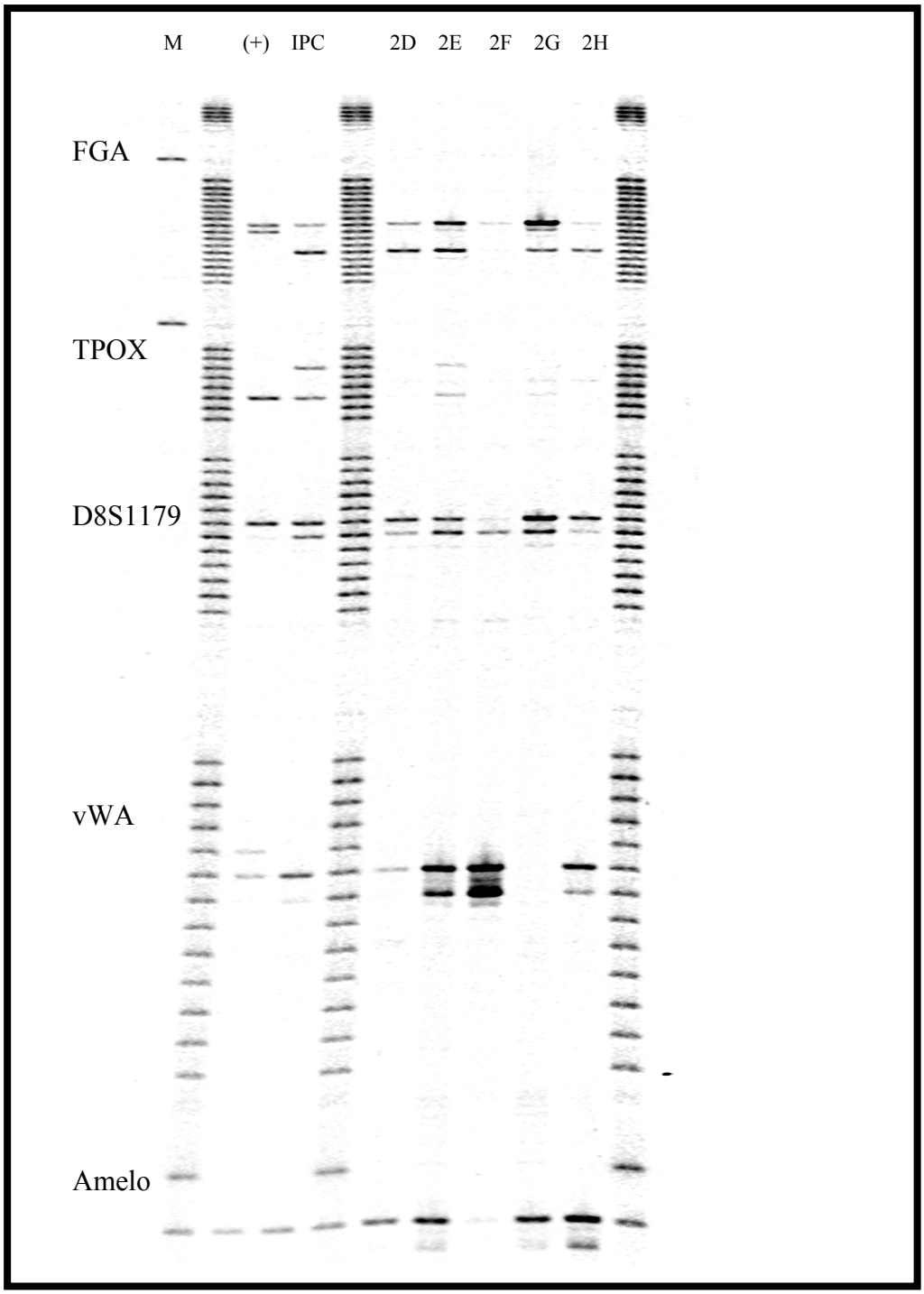

Figure 3.17: PowerPlex® 16 BIO amplified cell lysis WGA products-Rhodamine Red™-X (RRX) loci. Image color separation was optimized for RRX-labeled products, with locus designations indicated next to the corresponding ladders. Cell lysis product reaction designations are indicated for each lane; 2D=~141 cells; 2E=~70 cells; 2F=~35 cells, 2G=~18 cells and 2H=~ 9 cells. All samples were analyzed at standard volume. M=color separation matrix, (+)=positive amplification control (9947A), IPC=internal positive control (Microcon®-purified human DNA).

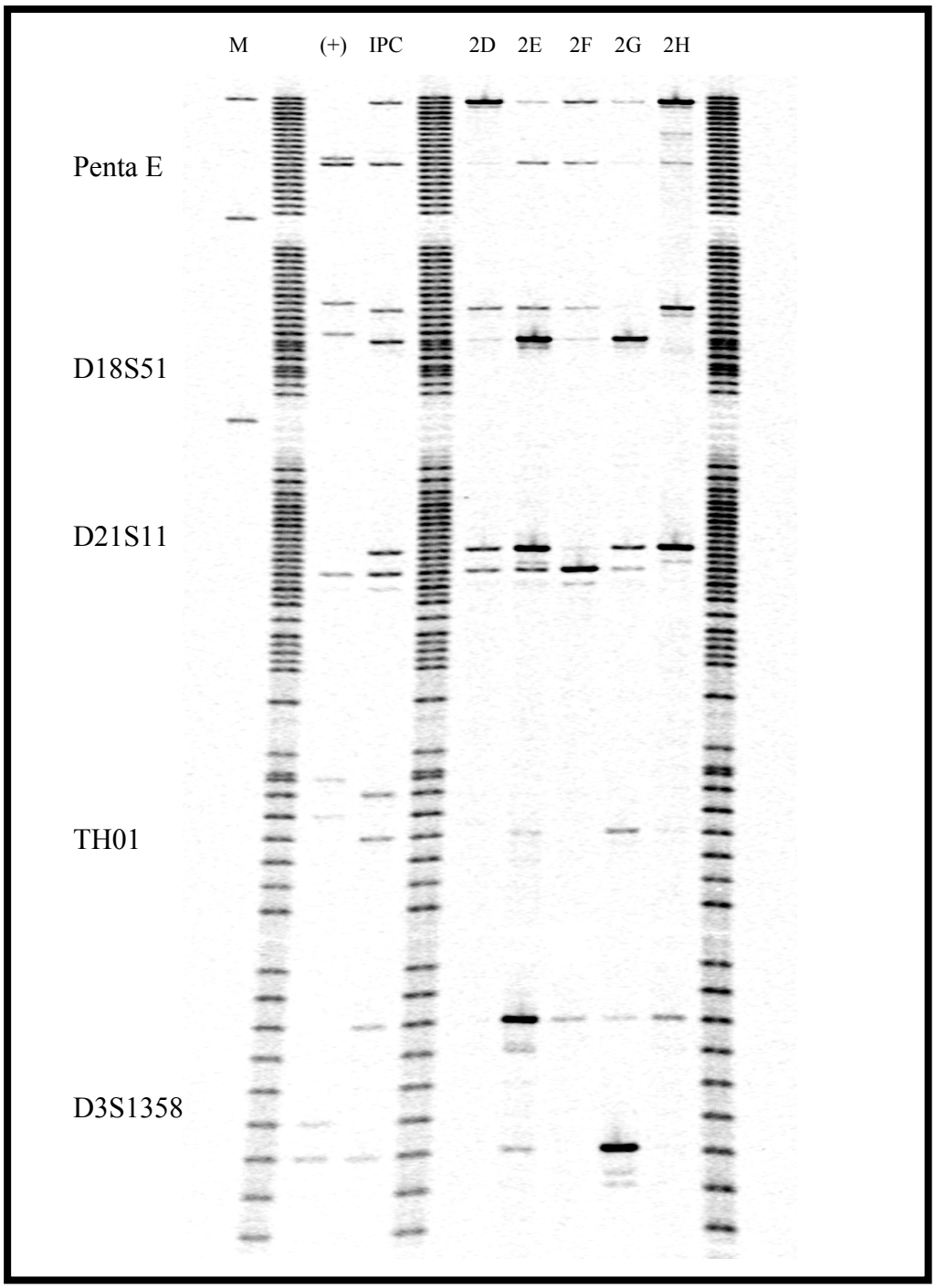

Figure 3.18: PowerPlex® 16 BIO amplified cell lysis WGA products-fluorescein (FL) loci. Image color separation was optimized for FL-labeled products, with locus designations indicated next to the corresponding ladders. Cell lysis product reaction designations are indicated for each lane; 2D=~141 cells; 2E=~70 cells; 2F=~35 cells, 2G=~18 cells and 2H=~ 9 cells. All samples were analyzed at standard volume. M=color separation matrix, (+)=positive amplification control (9947A), IPC=internal positive control (Microcon®-purified human DNA).

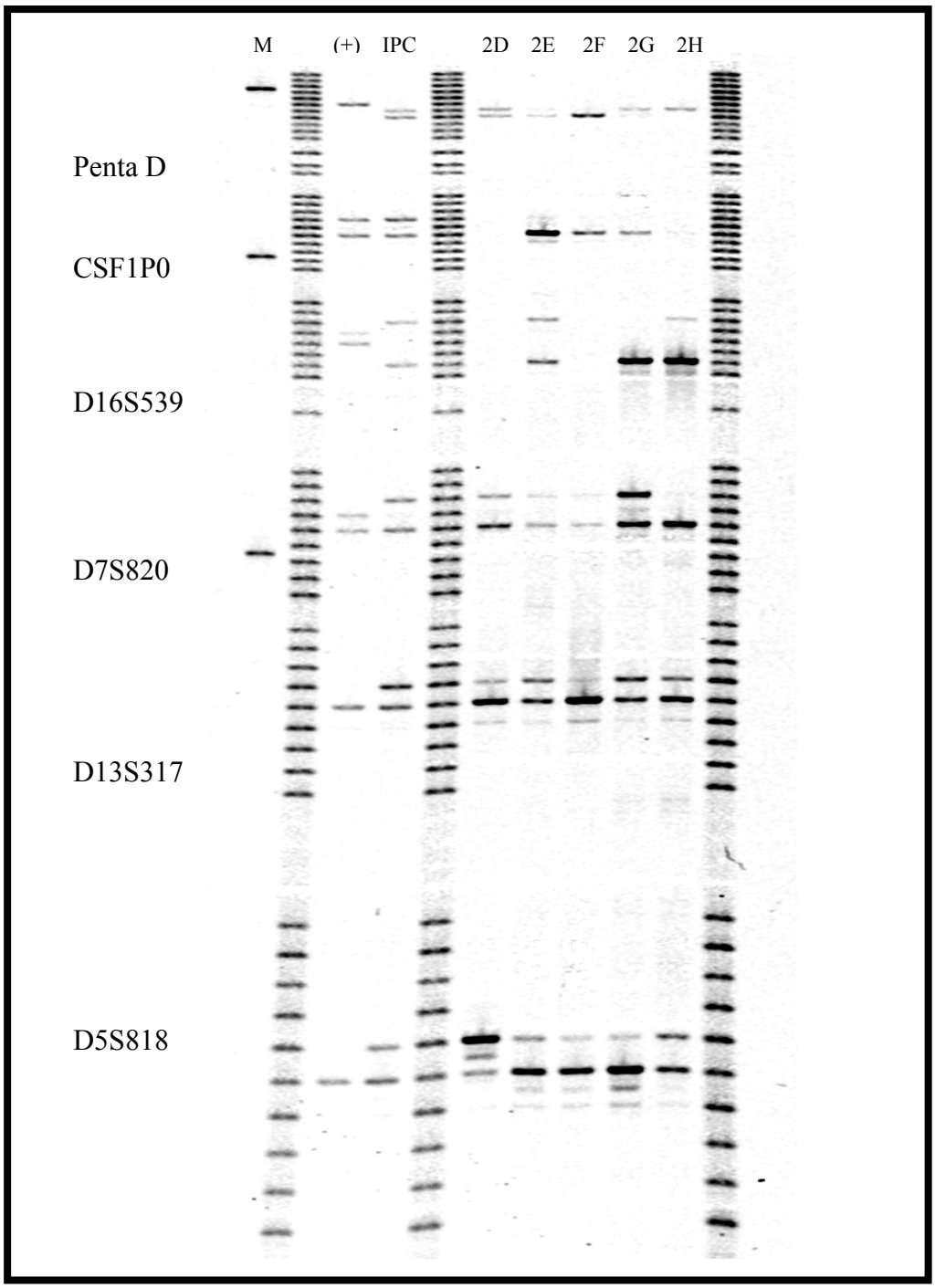

Figure 3.19: PowerPlex® 16 BIO amplified cell lysis WGA products-6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE) loci. Image color separation was optimized for JOE-labeled products, with locus designations indicated next to the corresponding ladders. WGA reaction designations are indicated for each lane; 2D=~141 cells; 2E=~70 cells; 2F=~35 cells, 2G=~18 cells and 2H=~ 9 cells. Cell lysis product reaction designations are indicated for each lane. All samples were analyzed at standard volume. M=color separation matrix, (+)=positive amplification control (9947A), IPC=internal positive control Microcon®-purified human DNA).

Visual inspection of the profiles following STR-PCR fragment separation revealed extensive drop out and imbalance both between and within loci. The template used for the cell lysis study originated from the same individual as the IPC, allowing for direct comparison between the WGA template reactions and the expected results. Percent heterozygote ratios were calculated for each locus within each profile. Those values, along with the average of all profile heterozygote ratios were tabulated and included in a results summary of the whole genome amplified cell lysates that underwent STR-PCR multiplex typing in Table 3.4.

Estimated number of cells (sample identifier)	141 (2D)	70 (2E)	35 (2F)	18 (2G)	9 (2H)
FGA	44.2	94.6	Missing 20	15.8	18.4
TPOX	-	75.2	-	-	-
D8S1179	35.5	84.9	12.4	40.8	18.1
vWA	N/A	46.8% ES	130.9% ES	-	26.2% ES
Amelogenin	N/A	N/A	N/A	N/A	N/A
Penta E	2.3	37.7	65.7	18.5	Extra 17
D18S51	17.5	12.1	57.1	Missing 18	Missing 14
D21S11	65.9	20.0	2.8	28.7	Missing 30
TH01	-	Missing 9	-	Missing 9	-
D3S1358	-	12.5	Missing 14	4.8	Missing 14
Penta D	86.4	19.8	Missing 11	Missing 10	Missing 10
CSF1PO	-	Missing 12	Missing 12	Extra 15 Missing 12	-
D16S539	-	56.2	-	Missing 13	4.9
D7S820	40.2	34.7	4.2	87.6	Missing 12
D13S317	12.6	64.6	2.2	88.8	31.8
D5S818	13.7	26.3	9.9	6.8	64.3
Average heterozygote ratio	35.4	44.9	22.0	36.8	24.0

Table 3.4: Summary of PowerPlex® 16 BIO amplified cell lysis WGA products. Estimated cell number and sample identifiers are above each column and a results summary for each is present beneath each sample. Loci are indicated in the far left column. Values reported without additional comment reflect percent heterozygote ratio of sister alleles for that locus, the values were averaged to calculate the average percent heterozygote ratio for each sample overall (reported in the bottom row). Where percent heterozygote ratios are not reported, absent alleles are noted. A dash indicates that bands were not detected for that locus. Alleles not attributable to the source template are indicated in red and ES (elevated stutter - greater that 20%) values observed at vWA are documented where applicable. ES=elevated stutter; N/A= not applicable (homozygotic type detected as expected).

The table indicates that there was no clear trend regarding number of cells lysed and quality of STR profile. Furthermore, allelic imbalance occurred somewhat randomly throughout the profiles. While TPOX and TH01 seemed to drop out first, other allelic imbalance or loss did not occur in a predictable manner. While most stutter percentage values were marginal or within acceptable ranges, vWA was the locus that most commonly exhibited elevated stutter percentages. Elevated stutter was observed in samples 2E (46.8%) and 2H (26.2%). Interestingly, the vWA locus dropped out of sample 2G, and the stutter band was actually more intense than the true allele at vWA for sample 2F (130.9%).

A certain degree of imbalance is tolerable in single source profiles, but detection of alleles that are not attributable to the template source is catastrophic. Alleles not expressed by the template source were detected in sample 2G (15 at CSF1PO) and in sample 2H (17 at Penta E). While a negative control may have been useful to explore the possibility of contamination, allelic drop-in is not observed in any other sample and, given the fact this occurred in two different samples at uncommon loci, results indicated that there is a strong likelihood these additional bands are artifacts. Drop-in was not observed in WGA reactions from purified DNA templates, which explored template quantities ranging from 50-1000 pg. The estimated amount of DNA present in the WGA reactions using the lysis procedure was 54-846 pg template. While not resolved by exhaustive studies, this could indicate that the presence of cellular debris, proteins, and a variety of inhibitors inherent to the lysate procedure are interfering with the WGA and resulting in the artifacts (rather than the mere lack of optimal amount of template).

Admittedly, the design of this whole cell lysate study did have assumptions. Namely, it must be acknowledged that the calculations for targeting a certain cell range are estimations. Since the instrumentation was not available for isolation of a single cell, this method was used to estimate cell number for lysis reactions. Regardless, the results do not indicate any direct relationship between typing success and cell concentration. While the exact number of cells is not known, the data suggests that WGA and downstream STR success is not related to input cell numbers and,

regardless of cell number input, the method was not successful in achieving a well-balanced DNA profile. Furthermore, because such a limited volume of lysate can be placed into the WGA reaction, and the lysis takes place in a very small volume of solution, the prospect of conducting the process on substrate-bound forensic samples is simply not feasible. While one could perform a cell extract to concentrate the forensic sample's cellular components into a pellet for lysis, this would introduce another step subject to transfer loss, the cell extraction would not be 100% efficient, and only 1/20 of this would ultimately go into the WGA reaction using this procedure.

Even though profiles were observed from all cell lysis WGA templates used as template for PowerPlex® 16 BIO amplification, the profile quality was severely compromised. A certain degree of sister allelic imbalance and/or drop-out is tolerable for forensic applications, but most WGA templates derived from cell lysis studies yielded profiles that exhibited loss at multiple loci accompanied by severe drop-out at others. Signal saturation was observed within profiles, and stutter interpretations could not be made. Most troubling was the detection of alleles not attributable to the DNA source. While one can never definitively determine whether additional alleles are PCR artifacts or the result of contaminating template, the occurrence of this in two profiles within the sample set is strikingly unusual. These occurrences raised the concern that random artifacts were resulting from unusual template structures produced during the WGA process on a limited number of cells.

In summary, due to the inherent problems with detection of additional loci that could result in wrongly including an individual as a possible contributor to a forensic profile, the application of this process in the forensic setting seemed limited at this point in the study. While detection of untrue alleles would alone dictate a severe shortcoming for forensic samples, the applications of this to forensic testing are further complicated by the fact that no observable trends could be identified with respect to untrue allele appearance or drop out, rendering the system next to impossible for refinement should further studies be pursued.

3.4 Conclusions of WGA for LCN Forensic Applications

Initial studies herein allowed for clear establishment of the current technological capacity for typing low quantity samples using two prevalent multiplex systems, and are summarized in Chapter 2. Subsequent research conducted exploring LCN applications of the commercially available GenomiPhi™ kit proved to offer little or no advantage to conducting WGA on template prior to conventional STR-PCR amplification. This was true for WGA of purified samples and WGA-amplified lysis products generated prior to DNA capture and purification.

It should be noted that the WGA aspects of this work were completed during the first several months of the course of this project. Since then, personal communication with WGA manufacturers reveals that the components of the kits are not prepared in clean rooms, which leaves the reagents subject to the introduction of human DNA [Nelson, 2007]. That said, it is acknowledged that a single molecule of DNA can be amplified by this system and result in drop-in events like those which occurred during these tests. Furthermore, the manufacturer has since advised that this is one primary reason why the kits are not highly recommended for use involving assays where less than 1 ng of template is available for amplification. While this fact presents another reason to discontinue the exploration of this particular kit for LCN forensic applications, it is important to note that new developments are focusing on methods to produce template-free kit components. Moreover, the prevalence of real time quantitation systems could potentially overcome the shortcomings of the blot quantitation systems which produce signal due to "primer-dimer" like occurrences which take place in WGA reactions [Nelson, 2007].

The results obtained using the GenomiPhi™ kit were disappointing with respect to measures explored for this project. Nevertheless, it appears that the results obtained for the purposes of this project are not unlike the results obtained later by groups investigating similar applications; details of other reports will be more fully addressed in Chapter 7. While it would have been exciting to explore the scope of a product that offers substantial advantages over current STR multiplex typing alone, affirmation of the limitations of the kit as made by other groups and the manufacturer's developing

scientists indicates that the determinations made herein are likely to be sound. The initial premise for investigating WGA in a pre-STR-PCR sense did not yield seemingly rewarding results, but it was apparent that the reaction is more forgiving than conventional multiplexing based on the fact that amplification takes place in crude cell lysate conditions. Therefore, future studies included in this project could explore the use of WGA to amplify samples that are not conducive to conventional typing because of challenging disposition. DNA testing was applied to many cases prior to the advent of PCR technology in the forensic community in the form of RFLP testing, which relied on binding restriction-cut DNA to a static support, usually nylon membrane. Therefore, where direct multiplex amplification of membrane-bound DNA proves difficult with conventional STR chemistries, the ability of WGA chemistry to replicate DNA bound to a static support is yet unknown. If WGA was found to provide free template that could be subsequently typed with STR analysis, this would provide a unique gap between two prevalent typing technologies. WGA is firmly established in the clinical and research fields, and while it did not offer the desired advantage for enhancing extracts prior to STR typing, establishing the limitations of the system will undoubtedly prove useful when considering the technique for other possible applications.

4.0
PCR Enhancement Techniques

4.1 STR-PCR Enhancement Approaches

The work performed in this chapter was aimed to investigate approaches for enhancing the STR-PCR process by template pre-treatment or PCR reaction additives to improve the quality and yield of end-point PCR product. Since the WGA work did not prove to offer an advantage over the standard multi-plex technology, the focus was shifted to enhancing the multiplex reaction directly. Using the same reaction components as the kits already approved for the database would expedite the validation and resulting profiles would likely be eligible for CODIS database entry if the core components of the reaction were those listed as acceptable by the FBI. At the very least, should approval be required, the review process by the FBI would be vastly expedited should the aforementioned key components remain unaltered.

Both the quality and the quantity of DNA are important for reliable profiling. Since the work herein progresses toward the removal of membrane bound DNA from membranes that contain samples that have undergone restriction digestion, possible UV irradiation, and a series of probings and strips, the focused goal of this project is not far removed from the daily challenges faced in the common casework lab. In fact, a membrane bound sample is not dissimilar to any DNA that has been bound by a substrate, degraded, and subjected to environmental insult. Therefore, the tests are applicable not only to this project, but potentially any forensic DNA sample encountered in the lab. The amount of damage imparted upon the DNA during the RFLP process will be critical when considering one's chances of using the DNA as a PCR template, either bound or recovered. Furthermore, recovery attempts will require further manipulation of the DNA which also could cause damage or substantial product loss. The degree of modification that will be caused by digestion of the DNA with the exonuclease alone is somewhat predictable given that these enzymes are well understood in their mechanism of action. Of greater uncertainty is the structural changes that are caused by binding to the membrane, either due to template interactions with surface moieties and/or the interactions induced or strengthened by UV irradiation of the membranes common in many RFLP protocols. The nature and extent of damage encountered by template will undoubtedly affect one's ability to PCR amplify the template given that template quality is a great factor

in successful processivity of a polymerase. Manipulations that involve acids, heat, light, extraction, and storage all impart damage, as may time alone [Walker, *et. al*., 2004].

This chapter will report on the evaluation of two recently released PCR enhancement systems. The first is tests conducted using a PCR additive, PCRboost™, which consists of a proprietary mixture of enhancers. In the reaction, the volume normally taken by water is supplanted by the PCRboost™ reagent.

The second is Restorase®, a novel polymerase blend manufactured by Sigma-Aldrich; it combines a DNA repair enzyme mixture with a polymerase and is designed to repair damaged templates, especially those that have undergone depurination or depyrimidation. While components of the system are proprietary, mechanisms similar to those that take place during normal genetic repair within the cell were likely considered when developing the reaction mixture.

Because both products to be tested have several proprietary components, the theory presented will be basic in the two areas of PCR enhancement additives and DNA repair systems.

4.1.1 PCR Enhancement Additives

With the introduction of PCR technology in the 1980's [Mullis and Faloona, 1987], it was not long after that the process had further refined and efforts began to focus on obtaining more DNA product from smaller, more challenging samples. This section will provide the theory and basis for many of these approaches since some combination of them undoubtedly comprise the proprietary enhancement cocktails marketed today.

Unlike the Phi 29 enzyme, *Taq* polymerase lacks 3' to 5' proofreading activity which means that it copies with relatively low fidelity [Lawyer *et. al*., 1993]. In fact, the polymerase incorporates a mismatched base pair once every 9000 bases assembled [Tindall and Kunkel, 1988]. Early improvements to the PCR process focused on

modifications to the enzyme itself in order to reduce artifacts while improving fidelity and processivity. One modification is hot start *Taq* polymerase, which renders the enzyme inactive until the reaction mixture reaches a certain temperature [D'Aquila, *et. al.*, 1991]. This prevents non-specific products and artifacts that can result from partial copying that occurs during reaction assembly. Early methods involved physical separation of template from PCR reagents by wax barrier that would vaporize upon heating [Chou *et. al*., 1992]. Some hot start products are based on antibody interactions with the enzyme; the antibodies denature at the high temperature, freeing and thus activating the polymerase [Kellogg *et. al.*, 1994]. Other approaches have included genetically modified *Taq* that has little or no activity at low temperature [Kermekchiev *et. al*., 2003]. As of spring 2009, commercial kits that are specified for use by the FBI have been optimized with Ampli*Taq* Gold® DNA polymerase. All Ampli*Taq*® polymerases are expressed recombinantly in E. coli, and the "Gold" is the trade reference for the hot-start property of the enzyme made possible by a bound inactive moiety that dissociates with long term heating to 95^{o} C [Applied Biosystems, 2007a]. The polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3' ends of the PCR products and, as cycling occurs, more enzyme is activated at the denaturation step to provide some newly activated polymerase with each cycle [Applied Biosystems, 2007a]. Today, a variety of proprietary *Taq* polymerases are available, chemically and genetically modified for polymerase activity and/or blended with other thermostable enzymes to achieve proofreading capability (by virtue of 3' to 5' exonuclease activity).

Other than modification to the polymerase, many additives have been shown to enhance PCR reactions either by reducing secondary structures or by reducing inhibitors. For example non-ionic detergents such as Triton X-100 and Tween have been shown to offer stability to the polymerase [Gelfand *et. al*., 1989]. Betain has also been successful for enhancing amplification across GC-rich DNA sequences, which are notoriously difficult to process [Henke *et. al*., 1997]. Other solvents that have proven to be very helpful have been DMSO and formamide [Sarkar *et. al*., 1990; Smith *et. al*., 1990; Pomp and Madrano, 1991]. Both betaine and DMSO enhance

strand separation and while DMSO interrupts base pairing in general, betaine is an isostabilizing agent that equalizes contribution of GC and AT base pairing to stabilize the DNA duplex [Henke *et. al*., 1997; Frackman *et. al*., 1998]. Formamide undoubtedly contributes to strand separation, as it is used as a denaturing agent in post amplification applications. Since the initial discovery that formamide was an enhancing additive, other amides such as acetamide and 2-pyrrolidone were found to equally or superiorly enhance PCR of difficult templates [Chakrabarti and Schutt, 2001]. One of the most widely used additives in the forensic field is bovine serum albumin (BSA), as it has been shown to induce non-specific interaction with proteins and inhibitors within the reaction, thus reducing the effects of inhibition; this was especially important to forensic scientists typing blood since heme is a major inhibitor to the PCR process [Akane, *et. al*., 1999]. BSA, due to high lysine content, binds lipids and anions and is believed to sequester phenolic compounds [Kreader, 1996].

Other substances such as tetra-methylammonium chloride (TMAC) and the T4 gene 32 protein have also shown to enhance PCR by elimination of non-specific priming. For example, the gp32 protein is a single-stranded binding protein that likely binds with denatured DNA strands to prevent secondary structure or non-specific priming events [Kreader, 1996].

These additives have all exhibited enhancement capabilities separately, and are not necessarily of increased function when used at higher levels or together. Polymerase storage buffers and commercial PCR buffers often contain some detergents and BSA to stabilize enzymes and reduce the effects of inhibition, respectively [Applied Biosystems, 2000a], therefore additives beyond those inherent to a commercial kit should be carefully investigated prior to use on casework. Furthermore, it should be noted that each additive has a defined mechanism, so while betaine might be useful on enhancing GC rich regions, if these are not prevalent in chosen STR amplification sites, this would not be a useful additive for forensic applications. Alternatively, BSA has the ability to overcome inhibition factors, which are often present in casework extracts.

Although the components of the PCRboost™ additive are proprietary, it would sensibly contain one or more of the above proven enhancement additives and is likely optimized for increased product from a broad variety of sample and template types. Studies presented by the manufacturer (available online), indicate that increased amplification occurs when the additive is used in PCR reactions instead of water [Biomatrica, 2009]. The manufacturer states that enhancement with this cocktail can produce up to five times greater amplified product than other additives currently available [Biomatrica, 2009]. The company markets the product for improvement of all sample types, including low copy number samples, inhibited specimens, and even degraded templates. Because the additive is compatible with a variety of cycling conditions, buffer systems, and polymerases [Biomatrica, 2009], it seems reasonable to test for use in conjunction with NDIS-approved typing systems.

4.1.2 DNA Repair Enzymes

While PCR additives can often enhance PCR, the mechanism is generally by induction of template denaturation or inhibitor sequestration. This serves to enhance the processivity on template regions containing difficult sequences and can help overcome inhibition, which are especially prevalent in forensic specimens. Often, forensic samples are also challenging for reasons related to degradation and deleterious change. The types of damage and modifications commonly encountered were presented in section 1.1.2. Today, one approach to improving typability of damaged template is *in vitro* repair, aimed at correcting the damaged areas via enzyme treatment. This would allow polymerase processivity through regions of the DNA that may otherwise not copy due to the presence of lesions, breaks, or structural defects [Sikorsky *et. al*. 2007]. One aim of this chapter is to test an enzyme mixture for this purpose, which may be of general use to forensic samples and also potentially useful for aspects of the project related to recovery of enzyme restriction and membrane bound DNA.

As described in the Introduction, radiation and chemical exposure induces damage to DNA resulting in modified bases. Mismatched bases cause structural defects in the strand, and replication through such lesions can be severely hindered or result in

misincorporation of nucleotides. Since STR typing is not nucleotide sequence specific, fidelity is usually not a concern. However, reduction in amplification efficiency can reduce the endpoint product, and if this occurs in an allele-selective manner during the early rounds of PCR, could impart inimical stochastic effects [Sikorsky *et. al*., 2007]. This chapter will expand upon damage that can be imparted upon the double helix and focus on the repair mechanisms that are used to reverse the damage and restore the integrity of the helical structure.

It is known that UV irradiation results in an abnormal covalent bond between adjacent thymidine bases and methylation of guanine bases. Forensic samples can suffer exposure to UV light in the form of sunlight, and many protocols for RFLP typing used nylon membranes that were UV irradiated to assist in the "permanent" bond between the DNA and the membrane. Therefore, correction for such damage could have both direct and ancillary importance to the forensic biologist.

Lesions induced by radiation include cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PP), both of which have been shown to block *Taq* polymerase [Wellinger and Thoma, 1995]. These structures are illustrated in Figure 4.1.

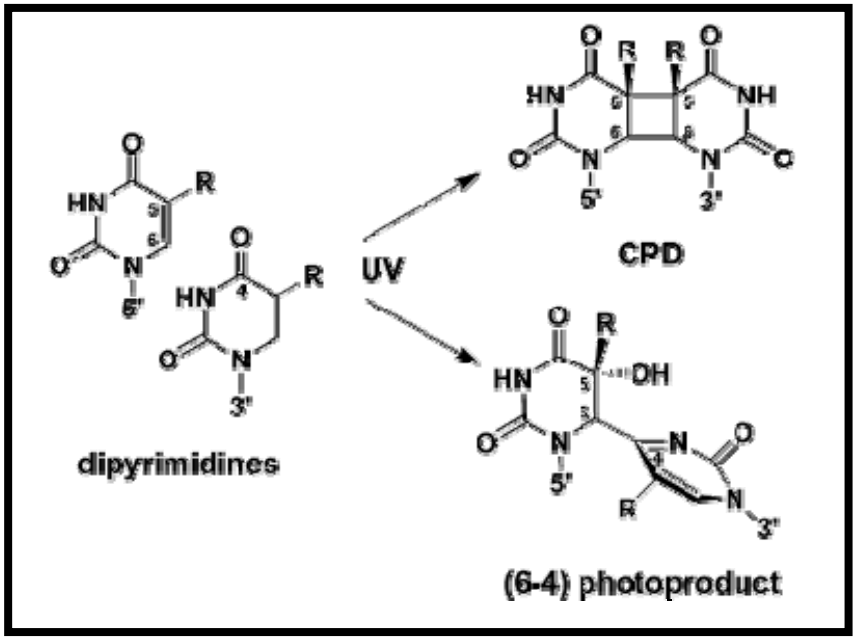

Figure 4.1: Structure of UV induced DNA pyrimidine dimer lesions. The figure illustrates the most stable UV induced lesions, cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PP), both cause conformational changes that inhibit processivity of *Taq* polymerase. R = H or CH_3 [adapted from Li, *et. al*., 2006].

While certain polymerases have been shown to bypass these and isomers of these lesions, *Taq* is only able to overcome the least severe *cis-syn* isomer of the dimer, which imparts the least disruption to the helical structure [Smith *et. al*., 1998]. While this is the most commonly induced form of damage, it is slow to repair and can still result in the incorporation of mismatched bases [Smith *et. al.*, 1998].

The natural process for reversing dimerization is photoreactivation and relies upon a catalytic reaction by photolyase. This reaction is described in Figure 4.2.

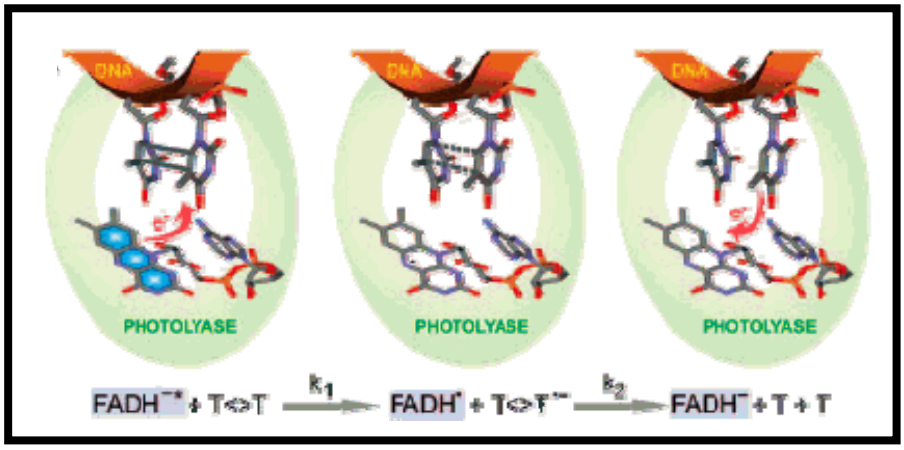

Figure 4.2: Diagram of photolyase repair of thymine dimer lesion. The figure is a schematic representation of repair of thymine dimers by CPD photolyase. First, FADH-, the cofactor for the enzyme, becomes excited by a photon (highlighted in blue and indicated as FADH-*), and transfers an electron to the dimer (T<>T), which has been extruded from the DNA double helix (red) by the photolyase. Next, the electron transfer breaks the cyclobutane ring of the dimer, leaving a ground-state reduced flavin radical (FADH*), a thymine, and a reduced thymine. Finally, electronic rearrangement restores both thymines to normal, the electron being transferred back to the ground-state reduced flavin radical (FADH*), restoring the original active-site cofactor (FADH-) [from Kao *et. al.*, 2005].

Recombinant DNA technology makes use of other enzymes to repair dimers, which includes the application of helicase, DNA polymerase, and ligase. The helicase removes a section of damaged DNA, a polymerase incorporates the new strand, and the nicks are then closed by the ligase. Figure 4.3 illustrates the process.

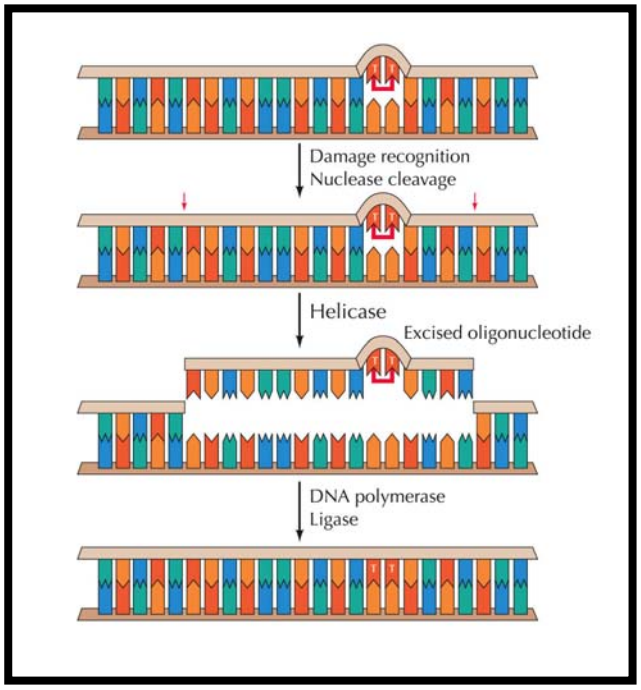

Figure 4.3: Nucleotide excision repair (NER) of damaged DNA. The diagram is a schematic of the NER process, where helicase recognizes the lesion and cleaves a single-stranded region from the strand containing the damage. A DNA polymerase incorporates the proper base pairs using the complimentary strand as template, and a ligase is used to re-establish the bonds between the flanking regions and the newly assembled strand [from Cooper and Hausman, 2007].

Aside from dimer malformations, UV irradiation can also induce guanine methylation, which, following secondary reaction also causes lesions that inhibit the processivity of *Taq* polymerase [Ludlum, 1990]. The methylated form of guanine is illustrated in Figure 4.4, along with the stoichiometric reaction that repairs methylation damage. This process is driven by the protein methyl guanine methyl transferase (MGMT), which is expended in the course of the reaction [Foote, *et. al*., 1980; Mitra and Kaina, 1993]. Expression of MGMT has proven variable among normal and tumor cell lines, and elucidation of the molecular mechanisms controlling MGMT expression has become of major interest in cancer research [Bhakat and Mitra, 2000] since normal expression levels must be maintained for proper cell regulation and proliferation.

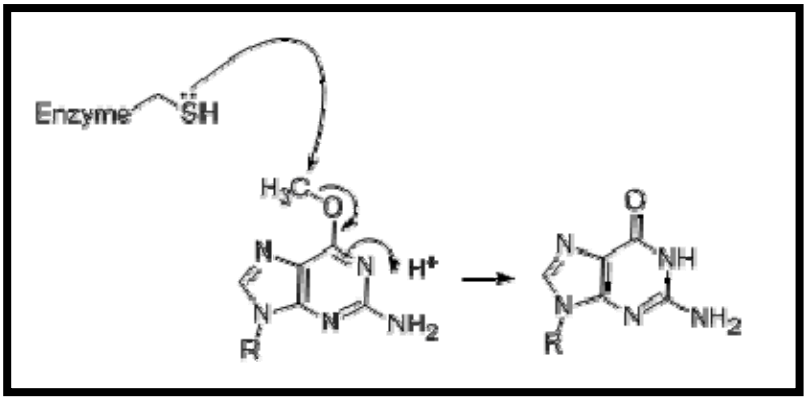

Figure 4.4: Repair with methyl guanine methyl transferase. The schematic shows electron transfer induced by the suicide enzyme (MGMT) which ultimately results in the removal of the methyl group from the base [image from ATDBio Ltd., School of Chemistry at the University of Southampton, 2004].

While MGMT is the leading mechanism whereby natural correction of methylated guanine occurs, base excision repair (BER) can also correct for the methylated product. Unlike nucleotide excision repair, where a section of nucleotides are excised and replaced, this process selectively removes and corrects a base. The process breaks the N-glycosylic bond between the base and sugar backbone of the DNA strand, release a base and leaving an apurinic or apyrimidinic (AP) site [Barnes *et. al.*, 1993]. Many glycosylases have an associated AP lyase which serves to complete cleavage of the phosphodiester bond 3' to the AP site; this is followed by a class II-endonuclease to create a nucleotide gap (class I nick at 3' side of AP site and class II nick at 5' side of AP site) [Barzilay and Hickson, 1995; Horton *et. al*., 2002]. A polymerase to follow can add the correct nucleotide where the excision has occurred [Singhal and Wilson, 1993]. The BER pathway is illustrated in Figure 4.5.

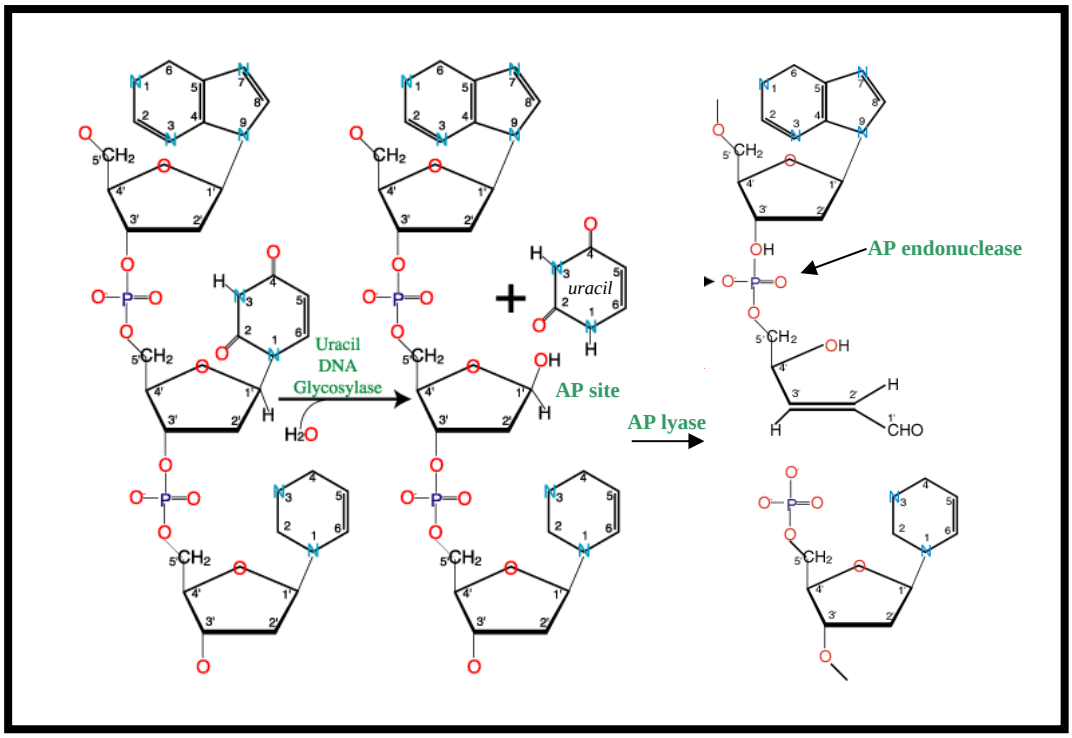

Figure 4.5: N-Glycosylase base excision repair (BER) pathway. In this example, uracil DNA glycosylase acts to break the N-glycosylic bond between the base and sugar of the DNA strand, releasing a base and leaving an apyrimidinic site. An AP lyase serves to complete cleavage of the phosphodiester bond 3' to the AP site. The site of a subsequent 5'-AP-endonuclease is indicated on the right side of the figure, which completes the excision and creates a single nucleotide gap [image from ATDBio Ltd., School of Chemistry at the University of Southampton, 2004].

While the pathways of DNA damage cannot be predicted for any given forensic sample, much work has been done to elucidate the types of damage imparted by UV irradiation, and these are expected in forensic samples as well as samples that have been bound to nylon for RFLP testing. Therefore, the mechanism, along with hydrolysis and other more specific chemical interference of the DNA structure, could potentially be applied to samples in vitro prior to the STR typing process in an attempt to "repair" template to a state more conducive to replication. This is the idea captured by Sigma's Restorase® DNA polymerase system.

The system comes with Restorase® DNA polymerase and a 10x reaction buffer. While all components of the buffer are disclosed and similar in composition to the buffer normally used in standard multiplex amplifications, the specific enzyme composition of the polymerase mixture is not known. It is speculated that the

mechanisms are even more straightforward than those already described and that apurinic and apyrimidinic (AP) sites on DNA may be acted upon by class I or II AP endonucleases, with an exonuclease serving to remove a short region of DNA and a DNA polymerase and ligase could then act to fill the gap. While the exact mechanism is unknown, most studies conducted by the manufacturer involve introduction of AP sites via formic acid treatment, and then recovery of amplifiability using the Restorase® system [Walker *et. al*., 2004]. Since the manufacturer boasts the system's ability to amplify highly degraded samples otherwise unable to be amplified by conventional methods, the system was considered for amplifying post-RFLP DNA samples and will be evaluated in this chapter for potential application for this specific project.

4.2 PCR Enhancement Materials and Methods

Data from preliminary tests used to evaluate the PCRboost™ and Restorase® polymerase systems' applicability to forensic samples, and specifically membrane bound DNA templates, will be presented based on the experiments described in this chapter.

4.2.1 PCRboost™ Experimental Methodology

DNA extracted from the blood of a male donor underwent standard organic extraction as described in Chapter 2, followed by Microcon® purification.

The sample was quantified using the Quantifiler™ Male and the Quantifiler™ Human Quantification Kits. Human genomic DNA (mixed male, Promega catalogue number G1471) was selected as the stock source of template DNA to prepare a standard series for these assays. After the initial preparation of a 50 ng/μL solution, a serial dilution was performed to achieve the standard series for the curve. The resulting concentrations are described in Table 4.1. All real time quantifications include a no template control (NTC), which consists of nuclease free water.

DNA Standard	Concentration (ng/μL)
1	50
2	16.7
3	5.56
4	1.85
5	0.62
6	0.21
7	0.068
8	0.023

Table 4.1: Dilutions for Quantifiler™ Kit standard curves. A stock solution of male DNA obtained commercially from Promega was used to prepare these dilutions which were then used when generating a standard curve with the Quantifiler™ Kit chemistry.

Samples were run on a 96 well optical plate format; for each well containing a standard or sample, 10.5 μL of Primer Mix and 12.5 μL PCR Reaction Mix were added (these components are supplied with the kits). To wells containing the reaction mix components, 2 μL of each sample was added. Samples were mixed, centrifuged, and amplified/detected using the Applied Biosystems 7500 Real-Time PCR System. Data was analyzed using Applied Biosystems SDS software and with the auto baseline parameters and a threshold setting of 0.2. The standard curve was analyzed to ensure a typical slope range between –2.9 and –3.5. For either kit, an R2 value of greater than 0.98 was verified. Based on an average quantification value, a 1 ng/μL concentration was made by diluting the extract 1:29 in NFW.

From this solution, the following concentrations were prepared: 0.5, 0.25, 0.125, 0.063, 0.031. A volume of 1 μL of each was incorporated into PCR reactions to achieve a sensitivity study of template quantities in the following amounts: 1.0, 0.5, 0.25, 0.125, 0.063, and 0.031ng. A volume of 0.5 μL of the 0.031ng/μL sample was also amplified to achieve a 0.015 ng template sample. Each template quantity was amplified in duplicate using the normal PowerPlex® 16 amplification procedure as described in Chapter 2. A single reaction cocktail generally consists of 2.5 μL Gold ST*R 10X buffer, 2.5 μL PowerPlex ®16 STR primers, 0.8 μL Ampli*Taq* Gold®

polymerase, and 4.2 µL NFW. Up to 15 µL of volume can then be added to the reaction which carries DNA template. For one sensitivity series, duplicate amplifications of each quantity were carried out in this manner and serve as the control sensitivity series. For the PCRboost™ assays, duplicate amplifications of each template quantity were carried out alongside the control series, however 4.2 µL of PCRboost™ reagent was substituted for the NFW. With the negative control in the amplification set, this consisted of amplification of twenty nine samples (duplicate amps of each dilution using water, duplicate amps of each dilution using PCRboost™, and a negative amplification control). Electrophoresis and detection were performed using the 3130 series genetic analyzers with Data Collection Software version 3.0. Data analysis was performed using GeneMapper™ ID Software version 3.1. Analysis method default settings were used with modifications to the Peak Detector parameters described in Chapter 2.

4.2.2 Restorase® Experimental Methodology

Amplification of 9947A (supplied with PowerPlex® 16 multiplex kit) was attempted to determine if the Restorase® system could be used in conjunction with the PowerPlex® 16 typing system One amplification of 9947A DNA employed Ampli*Taq* Gold® DNA polymerase and was carried out according to manufacturers protocol (as described in section 2.2) using 0.5 ng of template; this reaction served as the positive control. A second amplification of 9947A employed Restorase® DNA polymerase and was performed using approximately 5 ng of target template amount. The reaction was afforded an excess of template in an effort to obtain any profile at all since the modification to the amplification process was rather extensive and labor intensive. For this reaction, the following were added to the purified DNA: 19.2 µL nuclease-free water, 2.5 µL Gold ST*R 10X buffer, and 0.8 µL Restorase® DNA polymerase. The sample was incubated at 37° C for 10 minutes, followed by a secondary incubation at 72° C for 5 minutes. A volume of 2.5 µL primer mix (PowerPlex® 16) was added following the incubations. The 25 µL reaction then underwent 32 cycles of PCR using a modified version of the cycling parameters for the PowerPlex® 16 system with the omission of the 11 minute hot start normally used

for casework amplifications. The negative control for this amplification consisted of all PowerPlex® 16 typing components minus template. A negative control for Restorase® components was not performed specifically for this mini-study, but such a control will be later described when low copy number amplifications using the Restorase® system are conducted. Fragment detection was achieved with the ABI PRISM® 3100-*Avant* using Data Collection Software version 2.0. Data analysis was performed using GeneMapper™ ID Software version 3.1. Analysis method default settings were used with modifications to the Peak Detector parameters described in Chapter 2.

4.3 PCR Enhancement Results and Discussion

The results obtained from the PCRboost™ and Restorase® system assays were evaluated for potential enhancement of PCR profiling capabilities and/or potential for obtaining profiles from membrane bound DNA samples.

4.3.1 PCRboost™ Results and Discussion

RFU data was examined for each reaction. First, the aggregated percent heterozygote ratio was calculated for each profile by determining the average of the percent ratios observed at each of the 14 loci bearing heterozygotic types. Second, the RFU values for each allelic peak were collectively summed to create a "profile intensity", or total RFU value for each profile. And finally, the number of missing alleles was determined for each profile where drop out was observed. Data for samples amplified in the presence of PCRboost™ is reported in Table 4.2; data for samples amplified without the additive is reported in Table 4.3.

Template/reaction Description	Heterozygote Ratio (%)	Aggregated Heterozygote Ratio (%)	RFU Total	Mean RFU	Alleles Absent	Mean Alleles Absent
1 ng boosted	90.03		91055		0	
1 ng boosted	87.59	88.81	73922	82488.5	0	
0.5 ng boosted	85.47		47192		0	
0.5 ng boosted	86.75	86.24	40320	43756	0	
0.25 ng boosted	77.75		34096		0	
0.25 ng boosted	79.44	78.59	21173	27634.5	0	
0.125 ng boosted	74.48		13409		0	
0.125 ng boosted	76.91	75.69	16053	14731	0	
0.063 ng boosted	40.91		8965		1	
0.063 ng boosted	74.44	57.67	4485	6725	7	4
0.031 ng boosted	87.5		3724		12	
0.031 ng boosted	78.72	83.11	3499	3611.5	12	12
0.015 ng boosted	39.34		1737		19	
0.015 ng boosted	n/a	39.34	230	983.5	28	23.5

Table 4.2: RFU data from PCRboost™ sensitivity studies (with additive). The percent heterozygote ratios were determined for each profile (column two) and then averaged between profiles generated from common template quantities to arrive at an aggregated value for each concentration (column three). The overall profile intensities are reported in the fourth column, and the mean intensity between the duplicate template target amounts are in the fifth column. Finally, missing alleles noted per profile and the average number missing for each set of replicates are in the last two columns.

Template/reaction Description	Heterozygote Ratio (%)	Aggregated Heterozygote Ratio (%)	RFU Total	Mean RFU	Alleles Absent	Mean Alleles Absent
1 ng no boost	89.6		63412		0	
1 ng no boost	89	89.3	47275	55343.5	0	
0.5 ng no boost	82.98		37282		0	
0.5 ng no boost	84.55	83.77	53092	45187	0	
0.25 ng no boost	71.94		27583		0	
0.25 ng no boost	86.76	79.35	21881	24732	0	
0.125 ng no boost	75.16		12285		0	
0.125 ng no boost	71.57	73.37	15465	13875	0	
0.063 ng no boost	70.34		13276		1	
0.063 ng no boost	71.56	70.95	4459	8867.5	6	3.5
0.031 ng no boost	77.99		2731		14	
0.031 ng no boost	85.02	81.51	3007	2869	12	13
0.015 ng no boost	n/a		818		27	
0.015 ng no boost	n/a	n/a	964	891	23	25

Table 4.3: RFU data from PCRboost™ sensitivity studies (without additive). The percent heterozygote ratios were determined for each profile (column two) and then averaged between profiles generated from common template quantities to arrive at an aggregated value (column three). The overall profile intensities are reported in the fourth column, and the mean intensity between the duplicate template target amounts are in the fifth column. Finally, missing alleles noted per profile and the average number missing for each set of replicates are in the last two columns.

The data presented in these tables indicate that the results from these sensitivity series, with or without the additive, are fairly consistent with those previously presented in

Chapter 2 upon system validation. While there is some variation between heterozygote ratios and total RFU values obtained upon duplicate amplification of same template quantities using each system, the trends observed when the aggregated values are analyzed are consistent with those previously established. For example, the percent heterozygote ratios decline as the amount of template declines, and the overall observed RFU values also decline as less template is provided in the reaction. Drop out is observed at approximately the 0.060 ng template quantity, and very little profile information can be obtained at the 0.015 ng level. Because of the limited number of alleles detected, calculation of heterozygote imbalance was not possible in either of the 0.015 ng samples amplified without the additive; nor was it possible in one of the 0.015 ng samples amplified with the additive. In the 15 pg sample where sister alleles did exist, the observed ratio was at 39%, far lower than would be expected for optimal typing. The numbers presented in these tables also indicate the variation that one might expect in replicate amplifications, and the aggregated values from the duplicate amps were then compiled in a separate table so that the experimental and control series could more efficiently be compared. A summary of the aggregated values for the duplicate amplifications conducted with and without PCRboost™ comprises Table 4.4.

Template Amount	Aggregated Heterozygote Ratios (%)	Aggregated Heterozygote Ratios (%)	Aggregated Total RFU	Aggregated Total RFU	Mean Alleles Absent	Mean Alleles Absent
	No Boost	Boosted	No Boost	Boosted	No Boost	Boosted
1 ng	89.30	88.81	55343.5	82488.5		
0.5 ng	83.77	86.24	45187.0	43756.0		
0.25 ng	79.35	78.59	24732.0	27634.5		
0.125 ng	73.37	75.69	13875.0	14731.0		
0.063 ng	70.95	57.67	8867.5	6725.0	3.5	4.0
0.031 ng	81.51	83.11	2869.0	3611.5	13.0	12.0
0.015 ng	n/a	39.34	891.0	983.5	25.0	23.5

Table 4.4: PCRboost™ sensitivity comparison overview. The table reports the aggregated percent heterozygote ratios, profile intensities, and missing allele summary for sensitivity samples amplified with ("boosted" indicated in red) and without ("no boost" indicated in blue) the PCRboost™ additive.

This table highlights the similarity between the data sets, that is, the relative indifference between control and experimental series. First of all, the amount of drop-out observed at the lower template quantities is practically indistinguishable between

sensitivity series. While slightly less drop out occurred at the lowest two template levels in the series where the additive was used (experimental), the amount of drop out observed in the 0.063 ng range was slightly more for the experimental series. With respect to total RFU values within the profiles, at the 1 ng level, the experimental appears quite a bit higher than the control series. However, at the 0.5 ng level the experimental RFU value is slightly less; it is greater for the experimental series at 0.25 ng and 0.125 ng levels, but is less for the experimental series at the 0.063 template amount (and again more at the 0.031 and 0.015 ng quantities). Therefore, there is no overwhelming trend indicating that the profile intensity was enhanced by the additive. This is evident because most aggregated RFU values are within a couple thousand RFU of each other when same template quantities are compared with and without the additive; these differences are less than some differences noted between duplicate identical amplifications. For example, when the 0.5 ng quantity was amplified twice without the additive, the total RFU values obtained were 37282 and 53092 (a difference of 15,810 RFU). Likewise, when the 0.5 ng quantity was amplified twice with the additive, the total RFU values obtained were 47192 and 40320 (a difference of 6872). However, the differences between the RFU values of the 0.5 ng template for the control series and the experimental series was only 1431 RFU (45187-43756=1431). Therefore, one can conclude the control and experimental data are similar and that the additive did not serve to enhance intensity.

With respect to the heterozygote ratios observed, both the control and experimental series performed well at the higher template quantities and achieved average ratios above 70% when template quantities were 0.125 or greater. The average percent ratio declines as template quantity declines, with the exception of the 0.031 ng quantity, and this is likely due to the fact that alleles are only detected at the most robust loci at this level. As previously mentioned, the ratio cannot be calculated or is severely compromised at the 0.015 ng template amplification level. However, if one compares data between the control and experimental series directly at each quantity, there is no particular trend indicating the additive improved profile quality. The ratio was slightly lower at the 1.0 ng level with the additive, slightly better at the 0.5 ng level

with the additive, then slightly lower at the 0.25 ng level with the additive. This back-and-forth fluctuation between the data values continues as the template quantity declines indicating there is no difference in profile quality within the series amplified with the additive.

While this study is non-exhaustive, the data generated is fairly straightforward and shows no indication that PCRboost™ is effective in improving profile intensity or quality. One shortcoming of the study is that the amplification set-up for the series includes only 4.2 µL of the additive when substituted for NFW in the amplification cocktail. While most of these samples did contain additional water to make up the 25 µL reaction volumes, it is possible that the additive might be more effective if included at a level comprising a greater percentage of the reaction volume. However, in the true casework setting, the very samples where the additive would be needed would be those of limited quantity, at low concentrations, requiring the full 15 µL of sample be used in the reaction, affording no possible increase of PCRboost™ above quantities tested in this experimental design.

While this additive might be useful for improving profiles affected by other factors not investigated within the scope of this project (such as inhibition), the results herein indicate little promise for applications related to membrane bound template where template degradation and low copy number are the prevalent complications.

4.3.2 Restorase® Results and Discussion

The electropherograms obtained from samples amplified using the Restorase® polymerase were directly compared to electropherograms obtained from the control amplification of this template. The electropherograms from each fluorophore panel are presented in Figures 4.6a-c, JOE panel is zoomed for Figure 4.7 to more readily view artifacts.

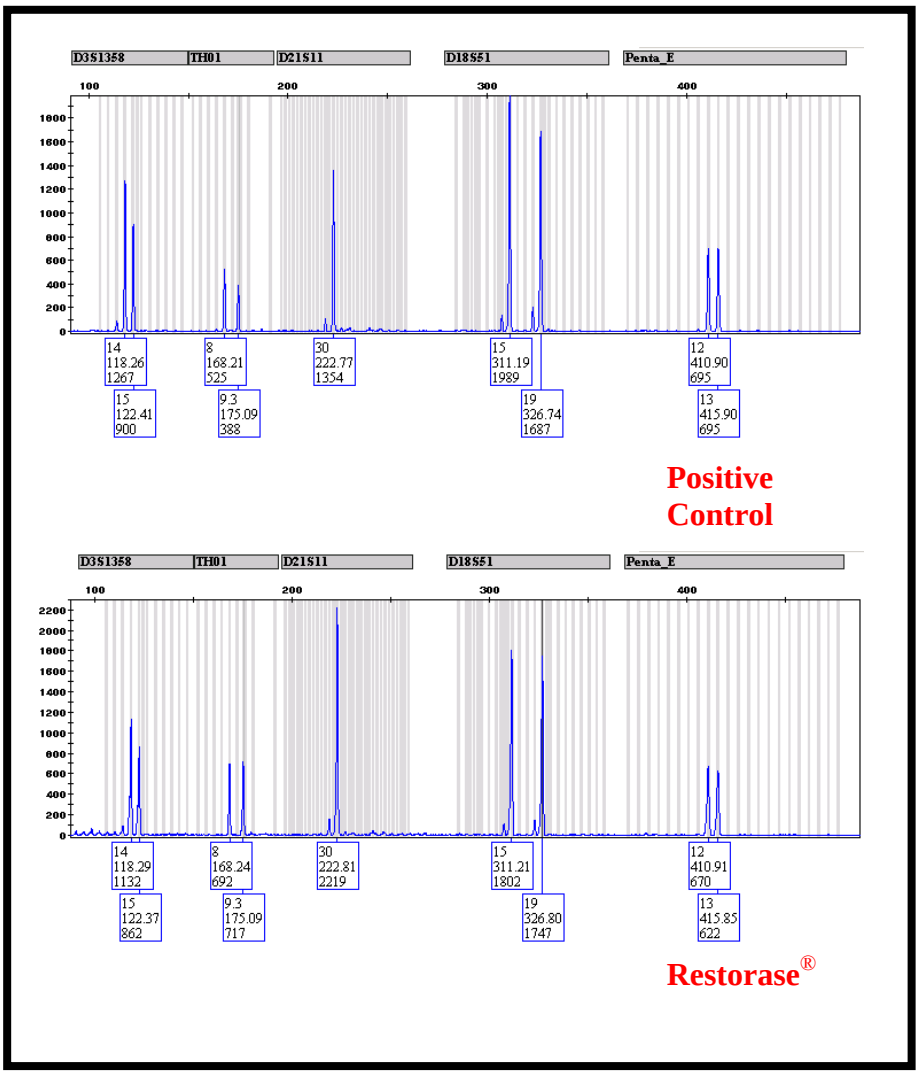

Figure 4.6a: Results of Restorase® amplification-FL. Fluorescein (FL) labeled loci are illustrated, with locus designations indicated above corresponding bins. Each peak label indicates allelic designation, base pair size, and peak height. The positive control 9947A (0.5 ng) amplified per current protocol with Ampli*Taq* Gold® is the top electropherogram, the lower electropherogram is that of the Restorase® polymerase amplified 9947A (5 ng).

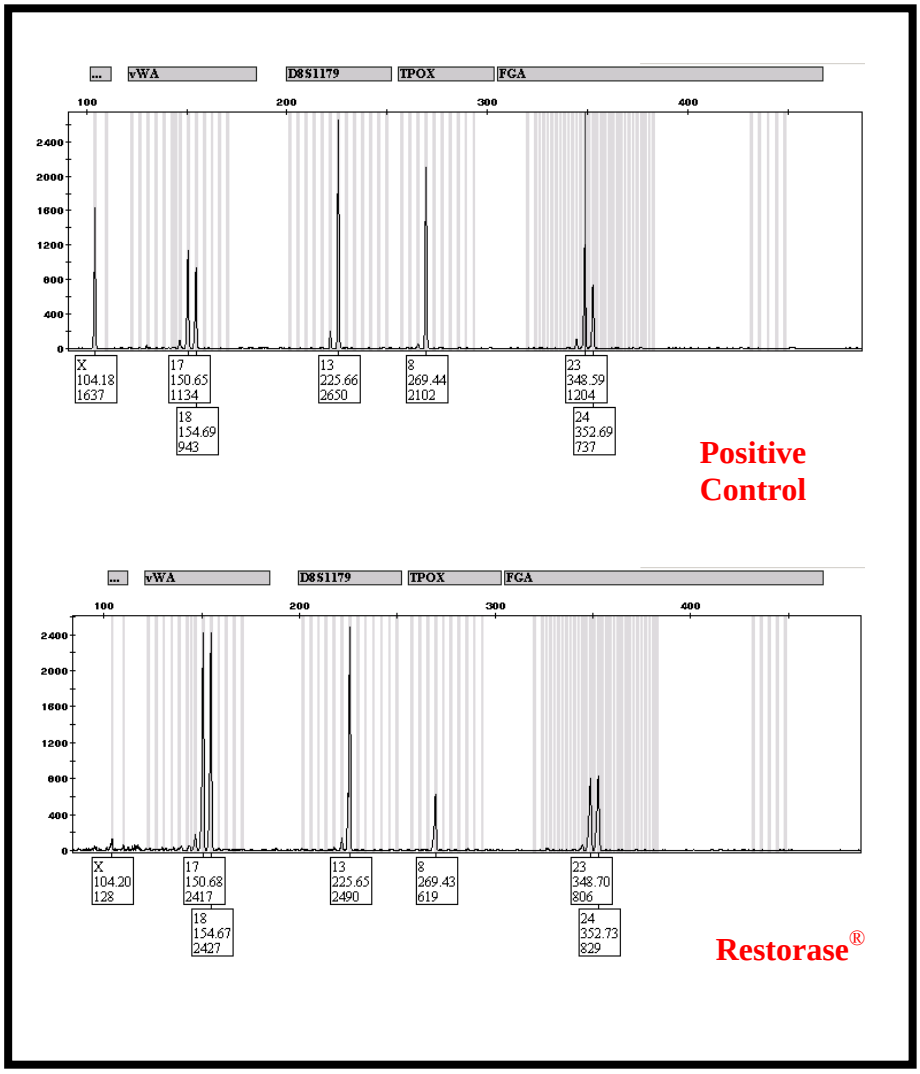

Figure 4.6b: Results of Restorase® amplification-TMR. Carboxy-tetramethylrhodamine (TMR) labeled loci are illustrated, with locus designations indicated above corresponding bins. Each peak label indicates allelic designation, base pair size, and peak height. The positive control 9947A (0.5 ng) amplified per current protocol with Ampli*Taq* Gold® is the top electropherogram, the lower electropherogram is that of the Restorase® polymerase amplified 9947A (5 ng).

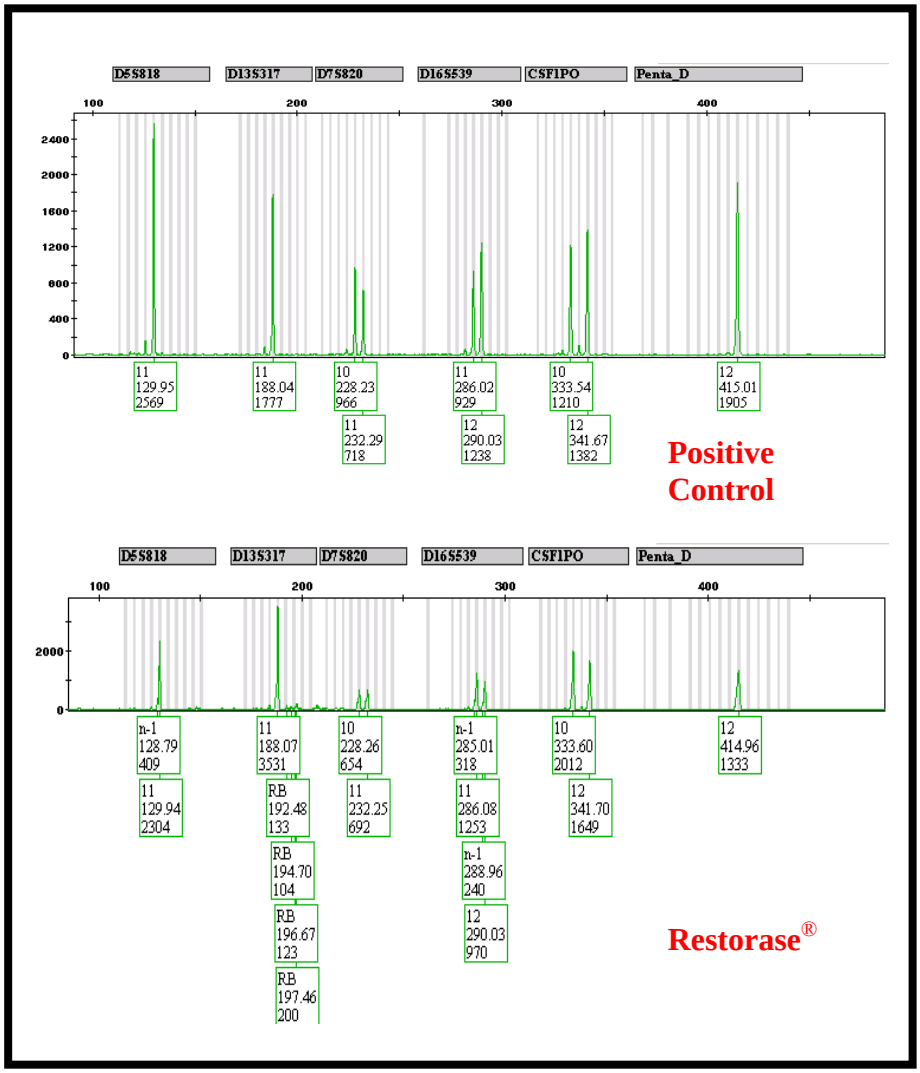

Figure 4.6c: Results of Restorase® amplification-JOE. 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE) labeled loci are illustrated, with locus designations indicated above corresponding bins. Each peak label indicates allelic designation, base pair size, and peak height. The positive control 9947A (0.5 ng) amplified per current protocol with Ampli*Taq* Gold® is the top electropherogram, the lower electropherogram is that of the Restorase® polymerase amplified 9947A (5 ng). [n-1] indicates possible minus-A amplicons; [RB] indicates raised baseline (noise) within the profile

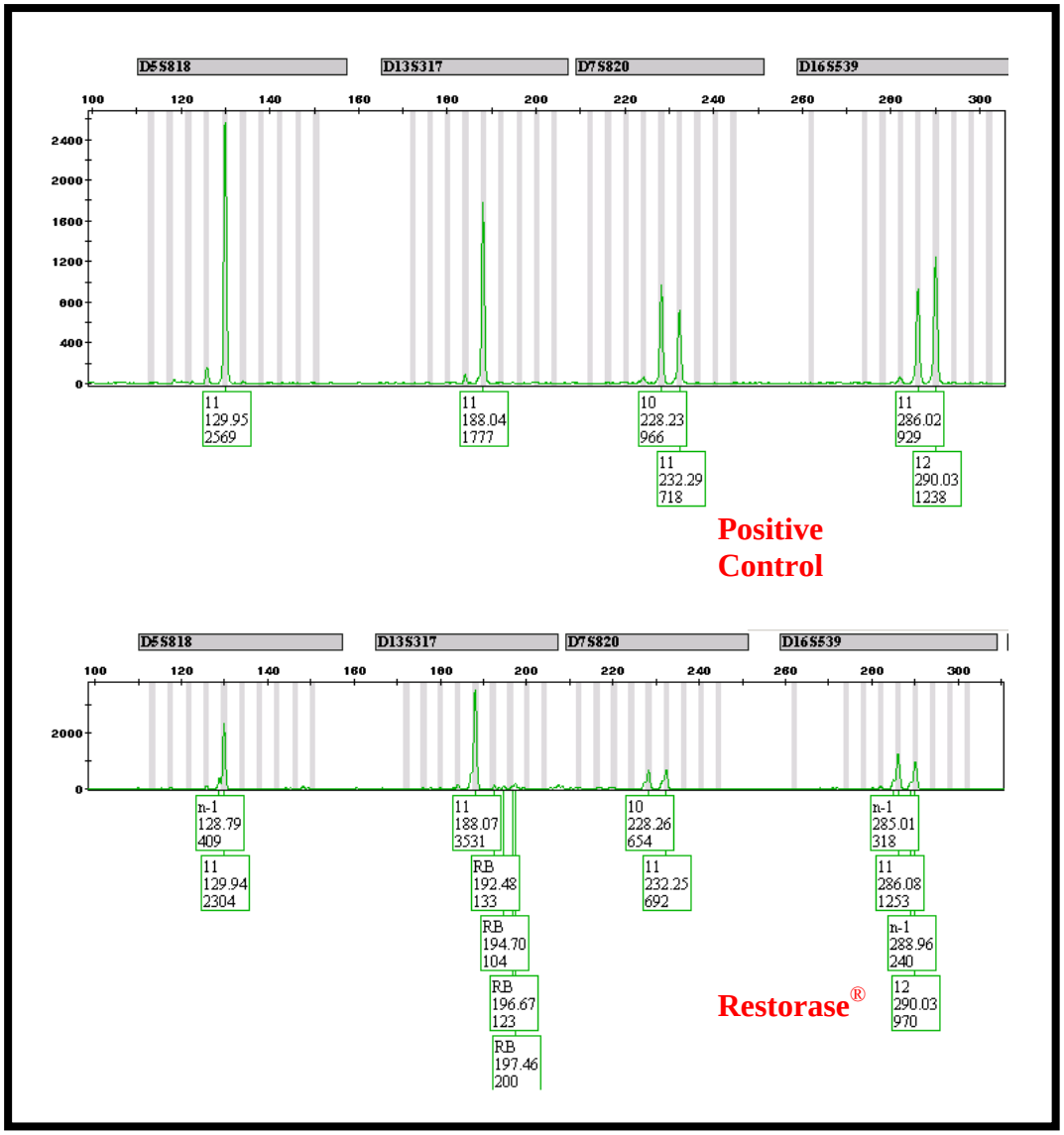

Figure 4.7: Results of Restorase® amplification-JOE artifacts. 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE) labeled loci are illustrated, showing enlarged view of D5S818, D13S317, D7S820, and D16S539 with scale zoomed to emphasize artifactual peaks present within the Restorase® polymerase system profile. Each peak label indicates allelic designation, base pair size, and peak height. The positive control 9947A (0.5 ng) amplified per current protocol with Ampli*Taq* Gold® is the top electropherogram, the lower electropherogram is that of the Restorase® polymerase amplified 9947A (5 ng). [n-1] indicates possible minus-A amplicons; [RB] indicates raised baseline (noise) within the profile

The data indicates that the Restorase® polymerase system, when used in conjunction with the PowerPlex® 16 primer and buffer system, did amplify the template and result in the correct profile. An excess of template was used to test the Restorase® polymerase system, therefore the peak heights from the experimental sample

(amplified with Restorase® polymerase system), and the control sample (amplified with the Ampli*Taq*® polymerase under casework validated conditions) cannot be directly compared for intensity. Because only a small amount of the Restorase® polymerase reagents could be afforded for the scope of this project, the study was designed to test only the functionality and feasibility for use with the current multiplex typing kit. It is noted that many of the artifacts observed may be due to the excessive template provided and these may not preclude correct genotyping. While the typing kits are specified by NDIS for database uploads, the guidelines do not specify any limitations with respect to the polymerase used; and since PowerPlex® kits are supplied without the polymerase, one could conduct necessary in house validation with an alternate polymerase and be eligible to upload data. This would not be possible with typing kits offered by Applied Biosystems since the polymerase is supplied with the kit.

The main goal of this study was to provide the Restorase® reaction with ample template. However, the performance level of the experimental system was completely unknown and, based on the results obtained, it appears that the reaction may have been more optimally executed under lower template conditions. The amplified product was injected at lower dilutions (50%, 25% and 10%), however the same trends were in diluted injections as were observed in the 100% sample, with peak heights diminishing accordingly with dilution (data not shown). Artifacts such as raised baseline noise were not detected above the 100 RFU threshold when lower volumes were injected, but the n-1 artifacts, or shouldering, that occurred at several loci were still identified as off ladder peaks by the analysis software. These artifacts are certainly a function of the amplification itself and it is well known that analysis of multiple dilutions could not correct for amplification artifacts, but only serve to optimize a profile for presentation purposes. The regions of the electropherogram that suffered most from artifacts are the focus of Figure 4.7, and consist mainly of n-1 shoulder peaks associated with the main alleles. While incomplete denaturation prior to injection can cause this, secondary structure is unlikely the cause; this was concluded since the proper heating and snap cooling procedure was executed, these artifacts were also evident in the diluted injections of the experimental amplified

product, and this shouldering was not observed in the positive control sample at any level.

While the correct allelic information was obtained, the profile balance within the Restorase® polymerase profile was poor. For example, within the fluorescein panel, the peak height ranged from 622 to 2219, the range in peak heights in the TMR panel was 123 to 2450, and the range in the JOE panel was 654 to 3531. Because a sensitivity study was not conducted, it cannot be determined if this imbalance would be due to the presence of excess template or if the polymerase is not as robust as the conventional Ampli*Taq* Gold® polymerase previously validated for casework. While many of the loci in the experimental profile exhibit peak heights exceeding those obtained from the control, some are exceptionally low. For example, the experimental profile peak at Amelogenin is less than 10% the value of that obtained from the positive control. However, the vWA peaks from the experimental sample in the same panel, exceed that exhibited by the control by an excess of two fold. Overall, these discrepancies between control and experimental are based on imbalance within the experimental profile, as more stability exists within the control between loci when heterozygotic and homozygotic loci are evaluated against each other for stability. For example, peaks at D13S317 and Penta D for the control amplification are each around 1500 RFU. When comparing the peaks at these loci for the experimental sample, Penta D is less than half the height of D13S317 for this homozygotic locus.

While imbalance and artifacts are prevalent in the experimental profile, these are not uncharacteristic of a high template reaction and the study does indicate that the polymerase can be used to achieve a comparable DNA profile. The optimal target template quantity for the Restorase® polymerase and PowerPlex® 16 reaction mixture was not explored during this preliminary assay in the interest of resource conservation. Instead, the applicability of the mixture will be further investigated for applicability to damaged template in the realm of membrane recovered template (section 6.2.4.1); this will be explored later in this work now that system feasibility has been established.

4.4 Conclusions of PCRboost™ and Restorase® Forensic Applications

Experiments in this chapter were designed to investigate two potential STR typing enhancement approaches, both are aimed at the PCR step of the analysis. The PCRboost™ reagent was investigated as a reaction additive. While the contents of the proprietary product are not reported, numerous additives were identified and described as introductory material to this rather common enhancement approach. Background was also presented with respect to DNA repair to offer a conceptual basis for the application of Restorase® polymerase, a product that reportedly repairs damaged template, returning it to a typable state. Simple studies were designed to test the feasibility of these reagents to properly type template and/or enhance results to a measurable degree.

Studies designed using the PCRboost™ additive in reactions of a duplicate sensitivity series did yield profiles, however the additive did not prove to enhance the profile quality or intensity when compared to results from duplicate sensitivity series where the additive was not included in the reactions. This simple study indicates that the additive mixture would probably offer little advantage to the project overall, given that membrane-recovered DNA samples, while degraded, are otherwise comparable to purified low copy number samples like those represented in low quantity samples within the sensitivity series. This determination is not meant to preclude any possible offerings of the additive with respect to inhibited samples, as the mixture may contain components useful for overcoming inhibitors in standard forensic DNA extracts. However, such investigations were beyond the immediate scope of the project at the juncture of these assays. Therefore, it was determined that the PCRboost™ additive would not be further investigated for this project.

Having discussed the results of this study and provided the data to Biomatrica, the manufacturer has since reformulated the mixture and, during the post-experimental aspects of this thesis, released a product for the multi-plex typing forensic community. The product formulation has been named "STRboost™", and preliminary poster data indicates that the new formulation improves sensitivity and amplification for multi-plex STR analysis [Le, *et. al*., 2009]. Efforts to develop a multi-plex specific

formulation would indicate that the results herein are consistent with those obtained by initial test laboratories, hence the need for improvement. Nevertheless, multiple formulations were not at the disposal of the scientist throughout the course of the experimental aspect of the thesis.

Regarding the second study within this chapter, the Restorase® polymerase did prove to generate a DNA profile using an experimental design that was essentially a hybridization of the Sigma protocol and multi-plex typing protocol validated in the laboratory. The goal of this approach was to preserve the essential components of the database approved typing kit, while testing the capability of the alternate polymerase to achieve the same result as Ampli*Taq* Gold®. The reaction was afforded an excess of template in an effort to obtain any profile at all since the modification to the amplification process was rather extensive and labor intensive. Surprisingly though, the reaction resulted in typable amplicons that could be analyzed with the conventional post amp reagents. While some artifacts were present in one dye panel of the electropherogram, most of these did not obliterate allele determinations for major peaks. Due to the limited number of reactions run/available, this preliminary study was not useful for determining if the artifacts are due to overwhelming amounts of template in the reaction, or if they will be inherent to the substitute polymerase system.

The results using the alternate polymerase indicate that the Restorase® product may have some promise for improving damaged template. Because a single kit was obtained for the trial and some level of feasibility demonstrated, the remainder was reserved for tests addressing membrane-recovered templates. In that sense, these preliminary feasibility studies were considered successful and the usefulness of this enzyme may be reconsidered for subsequent studies in this thesis.

5.0 Extraction Enhancement Techniques

5.1 Extraction Enhancement Approaches

Any aspect of the typing process, from extraction to detection, can be considered for pinpointing a technique that may ultimately result in an enhanced end result. Thusfar, this project has explored methods which amplify all template prior to STR typing (whole genome amplification), and approaches that may serve to improve STR multiplex PCR amplification (enhancement and polymerase/repair additives). This chapter is aimed at the exploration and validation of two methods that may serve to increase extraction yield, which redirects the focus to the earliest and most basic processes of the typing procedure – sample preparation and extraction.

5.1.1 Theory of Sample Processing and DNA Extraction

The extraction of nucleic acids from cellular material includes the dissociation of the cellular components, separation from associated proteins, and concentration of the nucleic acids into an aqueous solution. A procedure widely used in molecular biology labs, research and forensic, is organic extraction. First described over 20 years ago, the process is an efficient way to purify DNA from a cellular lysate [Chomczynski and Sacchi, 1987; Chomczynski and Sacchi, 2006]. The cell lysate is extracted with phenol or a 50/50 mixture of phenol and chloroform. Organic solvents solubilize hydrophobic molecules and denature proteins so that cell membranes and cellular proteins are either dissolved in the organic phase or trapped in the interface between the organic and aqueous phase. The organic phase and the interface are discarded and the aqueous phase containing the DNA is retained [Ausubel, 1996].

The DNA can be concentrated by precipitating it out of solution with ethanol. The ethanol causes an electrical attraction between phosphate groups present on the DNA and any positive ions present in solution and forms a precipitate. The precipitate can be centrifuged into a pellet that is then resolubilized into an aqueous DNA solution [Ausubel, 1996]. An alternative, used by many laboratories, are centrifugal devices such as the Microcon® filtration device. The solution containing DNA is passed through a cellulose filter where the DNA and protein bind to the filter while other components pass through. The device is then inverted and centrifuged again, as the DNA is pulled from the filter it is captured in a tube [Millipore, 2000]. This method

is fast and generally thought to offer higher recovery than precipitation methods, however ethanol precipitation can achieve greater purity from certain samples. Furthermore, the binding capacity of the cellulose filters, can be affected by solvents or the pH of the extract. The best practice for DNA concentration and purification is somewhat sample specific.

More recently, paramagnetic capture of DNA following cell lysis has become a popular method over organic extraction in the casework laboratory. The popularity is prompted by the fact that, unlike phenol/chloroform extraction, the methods do not employ hazardous chemicals and exposure risk. Furthermore, because the capture is based on a binding column/elution buffer mechanism, these techniques are conducive to automation. One of the earliest commercially available products using this technology was MagneSil™ Paramagnetic Particles, which were clad silica particles with a magnetite core [Otto, 2002]. In a chaotropic salt solution, the particles selectively bind nucleic acids, and the high magnetite concentration makes the particles very susceptible to a magnetic field. The paramagnetic nature of the particles render them attractively "inert" outside a magnetic field, and the selective application of magnetism can serve to collect the particles for washing and elution without clumping during the binding process as would be the case if they retained magnetic memory [Otto, 2002]. The DNA can then be washed and eluted using a second low salt buffer [Bitner, *et. al*., 2004].

5.1.2 Automated Extraction Systems in Forensic DNA

Automated DNA extraction systems, both large and small scale, began to emerge in the forensic science community during the past decade with increasing emphasis on validation in recent years [Greenspoon, 2004b; Krnajski *et. al*., 2007; Cowan, *et. al*., 2009]. Legislation concerning crimes eligible for CODIS collection and the introduction of all-arrestee laws in many states has been a driving factor for many labs in the United States to introduce automation. This has included systems for extraction, dilution, quantification, and amplification of known reference samples. Small scale automation for extraction has recently become popular among casework laboratories, and a variety of 6-16 sample extraction "robots" are now marketed in the

United States. One such system, the Maxwell® 16, uses paramagnetic bead capture technology for the extraction of DNA samples. The unit is designed for use with the DNA IQ™ extraction reagents, which are available in pre-loaded cartridges designed for the Maxwell® 16 stage. An example of The DNA IQ™ Reference Sample Kit cartridge is illustrated in Figure 5.1.

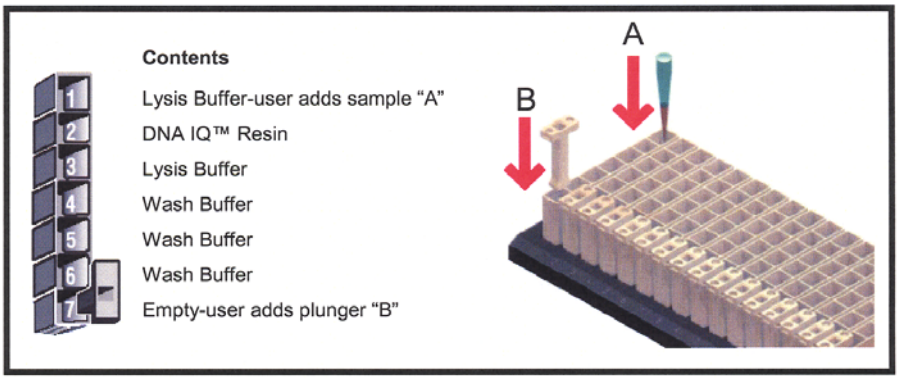

Figure 5.1: DNA IQ™ Reference Sample Kit cartridge. Samples are incubated in lysis buffer; the buffer solution is then loaded into well #1 (see "A"). A plunger (supplied with sample kit) is then placed in well 7 (see "B"). Cartridges are assembled on the stage as illustrated, which is retracted into the instrument for the run. When the run initiates, the magnetic rod apparatus retrieves the plunger, which serves as a sheath for the metal rod. The plungers are then transferred between wells and up-and-down motion is used to achieve complete lysis, bind the paramagnetic resin to the nucleic acid, and to wash the bound resin. An elution tube is assembled by the user on the stage for each sample (not pictured here), which contains the desired volume of buffer for elution and storage. Upon completion of the run, the used plungers are ejected back into well #7 by the instrument and can then be disposed of with the cartridge [Promega, 2008a; Krnajski, *et. al*., 2007].

The Maxwell® 16 is available in two platforms, one is a high elution volume (HEV) unit that performs final elution in approximately 300 µL, the other is the low elution volume (LEV) unit that is optimized for 25-50 µL final elution volumes. The HEV unit was introduced in 2003 with the LEV unit just a few years to follow; the HEV can be converted to an LEV instrument quite easily and cost effectively. The LEV and HEV systems have similar DNA capture capability, the main difference being the final volume of buffer into which the DNA is eluted. The LEV employs smaller rods and plungers designed for elution into smaller tubes with reduced volume of elution buffer. This results in higher concentrations of DNA per unit volume, but not necessarily more total DNA per sample. For limited quantity samples, the LEV is desirable because it maximizes DNA concentration of the extracted sample.

At the commencement of this project, the host laboratory was not equipped with paramagnetic extraction technology, but did purchase and publish the validation of the HEV instrument when it was released [Krnajski, *et. al*., 2007]. During the final stages of this work, the LEV instrument did become available for testing. One objective of the work presented in this chapter is the performance verification of the LEV instrument and extraction yield comparison between the Maxwell® 16 eluates and that achieved by standard organic extraction with Microcon® purification and concentration. A foundational verification is useful to ensure the LEV configuration performs to expectation and can be comparably substituted for the previously validated HEV instrument. The performance study serves to check STR profile concordance of sample extracts generated by the Maxwell® 16 LEV and to verify that cross contamination does not occur during the automated processing of samples within or between runs. Concordance of yield between samples was previously investigated for the HEV configuration [Krnajski, *et. al*., 2007], but this aspect of the verification is not of concern for the LEV since the lab now intends to conduct quantification on sample extracts (as maximum binding capacity is not an essential design component of the DNA IQ™ Casework Sample Kits).

The LEV unit was verified using a procedure modified slightly from the manufacturer's recommendation and was guided by previous validation efforts on the HEV instrument. Because the performance verified procedure is designed for the extraction of known reference samples, the method has shortcuts at the expense of recovery yield. Recognizing this, yield comparison studies were conducted in accordance with the manufacturer's suggested protocol for the extraction of trace samples; this method uses a more aggressive lysis approach and a more time consuming sample preparation procedure in an effort to maximize recovery yield.

5.1.3 Freezer Mill Theory and Application

Another approach to increasing yield involves the manner in which the substrate and sample are prepared prior to cell lysis. Cryogenic laboratory mills have experienced increased popularity in the forensic lab and are mainly used for pre-processing of skeletal remains since bones are inherently difficult to clean and cut. Of interest

related to this project is if sample yield could be increased by freezer mill processing of samples prior to extraction in an effort to dissociate biological samples from substrate as much as possible and increase surface area of the biological material for treatment with cell extraction buffer. An ancillary study was conducted alongside the extraction yield comparison study for the Maxwell® 16 to also include freezer-mill pulverized samples. This included a brief performance verification of the Spex 6770 freezer mill and subsequent processing and extraction of blood swabs for the yield comparison.

5.2 PCR Enhancement Methods and Materials

Experimental design for studies related to the performance verification of the Maxwell® 16 LEV and the Spex 6770 Freezer Mill and associated DNA recovery comparison studies are described herein.

5.2.1 Maxwell® 16 LEV Performance Verification and Yield Study

This work will be described in two subsections. First a performance verification consisting of contamination and concordance studies will be performed. Thereafter, two different LEV extraction protocols (a "quick" method and a maximum recovery "trace" method) will be compared to each other and to the recovery achieved by standard organic extraction methods.

5.2.1.1 Maxwell® 16 LEV Performance Verification

For the performance verification (contamination and profile concordance) studies, samples from previously typed individuals were prepared. For buccal swab extractions, 16 oral standards from 8 different individuals were obtained on cotton swabs (8 swabs) and also on polyester swabs (8 swabs). A total of 11 blood samples were processed. Extraction was conducted on 8 samples derived from 8 different individuals deposited on cotton swatches. Blood samples also included 2 samples from individuals deposited on FTA® (Whatman) cards. Portions of the dried samples were excised from the stained area of the swatches and were each approximately 5x10mm. One additional blood sample was a NIST traceable sample on cotton, the cutting was approximately 5x5mm. While the oral standards on cotton and polyester

were duplicates from the same 8 individuals, these same 8 volunteers were not all represented in blood extracts. Therefore, a pool of 8 previously typed blood donors was used; some of whom had also submitted buccal swabs. From these two body fluid types, 12 different previously typed individuals were represented.

Performance verification samples were processed using the LEV quick method. Whole oral swabs were placed in 2.0 mL microcentrifuge tubes and treated with a lysis solution consisting of 485 µL of DNA IQ™ Lysis Buffer, 10 µL 20 mg/mL proteinase K (proK) and 5 µL of 1M dithiothreitol (DTT). Alternatively, blood stains deposited on cotton or FTA® cards were excised entirely from the matrix, placed into 2.0 mL microcentrifuge tubes, and treated with the aforementioned quantity of DNA IQ™ Lysis Buffer/proK/DTT solution. Negative controls consisted of DNA IQ™ Lysis Buffer/proK/DTT solution only. All samples were then heated to 70° C for 30 minutes. Following incubation, tubes underwent a quick spin, and the lysis solution was then drawn off the substrate and placed into the specified well of the purification cartridge. The step involving filter basket suspension and centrifugation of substrates following lysis was omitted for the purposes of this study.

For all Maxwell® 16 processed samples, cartridge/instrument preparation was carried out by placing a plunger into the appropriate well of each sample cartridge, and assembling Elution Tubes containing 25 µL of DNA IQ™ Elution Buffer into the front of the platform for each sample. Cartridges were then placed into the Maxwell® 16 LEV Instrument, and processed using the Forensic Mode. Following completion of the run, Elution Tubes were removed and closed for long-term storage. Cartridges and plungers were discarded following the runs.

The performance verification (contamination and profile concordance) study samples comprised three Maxwell® 16 run batches; extraction method yield comparisons were carried out in a fourth run (comprising section 5.2.1.2). The first run consisted of six oral standards on cotton, which were placed in odd numbered wells 1 to 11. A negative control was run between each sample, and was therefore located in even numbered wells 2 to 12. Elution buffer was inadvertently omitted from elution tubes

in positions 13-16 for this run so those samples were re-extracted in these rack positions with the third run. Each sample was individually referenced by a "Run#-Rack#" identifier, therefore the oral swab extracted in well 5 of the first batch is denoted as "Sample 1-5". The second batch consisted of eight oral standards on polyester, which were placed in even numbered wells 2 to 16. Negative controls were run between each sample, and were therefore located in odd numbered wells 1 to 15. Finally, the third batch consisted of eleven blood samples (two of which were from FTA® card stains and one of which was a NIST traceable standard) and the completion of the contamination study samples omitted from the first run (oral standard on cotton in each of well 13 and 15 with negative controls run in positions 14 and 16). The final sample of the third run consisted of an additional negative control in position 2. The performance verification runs are illustrated in Figure 5.2.

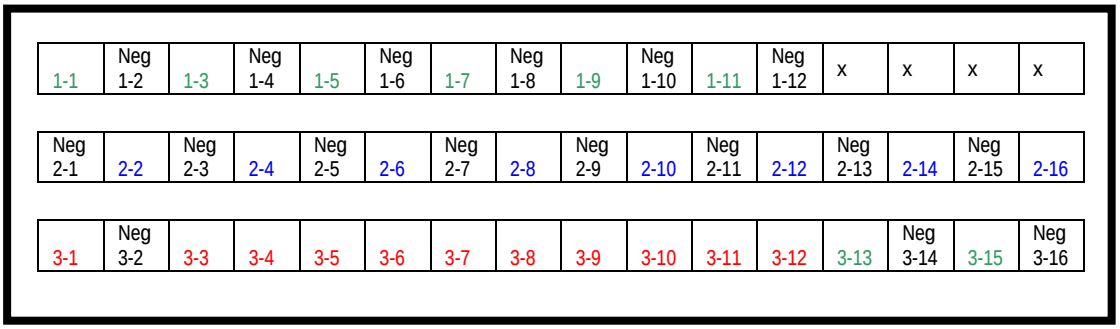

Figure 5.2: Maxwell® 16 performance verification run overview. Samples within each run are individually referenced by a "Run#-Rack#" identifier within each square. The top row indicates loading order for the first run, samples indicated in green are representative of oral swabs on cotton. The second row indicates loading order for the second run, samples indicated in blue are representative of oral standards on nylon swabs. The bottom row indicates loading order for the final performance verification run. Samples in red indicate bloods; sample 3-10 and 3-11 were on FTA® cards and sample 3-12 represents a NIST traceable blood sample on cotton. Since elution buffer was omitted from racks 13-16 during the first run, completion of contamination study was achieved by re-extracting two of the eight cotton oral swabs via samples 3-13 through 3-16 of the final run. Negative control racks are indicated in black and unused cartridges are indicated with "x".

A quantity of 2 µL of each sample and negative control extract then underwent Quantifiler® Human DNA Quantification using the ABI 7500 Real-Time PCR System (Applied Biosystems, 2005a). Samples were appropriately diluted following quantification and each extract was then amplified with the PowerPlex® 16 multi-plex system. A volume of 1 µL each negative control, undiluted, was incorporated into a PCR reaction prepared at a final volume of 25 µL. Because the quantification

indicated the possible presence of trace amounts of DNA in one of the negative controls (Sample 2-15), 15 μL of this sample was also amplified as a conservative measure. Amplified product was electrophoresed using the 3130 genetic analyzer and data was analyzed using GeneMapper™ ID Software as described in Chapter 2.

5.2.1.2 Maxwell® 16 LEV Yield Study

For the extraction method yield comparison, triplicate samples of blood stains consisting of 10 μL whole blood spotted on a suspended cotton swatch were extracted by each method. The first triplicate set was extracted using standard overnight incubation in stain extraction buffer working solution followed by PCI/ Microcon® purification and concentration as described in Chapter 2 [Laber, 1992; Steadman 2002a]; a reagent negative control was carried out alongside sample extractions. Extracts were brought to 25 μL so that concentrations could be directly compared to extracts from the Maxwell® 16 runs. A second triplicate set was extracted using the LEV quick method and the third triplicate set was extracted using the LEV trace method; these methods were guided by the manufacturer's suggested protocol [Promega, 2008a] and are fully described below.

For the LEV quick method, blood spots were excised from the cotton swatch and placed in 2.0 mL microcentrifuge tubes and treated with a lysis solution consisting of 485 μL of DNA IQ™ Lysis Buffer, 10 μL 20 mg/mL proteinase K (proK) and 5 μL of 1M dithiothreitol (DTT). The negative control consisted of DNA IQ™ Lysis Buffer/proK/DTT solution only. Samples were incubated and processed in the Maxwell® as described for LEV extraction method in section 5.2.2.1.

For the LEV trace method, each blood stain was placed in 2.0 mL microcentrifuge tubes. These samples, and an empty negative control tube, were treated with 190 μL Incubation Buffer (supplied commercially with Promega's tissue and hair extraction kit) plus 10 μL proK. Each tube was incubated at 56^{o} for 1 hour. Following incubation, 198 μL of Lysis Buffer and 2 μL of 1M DTT were added to each tube. Each sample was vortexed briefly and held at room temperature for 5 minutes.

Following a brief centrifugal spin, the cuttings were removed from solution and suspended in a filter basket. The tubes were centrifuged at 15,000 g for 5 minutes; filter baskets containing substrates were discarded. The sample solutions were then transferred into the specified well of the purification cartridge and processed in the Maxwell® as described for LEV extraction method in section 5.2.2.1

A quantity of 2 μL of each sample and negative control extract then underwent Quantifiler® Human DNA Quantification using the ABI 7500 Real-Time PCR System (Applied Biosystems, 2005a). Samples were appropriately diluted following quantification and each extract were then amplified with the PowerPlex® 16 multi-plex system. A volume of 1 μL each negative control, undiluted, was incorporated into a PCR reaction prepared at a final volume of 25 μL. Amplified product was electrophoresed using the 3130 genetic analyzer and data was analyzed using GeneMapper™ ID Software as described in Chapter 2.

5.2.2 Spex 6770 Freezer Mill Performance Verificiation and Yield Study

For the freezer mill verification, a human femur was obtained commercially from The Bone Room, a natural history store in Berkeley CA, USA. The time of death of this donor was not available, therefore the age of the skeletal remains are unknown. The STR profile of the sample was available from testing carried out independently of this study. Prior to collection of any material, the sampling area was sanded and decontaminated by with 10% bleach followed by ethanol wiping. Following drilling, sampling of the specimen continued with collection of fragments with a Dremel® tool equipped with a saw blade. The excised fragment underwent cleaning with sonication and was washed with water and then ethanol. Once dry, manual crushing was performed using a hammer, but the manually crushed sample did not result in a DNA profile following standard casework extraction, quantification, and amplification. The prior processing serves as a basis for comparison for any data obtained via freezer mill pulverization. A similar sampling was performed using the Dremel® tool, the specimen was photographed before and after excision of material for the freezer mill assay (see Figure 5.3). Figure 5.4 is a photograph of the fragments collected for cryogenic pulverization.

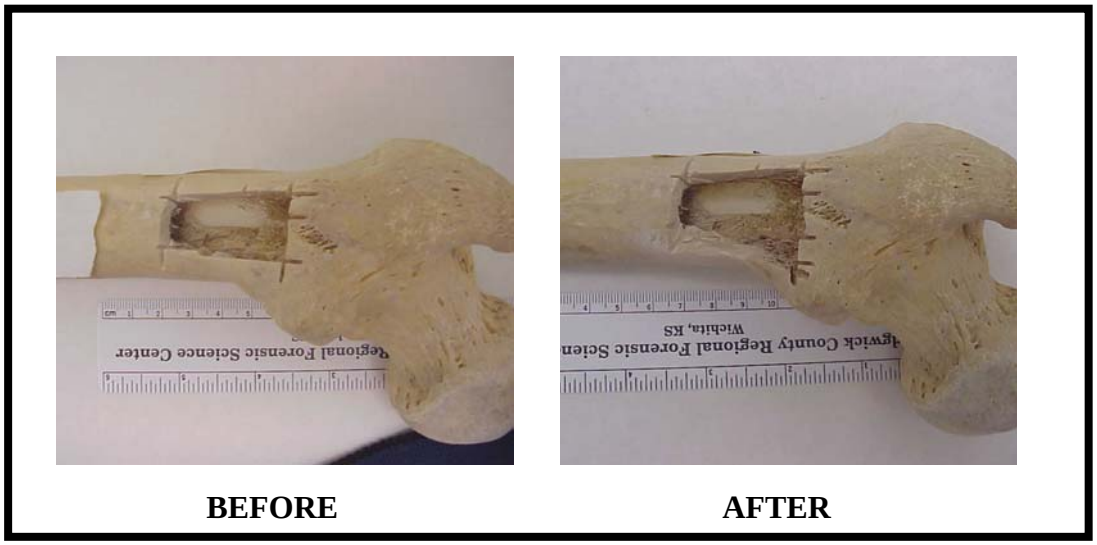

Figure 5.3: Photograph of bone sampling. The image on the left indicates the condition of the specimen prior to sampling for the freezer mill sampling. The image on the right was captured following the removal of additional material for pulverization and documents the amount of additional excised area. Collected material is documented in Figure 5.4.

Figure 5.4: Photograph of sample used for freezer mill pulverization. The image indicates the amount of bone that was pulverized and extracted for the freezer mill performance verification. Total mass of these fragments was ~0.7 grams.

Fresh samples were also prepared for pulverization and this was done to provide samples for the extraction yield study and to serve as supplemental performance verification samples should no STR type be obtained from the bone (due to the unknown nature of the skeletal remains STR profiling may not be possible). The fresh samples consisted of a triplicate set of polyester swabs, each treated with 10 μL

of whole blood from a male volunteer. A second triplicate set was prepared for extraction straight away (no pulverization). Additionally, to fully address possible product loss of sample due to transfer, two additional sets of triplicate swabs were prepared, each swab was treated with 161 ng genomic male mixed DNA (obtained commercially from Promega).

A fragment of nylon membrane was also subjected to grinding; the size was determined to mimic that of a single RFLP membrane lane.

Polycarbonate vials, end plugs, and stainless steel impactor were cleaned with mild SDS, bleach, and water followed by ethanol rinse and autoclaving. Vials were assembled to contain impactor and sample. The bone sample was processed using a 10 minute pre-cool followed by two 2-minute grinding cycles at 13 CPS. The cool between grinding cycles was 2 minutes. Bone powder was divided into two microcentrifuge tubes prior to overnight incubation with stain extraction buffer working solution; standard organic extraction with PCI and Microcon® concentration/purification were carried out as described in Chapter 2; sample tube aqueous phases were combined over one filtration device to combine and concentrate yield from the two separate tubes. Blood swabs and DNA swabs were processed consecutively by tube in the same vials (one vial used to extract blood swab #1, #2, and then #3; a second vial was used to extract DNA swab #1, #2, and then #3), but resulting powder was collected into individual microcentrifuge tubes for subsequent extraction following each run. The pulverized swabs were extracted as were triplicate samples of blood and DNA swabs that did not undergo pre-processing; this was achieved by standard organic methods described in Chapter 2. Final volume for all samples, including bone, was 17 μL). Samples were quantified using Quantifiler® Human DNA Quantification using the ABI 7500 Real-Time PCR System (Applied Biosystems, 2005a). The bone sample was consumed for amplification using the PowerPlex® 16 multi-plex system and electrophoresed using the 3130 genetic analyzer and data was analyzed using GeneMapper™ ID Software as described in Chapter 2. Blood and genomic DNA swab samples did not undergo STR profiling.

5.3 PCR Enhancement Results and Conclusions

Results obtained in this chapter will be presented in two major sections. The first is regarding the automated extraction (5.3.1) and is followed by studies concerning the freezer mill pulverization (5.3.2).

5.3.1 Maxwell® LEV Performance Verification and Yield Study Results and Discussion

The Maxwell® LEV automated extraction work is divided so that performance verification is first established (5.3.1.1), followed by a second aspect concerning extraction method yield comparisons (5.3.1.2).

5.3.1.1 Maxwell® LEV Performance Verification Results and Discussion

The amount of DNA recovered from each sample from each of the three performance verification runs (described section 5.2.1.1) was recorded and average concentrations along with the standard deviation of concentrations were determined for the various sample/substrate types. The quantifications for each sample are reported in Table 5.1. The sample-substrate means and standard deviations are reported in Table 5.2.

Sample ID (Run#-Rack#)	Sample-Substrate	DNA Yield (ng/uL)
1-1	oral-cotton swab	3.71
1-3	oral-cotton swab	26.39
1-5	oral-cotton swab	8.81
1-7	oral-cotton swab	3.33
1-9	oral-cotton swab	28.56
1-11	oral-cotton swab	9.57
3-13	oral-cotton swab	16.78
3-15	oral-cotton swab	24.06
2-2	oral-polyester swab	3.39
2-4	oral-polyester swab	22.37
2-6	oral-polyester swab	14.39
2-8	oral-polyester swab	7.99
2-10	oral-polyester swab	20.29
2-12	oral-polyester swab	22.01
2-14	oral-polyester swab	8.12
2-16	oral-polyester swab	22.33
3-1	blood-cotton swatch	3.71
3-3	blood-cotton swatch	1.11
3-4	blood-cotton swatch	1.26
3-5	blood-cotton swatch	0.929
3-6	blood-cotton swatch	2.88
3-7	blood-cotton swatch	5.02
3-8	blood-cotton swatch	4.78
3-9	blood-cotton swatch	2.38
3-10	blood-FTA®card	2.39
3-11	blood-FTA®card	1.58
3-12	NIST traceable blood	1.48

Table 5.1: Quantifications obtained from Maxwell® 16 LEV contamination and profile concordance study samples. Quantification in ng/µL is listed for each sample tested. Note that negative controls were run in alternating rack positions between runs; (even numbered wells of run 1, odd numbered wells of run 2, and wells 3-2, 3-14, and 3-16). Samples 1-13 through 1-16 could not be used for the study since elution buffer was inadvertently omitted from the tubes. All negative controls performed to expectation with undetermined quantification results using Quantifiler® Human except sample 2-15 which indicated a trace quantification value of 0.000407 ng/µL.

	Average Yield (ng/μL)	Standard Deviation (ng/μL)
Oral swabs on cotton	15.2	10.2
Oral swabs on polyester	15.1	7.7
Blood on cotton	2.76	1.6
Blood on FTA® paper	1.99	0.5

Table 5.2: Mean and standard deviation for the Maxwell® 16 LEV contamination and profile concordance study samples by sample-substrate category. This table reports the average concentration of extracts resulting from extraction of oral samples on cotton (n=8), oral samples on polyester (n=8), blood samples on cotton swatch (n=8), and blood samples on FTA® paper (n=2). The NIST traceable sample cutting was not included in these calculations because the cutting size was intentionally reduced to conserve sample.

The average amounts of DNA recovered from the oral swabs on cotton vs. polyester were very similar (approximately 15 ng/μL) and while there was less variation observed between the polyester swab quantifications, the difference in standard deviations was not of magnitude (standard deviation of approximately 7.7 vs. 10.2) and may be considered negligible.

Each profile was evaluated for the presence/absence of expected alleles for the known sample donor. All samples gave correct profiles and were found to be in concordance.

All reagent negative controls were evaluated for the presence of possible contamination. Sample 2-15 resulted in a Quantifiler® Human quantification of 0.407 pg/μL. To determine if this was truly indicative of a contaminant or whether it was an artifact of the detection system, the sample was amplified in two quantities. A volume of 1 μL was amplified (in accordance with the volume of all other samples amplified), and a volume of 15 μL (maximum template volume accommodated by the amplification set-up protocol) was also amplified as a conservative measure. No profile was detected for this sample or any other negative control sample in this study. The electropherogram for sample 2-15 was analyzed below the 100 RFU threshold for any possible low level peaks and none were noted. Therefore it was concluded that

well-to-well contamination did not occur within runs. The study also shows that the disposable plungers effectively prevent contamination between runs when the same magnetic bar is used to process subsequent samples.

Results for the verification of the instrument performance, based on contamination and concordance studies, were straightforward. With respect to the contamination study, all negative controls run yielded no profile and given the alternating well position design of the verification runs, this indicates that contamination does not occur within or between runs. One sample did indicate the possible presence of trace quantities of DNA based on the quantification, but full amplification of the sample did not result in the detection of a profile. Both the 1 µL and 15 µL amplification volumes of that sample resulted in no detectable alleles; furthermore, no peaks were detected below the threshold. As for the concordance study, all profiles were the same as previously typed for all sample donors. Performance of the Maxwell® 16 LEV instrument was comparable to that of the previously validated HEV instrument; therefore, the Maxwell® 16 LEV was deemed suitable for extraction of reference samples.

5.3.1.2 Maxwell® LEV Yield Study Results and Discussion

For the extraction yield comparison study, a volume of 2 µL of each sample and the respective negative control extract underwent Quantifiler® Human DNA Quantification system as described in Chapter 4. The quantifications were carried out in duplicate on two different quantification plates, resulting in four quantification values for each extract. The average values of each the quadruplicate quantifications for each sample/extraction type are reported in Tables 5.3-5.5. Overall method yield comparison is summarized in Table 5.6, to include the averages and standard deviation resulting between all obtained values for all stain extract quantifications. Negative controls for each extraction method resulted in undetermined results using the Quantifiler® Human DNA Quantification system as expected.

Quant Identifier	Stain 1-organic (ng/uL)	Stain 2-organic (ng/uL)	Stain 3-organic (ng/uL)
081027ss1	9.35	12.56	12.11
081027ss1	10.26	12.58	11.68
081027ss2	9.12	13.02	11.86
081027ss2	9.36	13.45	12.37
sample yield mean	9.52	12.90	12.01

Table 5.3: Quadruplicate quantification values for stains extracted with standard organic extraction. Quantification data obtained from 10 µL blood stains extracted using standard organic method is recorded. Average recovery for each sample, calculated from the four replicate quant wells, is expressed in the bottom row of the table.

Quant Identifier	Stain 1-trace LEV (ng/uL)	Stain 2-trace LEV (ng/uL)	Stain 3-trace LEV (ng/uL)
081027ss1	6.80	4.58	3.83
081027ss1	6.64	5.04	3.73
081027ss2	6.60	4.71	3.79
081027ss2	6.35	4.72	3.64
sample yield mean	6.60	4.76	3.74

Table 5.4: Quadruplicate quantification values for stains extracted using the trace LEV protocol. Quantification data obtained from 10 µL blood stains extracted using the Maxwell® 16 LEV trace extraction protocol is recorded. Average recovery for each sample, calculated from the four replicate quant wells, is expressed in the bottom row of the table.

Quant Identifier	Stain 1-quick LEV (ng/uL)	Stain 2-quick LEV (ng/uL)	Stain 3-quick LEV (ng/uL)
081027ss1	1.42	1.39	1.39
081027ss1	1.63	1.39	1.36
081027ss2	1.51	1.44	1.31
081027ss2	1.59	1.42	1.27
sample yield mean	1.54	1.41	1.33

Table 5.5: Quadruplicate quantification values for stains extracted using the modified quick LEV protocol. Quantification data obtained from 10 µL blood stains extracted using the Maxwell® 16 LEV quick extraction protocol is recorded. Average recovery for each sample, calculated from the four replicate quant wells, is expressed in the bottom row of the table.

	Average Yield (ng/μL)	Yield Standard Deviation (ng/μL)
Organic method	11.5	1.54
Trace LEV method	5.0	1.25
Quick LEV method	1.43	0.10

Table 5.6: Mean and standard deviation for sample concentration of triplicate extracts using standard organic extraction, trace LEV extraction, and modified quick LEV extraction methods. This table reports the average concentration of extracts resulting from extraction of stains using the organic method, the Maxwell® 16 LEV trace method, and a modified Maxwell® 16 LEV quick method.

Samples extracted using the LEV trace method were appropriately diluted following quantification and each extract was then amplified with the PowerPlex® 16 multi-plex system; 1 μL of the corresponding negative control was also amplified. Amplified product was electrophoresed using the 3130 genetic analyzer and analyzed using GeneMapper™ ID Software as described in Chapter 2. All samples resulted in high quality STR profiles concordant with the previously determined profile for this donor. Samples extracted using organic and quick LEV methods were not amplified as verification of these methods was previously demonstrated.

Studies with the LEV configuration concerning yield are pertinent for various reasons. If the laboratory should consider the use of personal automation for the extraction of casework samples, it is important to understand how the extraction yield compares to the method currently in place. Many approaches could ensue and this would require the extraction of a wide variety of sample tissues and types as well as multiple substrate tests. Should the yield be equal to or greater than that obtained from standard organic extraction for all tissues/substrate combinations tested empirically, one may rationalize immediate application of the automated approach for all samples that do not require differential extraction. Should the yield be lower than that achieved by current methods, one would need to further investigate which sample types might be suitable for robotic extraction. For example, even if yields are lower with the paramagnetic technology, samples containing inhibitors may result in better quantification and STR amplification when processed in this manner. Alternatively, the lab may choose to apply automated extraction to samples that are likely to result

in high yields regardless of the extraction method used (blood samples from property crimes). With respect to this project, the goal is to determine how the yield compares to standard organic extraction in a general manner, since it is unknown if the paramagnetic technology could offer advantages for DNA extraction from nylon membranes. Regardless, one must understand probable cost-benefit associated with this method.

The modified quick LEV approach used for the performance verification makes the addition of proteinase K to the lysis solution but omits a substrate basket filtration step that is generally used to remove all lysis solution from the substrate. While the target incubation temperature of the samples exceeds that of proteinase K activity, the decision to add the enzyme was based on the fact the tubes do not immediately equilibrate to this temperature after addition of lysis cocktail. It is also well known that this level of proteinase K will not adversely affect the extraction. Therefore, during the several minutes of preparation and tube mixing, as well as the period of ramping to the desired lysis temperature, the proteinase K will have some activity. Omission of the filter basket step is merely a convenience factor in that it prevents additional entry into the sample (which reduces the possibility of contamination); this method also omits the need to clean forceps between samples and reduces the overall processing time substantially. This method was never intended to enhance yield and exemplifies a rational cost-benefit analytical approach to processing reference samples. However since the quantification data was already available from the performance verification it was certainly of interest to compare yield.

The LEV trace method did employ more extensive pre-processing but did result in much greater yield than did the modified quick LEV protocol. This is undoubtedly due to the extended incubation with proteinase K at a temperature optimal for the enzyme activity as well as the spin basket centrifugation of all lysis buffer out of the substrate. The trace LEV extraction generally resulted in 3-5 times more DNA from a sample than did the quick method.

Of most interest to this project is the difference in yield between the trace LEV method and standard organic extraction. The trace LEV method resulted in average recovery of 5 ng/μL, but the average recovery using the organic method was 11.5 ng/μL from the same sample types. Therefore, one can expect approximately 50% product loss beyond that which would be achieved by the conventional extraction method. Little variation was noted between samples within any given test group; therefore, assuming samples were consistent in preparation, this consistency indicates analyst and instrument precision. However, this is limited in scope to extraction of bloods on cotton, as this may vary from sample to sample, or be affected by substrate, as discussed previously. That said, the chances that this method would achieve greater yield from membrane-bound samples is unlikely but the probability cannot be elucidated without empirical testing.

5.3.2 Freezer Mill Performance Verification Results and Discussion

The cryogenic grinding process very adequately pulverized all samples tested. Following the processing of the bone, which resulted in a fine powder, similar techniques were successfully applied to all swab samples. Furthermore, the fragment of nylon on which grinding was attempted was also successfully pulverized. However, the main difficulty in processing samples is recovering the powder from the large surface area of the interior of the vial. All samples resulted in recoverable quantities of powder with the exception of the membrane fragment, which was adhering to all aspects of the vial interior with little or no aggregation of product that could be transferred. Due to the insolubility of the nylon and low extraction volume relative to the large capacity of the vial, rinsing/liquid transfer was not attempted.

Quantification of the bone extract was performed in a single well since replicate sampling would result in appreciable PCR template loss in this low copy number sample. The extract quantified at 0.000527 ng/μL. Given that the remaining 15 μL of this extract would contain far less than optimal amount of template, the sample was consumed for multiplex typing. The initial injection of the amplified sample resulted in the detection of a single allele above 100 RFU, a 14 at vWA. An extended injection duration (11 sec) resulted in the detection of additional peaks above the

threshold; these results and peaks noted below 100 RFU were compared to the profile previously achieved by drilling and are described in Table 5.7.

	Drillings	Freezer Mill Pulverized
D3S1358	16,18	*
TH01	7	*
D21S11	29,32.2	*
D5S818	9,11	11 (+)
D13S317	8,10	8
D7S820		10
Amelo	X,Y	*
vWA	14,18	14 (+)
D8S1179	15,16	15

Table 5.7: STR PCR alleles obtained from bone via drillings and freezer mill pulverization pre-processing. The table lists the peaks detected above 100 RFU for each of these injections; both were carried out using 11 second duration parameters. Peaks detected below the threshold consistent with those detected from the drillings are indicated by (*); heterozygote alleles visible below the threshold consistent with those obtained from the drillings are indicated with (+) at D5S818 and vWA.

Less profile information was detected from the pulverized sample, however concordance existed between the two profiles using the different pre-processing methods. Since the drillings were conducted for training purposes and were executed prior to this study, the mass of the material extracted is unknown. However, the drillings are estimated to be comparable in mass based on number of microcentrifuge tubes required for overnight incubation in extraction buffer. Nonetheless, the mass of the bone pulverized was far less than that which could be accommodated by the grinding vial, therefore increasing the amount of sample pulverized may have been useful in achieving more STR markers. Instrument verification is documented by the adequate grinding action of the mill, and was observed by reduction of the bone to a fine powder; the results from the sample are consistent with profiling results previously obtained. However, this method should be used on large samples or samples which require extensive exterior and interior washing prior to pulverization and extraction. It may also be useful on small fragments where drilling may not be

possible due to small surface area. Regardless, an increase in the amount of bone pulverized would be recommended for future casework samples.

To investigate transfer loss trends, the blood swabs were processed one after the other in the same vial, with powder from each harvested in between swabs. The first blood swab processed is denoted as #1 and the last as #3; the DNA swabs used this nomenclature as well. Sample quantification occurred over three plates and each sample was quantified from five different wells. Samples extracted without pulverization were also quantified in this manner. The average yield for each sample based on the mean of the five quantifications and the overall mean for the blood swabs vs. pulverized blood swabs are presented in Tables 5.8 and 5.9.

Quant ID	Control #1 conc (ng/μL)	Control #2 conc (ng/μL)	Control #3 conc (ng/μL)
081027ss1	13.52	14.51	19.41
081027ss1	12.44	14.47	18.93
081027ss2	14.65	13.92	20.20
081027ss2	13.04	14.91	20.35
081023ss1	12.93	12.28	16.72
mean sample conc	13.41	14.02	19.12
Control Swabs Mean Yield	**15.49**		
Control Swabs Standard Deviation	2.88		

Table 5.8: Quantifications for replicate control blood swabs (not pulverized prior to extraction). Swabs pre-treated with 10 μL of whole blood and extracted without any pre-processing. The average value and standard deviation across all quantifications for this sample type are listed at the bottom of the table.

Quant ID	Pulverized #1 conc (ng/μL)	Pulverized #2 conc (ng/μL)	Pulverized #3 conc (ng/μL)
081027ss1	2.04	2.95	5.28
081027ss1	2.23	3.05	5.67
081027ss2	2.26	2.83	5.69
081027ss2	2.32	2.94	5.51
081023ss2	2.14	3.01	5.36
pulverized sample conc	2.20	2.96	5.50
Pulverized Swabs Mean Yield	**3.55**		
Pulverized Swabs Standard Deviation	1.47		

Table 5.9: Quantifications for replicate blood swabs pulverized prior to extraction. Swabs pre-treated with 10 μL of whole blood were pulverized consecutively in one grinding vial; powder from each swab was extracted separately. Resulting extracts underwent five quantifications, each value is reported as is the mean for each swab. The average value and standard deviation across all quantifications for this sample type are listed at the bottom of the table

The average yield for each sample based on the mean of the five quantifications overall mean for the DNA swabs vs. pulverized DNA swabs are presented in Tables 5.10 and 5.11.

Quant ID	Control #1 conc (ng/μL)	Control #2 conc (ng/μL)	Control #3 conc (ng/μL)
081027ss1	5.24	4.34	4.40
081027ss1	5.04	4.53	4.62
081027ss2	5.27	4.14	4.95
081027ss2	5.31	4.47	4.72
081023ss1	4.79	3.94	4.11
mean sample conc	5.13	4.28	4.56
Control Swabs Mean Yield	**4.66**		
Control Swabs Standard Deviation	0.439		

Table 5.10: Quantifications for replicate control DNA swabs (not pulverized prior to extraction). Swabs pre-treated with 1 μL of 161 ng/μL genomic DNA were extracted without any pre-processing. Resulting extracts underwent five quantifications, each value is reported as is the mean for each swab. The average value and standard deviation across all quantifications for this sample type are listed at the bottom of the table.

Quant ID	Pulverized #1 conc (ng/μL)	Pulverized #2 conc (ng/μL)	Pulverized #3 conc (ng/μL)
081027ss1	0.394	0.492	0.581
081027ss1	0.344	0.523	0.542
081027ss2	0.388	0.480	0.559
081027ss2	0.392	0.516	0.565
081023ss2	0.394	0.508	0.590
pulverized sample conc	0.382	0.504	0.567
Pulverized Swabs Mean Yield	**0.484**		
Pulverized Swabs Standard Deviation	0.0813		

Table 5.11: Quantifications for replicate DNA swabs pulverized prior to extraction. Swabs pre-treated with 1 μL of 161 ng/μL genomic DNA were pulverized consecutively in one grinding vial; powder from each swab was extracted separately. Resulting extracts underwent five quantifications, each value is reported as is the mean for each swab. The average value and standard deviation across all quantifications for this sample type are listed at the bottom of the table.

The results from this indicate that an appreciable amount of product loss occurs from sample transfer. This conclusion is based on the fact that yields differed between control and pulverized samples by three fold for the blood swabs and approximately ten-fold for the genomic DNA swabs. This was expected throughout the execution of the processing because an appreciable amount of powder remains on the vial walls after the transfer and was not accessible for extraction. It is also evident by the fact that a yield increase trend is apparent between samples collected following the first pulverization versus that from the third collection. As the vials become coated with sample, the amount transferred from the vial following subsequent grinds increased with each sample. While there is not excessive data to support this, the trend is apparent for both ground sample types; blood samples 1, 2 and 3 yielded 13.41, 14.02, and 19.12 ng/μL respectively and DNA samples 1, 2, and 3 yielded 0.382, 0.504, and 0.567 ng/μL (respectively). This trend coincidentally occurred for control blood swabs but was not apparent for the DNA samples. So while this may have been random happenstance, it could reasonably be due to the compounding material sticking to the inner walls of the vial.

While the freezer mill undoubtedly pulverizes samples adequately, the mechanics of the device and the large volume of the grinding vials make it of limited use for trace samples unless the nature of the sample is the matrix itself (bone). Even in the case of bone, drilling methods achieved more information than did the pulverized sample. However, drilling is not possible on some sample types and this method is of great application should tiny fragments be recovered or if extensive cleaning of the bone is required as may be the case of comingling remains. Regardless, the method did not offer advantages to recovery of swabs in general and this is presumably due to the large transfer loss that occurs when small samples are crushed in the large vial and then transferred to microcentrifuge tubes. Also to note, the vial cleaning and transfer processes are both areas of the protocol that could give rise to the entry of contaminants. Therefore, since the pulverization is not serving to break molecular bonding interactions (such as those between DNA and surface moieties on nylon

membrane), this is not an optimal pre-processing method for the applications of this project.

5.4 Conclusions of Maxwell® LEV and Freezer Mill Forensic Applications

Overall, the results obtained throughout the course of the Maxwell® LEV performance verification were quite expected. The instrument functionality was easily verified by acceptable yields, robust STR profiles, and the lack of contamination. The results of the yield comparison were also not surprising given that personal automation is slowly emerging in the lab and is still primarily used for reference sample extracts. However, with the reduced elution volume as a marketing strategy for use on casework specimens, the Maxwell® 16 LEV has undergone validation for casework in some labs. Research is ongoing for improvement of yield, including the use of different plastics to achieve less transfer loss and greater elution yield [Green, 2009]. The studies conducted here offer a crude comparison of sample recovery but cannot necessarily be ubiquitously applied to all sample types, therefore further consideration may be made specifically for nylon-bound template extraction since the yield comparisons offered in this chapter are limited to blood on swatches of cloth and do not address specific sample or substrate types and do not address special concerns, such as removal of inhibitors or introduction of DNA damage during extraction, both of which can affect one's ability to type a sample.

As for the freezer mill studies, it is clear that sample loss is a much more critical issue; as much as a 10 fold transfer loss may be experienced prior to additional loss imparted by the chosen extraction procedure. While advantageous to certain sample types that cannot be processed directly, the brief investigation presented here indicates sample loss far too substantial for trace or low copy number DNA samples. Although the nylon membrane fragment was adequately pulverized by the cryogenic grinding process, very little of the powder could be efficiently recovered due to do adherence to the instrumental components themselves. Therefore, this processing method will not be further investigated as it applies to removal of membrane bound DNA template.

6.0
DNA Typing of Membrane Bound Forensic Samples

6.1 Introduction and Theory for Recovery of Bound Samples

The development of DNA analysis by multiplex amplification of short tandem repeats marks a great advance over the prior methodology, restriction fragment length polymorphism (RFLP) analysis. STR analysis requires approximately 1000 times less DNA template and is more likely to produce results with degraded DNA. Moreover, PCR analysis can be completed in a single day compared to days or weeks for RFLP analysis [Davis, 1997a/b; Inman and Rudin, 1997]. The current standard for PCR STR analysis was fully described in Chapter 2 and the principles of the RFLP procedure are given in section 1.2.1.

6.1.1 Background and Relevance of the Procedure

To date, a method for comparing RFLP profiles to STR profiles has not been developed because the two systems interrogate different regions of the DNA sequence and use fundamentally different analytical platforms. Since the shift in technology from RFLP to STR-PCR, most laboratories, including the Federal Bureau of Investigation (FBI) and Forensic Science Service (FSS), have discontinued RFLP analysis. Moreover the commercial reagents used for RFLP analysis are becoming scarce, further restricting the opportunity for the forensic community to maintain the technology, even in the private sector [Beckwith, 2005]. Because results cannot be compared between systems, and RFLP analysis has become uncommon, it is now difficult to continue investigations where the primary methodology employed was RFLP. This is especially true in cases where the DNA from evidentiary items was consumed to generate a profile. Consumption was not uncommon because RFLP technology required large quantities of DNA and evidentiary DNA samples were often entirely used in an effort to obtain a profile for comparison. It is possible that DNA left from the exhibit in an RFLP case would be preserved in a restriction-cut form bound to a nylon membrane. Where post-analysis membranes have been archived, a theoretical opportunity exists for the sample DNA to be recovered from the membrane and exploited as a template for STR PCR analysis. Should a procedure be developed that would allow for STR typing of membrane bound DNA, it is possible that the technique could be validated in the forensic laboratory so that profiles generated in this manner could be searched against current criminal

intelligence databases. Furthermore, should membrane bound template be found viable for additional testing, technology may be applied to forensic exhibits that was not available in the past, such as SNPs or YSTR testing.

6.1.2 STR PCR Amplification of Restriction-Cut Templates

Prior to considering the implications of membrane binding and/or removal of potential template from a membrane, one should consider template damage resulting from the restriction digestion carried out during early steps of the RFLP process. In the United States, the restriction enzyme most commonly used for restriction digestion prior to fragment separation during the RFLP process has historically been *Hae*III [Budowle, *et. al*., 1990], therefore potential amplified fragment sequences were searched for *Hae*III recognition sites. The search included primer binding sites, STR repeat units, and the sequence intervening. Figures 6.1 through 6.3 illustrate locations of found cut sites, which were limited to the TPOX, TH01, and Penta E loci.

```
001   GGCACAGAAC AGGCACTTAG GGAACCCTCA CTGAATGAAT GAATGAATGA
      CCGTGTCTTG TCCGTGAATC CCTTGGGAGT GACTTACTTA CTTACTTACT
051   ATGAATGAAT GAATGAATGA ATGAATGTTT GGGCAAATAA ACGCTGACAA
      TACTTACTTA CTTACTTACT TACTTACAAA CCCGTTTATT TGCGACTGTT
101   GGACAGAAGG GCCTAGCGGG AAGGGAACAG GAGTAAGACC AGCGCACAGC
      CCTGTCTTCC CGGATCGCCC TTCCCTTGTC CTCATTCTGG TCGCGTGTCG
151   CCGACTTGTG TTCAGAAGAC CTGGGATTGG ACCTGAGGAG TTCAATTTTG
      GGCTGAACAC AAGTCTTCTG GACCCTAACC TGGACTCCTC AAGTTAAAAC
201   GATGAATCTC TTAATTAACC TGTGTGGTTC CCAGTTCCTC CCCTGAGCGC
      CTACTTAGAG AATTAATTGG ACACACCAAG GGTCAAGGAG GGGACTCGCG
251   CCAGGACAGT AGAGTCAACC TCACGTTTGA GCGTTGGGGA
      GGTCCTGTCA TCTCAGTTGG AGTGCAAACT CGCAACCCCT
```

Figure 6.1: *Hae*III restriction site analysis of PowerPlex® 16 TPOX amplicon sequence. The *Hae*III restriction cut site found in the PowerPlex® 16 TPOX locus amplicon is indicated in green over residues 110-113 [Huston, 2006].

```
001   GTGATTCCCA TTGGCCTGTT CCTCCCTTAT TTCCCTCATT CATTCATTCA
      CACTAAGGGT AACCGGACAA GGAGGGAATA AAGGGAGTAA GTAAGTAAGT
051   TTCATTCATT CATTCATTCA TTCACCATGG AGTCTGTGTT CCCTGTGACC
      AAGTAAGTAA GTAAGTAAGT AAGTGGTACC TCAGACACAA GGGACACTGG
101   TGCACTCGGA AGCCCTGTGT ACAGGGGACT GTGTGGGCCA GGCTGGATAA
      ACGTGAGCCT TCGGGACACA TGTCCCCTGA CACACCCGGT CCGACCTATT
151   TCGGGAGCTT TTCAGCCCAC AGGAGGGGTC
      AGCCCTCGAA AAGTCGGGTG TCCTCCCCAG
```

Figure 6.2: *Hae*III restriction site analysis of PowerPlex® 16 TH01 amplicon sequence. *Hae*III restriction cut sites found in the PowerPlex® 16 TH01 locus amplicon is indicated in green over residues 13-16 and residues 136-139 [Huston, 2006].

```
001   AAATACATTT TACCAACATG AAAGGGTACC AATAACAAGA AAATTGTGGC
      TTTATGTAAA ATGGTTGTAC TTTCCCATGG TTATTGTTCT TTTAACACCG
051   CAGGTGCGGT GGTTCACGCC TGCAATCCTA GCACTTTGGG AGGCCGATGC
      GTCCACGCCA CCAAGTGCGG ACGTTAGGAT CGTGAAACCC TCCGGCTACG
101   AGGTGTATTA CCTGAGCTCA GGAGATCAAG ACCAGCCTGG GCAACATGGT
      TCCACATAAT GGACTCGAGT CCTCTAGTTC TGGTCGGACC CGTTGTACCA
151   GAAACCCCGT CTCTACTAAA ATACAAAAAA TTAGCTGGGT GTGGTGGTAG
      CTTTGGGGCA GAGATGATTT TATGTTTTTT AATCGACCCA CACCACCATC
201   GCACCTATAA TCCCAGCTAC TCTGGAGGCT GAAACAGGAG AATCACTTGA
      CGTGGATATT AGGGTCGATG AGACCTCCGA CTTTGTCCTC TTAGTGAACT
251   ACCCAGGAGG TGGAGATTGA AGTGAGCCGA GATCACGCCA TTGCACTCCA
      TGGGTCCTCC ACCTCTAACT TCACTCGGCT CTAGTGCGGT AACGTGAGGT
301   GCCTGGGCGA CTGAGCAAGA CTCAGTCTCA AAGAAAAGAA AAGAAAAGAA
      CGGACCCGCT GACTCGTTCT GAGTCAGAGT TTCTTTTCTT TTCTTTTCTT
351   AAGAAAAGAA AAGAAAAGAA AAGAAAAGAA AAGAAAAGAA AATTGTAAGG
      TTCTTTTCTT TTCTTTTCTTVTTCTTTTCTT TTCTTTTCTT TTAACATTCC
401   AGTTTTCTCA ATTAATAACC CAAATAAGAG AATTCTTTCC ATGTATCAA
      TCAAAAGAGT TAATTATTGG GTTTATTCTC TTAAGAAAGG TACATAGTT
```

Figure 6.3: *Hae*III restriction site analysis of PowerPlex® 16 Penta E amplicon sequence. *Hae*III restriction cut sites found in the PowerPlex® 16 Penta E locus amplicon is indicated in green over residues 48-51 and residues 92-95 [Huston, 2006].

The presence of the restriction cut sites will affect the ability to obtain a profile at the three affected loci regardless of whether direct amplification from the membrane is attempted, or whether the DNA is removed from the membrane prior to typing. Therefore, some of the preliminary experiments served to confirm that these amplicons would be lost due to restriction digestion. Amplification of restriction

digested template empirically tests these theoretically identified cut sites and insures other loci are not affected in practical application.

6.1.3 Structural Properties of Nylon Static Supports

In 1963, Nygaard and Hall first reported techniques based upon the binding of DNA and RNA to nitrocellulose [Nygaard and Hall, 1963]. This work served as an important foundation in molecular biological techniques involving immobilization of nucleic acids on static supports and is the basis for Southern/Northern transfer, dot and slot blotting techniques, and a variety of other applications that have provided a wealth of qualitative and quantitative information for the past fifty years. It is known that molecular weight, nucleic acid conformation, and ionic forces all play an important role in binding of macromolecules to static supports, but the exact underlying mechanism of adsorption is not well described [Jones, 2001].

While early work focused on modifications of buffer composition and conditions in place during the adsorption process (high salt and high pH being optimal for inducing binding), commercialization of other supports that offered increased binding efficiency became prevalent. Because nitrocellulose membranes are electrostatic, brittle, and less conducive to repetitive probing, the use of nylon membranes made from 6,6 polymer (see Figure 6.4) were developed for binding of nucleic acids and other biomolecules [Bartelsman and Fost, 1984]. Because the nylon membranes can be easily manipulated and subjected to multiple probings, most laboratories selected nylon when conducting RFLP testing due to superior performance in hybridization-based assays [Bartelsman and Fost, 1984]. Furthermore, nylon can be surface-modified so that charged groups present on the membrane can interact with the phosphate backbone of the nucleic acid [Wang, 1992]. When the DNA is dried to the membrane, thymine residues reportedly cross-link to the amine groups on the membrane surfaces, and this can be furthered by exposure to UV radiation [Kalachikov, *et. al*., 1992]. While there are different opinions regarding the exact nature of the interactions, one consensus that exists in the manufacturing industry of supports is that production methods generally remain proprietary and have focused on increasing binding and not the ability to remove the nucleic acid once fixed

[Bartelsman and Fost, 1984; Jones, 2001]. Nylon binds nucleic acids primarily electrostatically, but also by covalent binding following UV fixation which makes the interaction fairly irreversible and allows for repeated probing of bound samples [Meinkoth and Wahl, 1984]. Charged membranes advanced binding chemistry because surface modifications to the microporous substrate allowed for increased binding capacity, versatility, and decreased analytical processing. As summarized by Jones, the most common surface modifications are based on amine, carbonyl, carboxyl, or thiol chemistries [Jones, 2001]. The chemistries can be further controlled with linkers which introduce thiol or amino groups to the 5' end of bound nucleic acid to ensure that the binding of the macromolecule offers the best orientation for downstream probe annealing and signal detection [Jones, 2001]. More recently, positively charged membranes usually consist of a porous, hydrophilic substrate comprised of any suitable material which may include polyaromatics and polystyrenes. They are then coated with a copolymer and a suitable cationic group, ammonium groups being most common. Spacial charge separation is achieved across the surface by the use of spacer groups, which usually consist of hydroxyl, hydroxyalkyl, amino, imino, aminoalkyl, amido, alkylamido, urea, ester, alkoxyalkyl or another polar moiety [Wu, *et. al*., 2006].

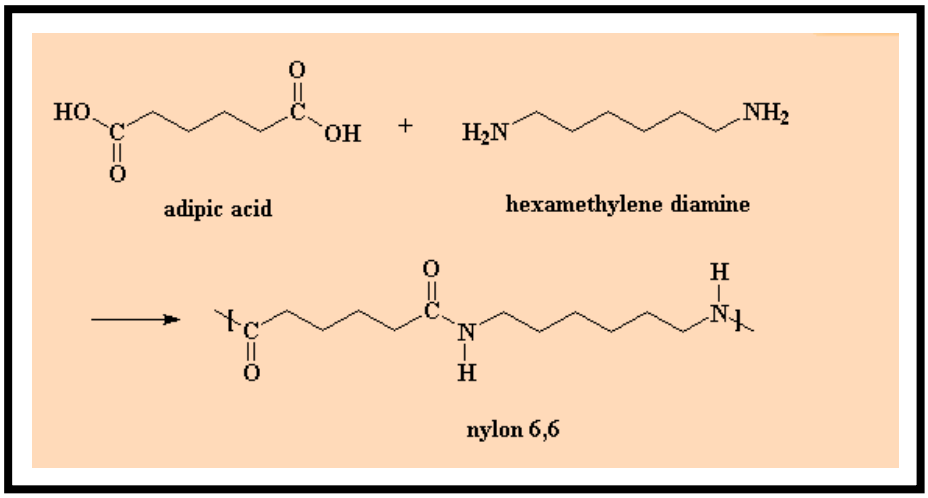

Figure 6.4: Structure of 6,6 nylon membranes. Illustrated is the reaction involved in the formation of 6,6 nylon. This is the chemical structure of the 6,6 nylon polymer used to manufacture membranes used for binding biomolecules [courtesy of the Polymer Science Learning Center (PSLC) at the University of Southern Mississippi, 2005].

A number of laboratories validated RFLP methods with Biodyne® brand nylon membranes manufactured by Pall Corporation. Most common to radioactive applications, Biodyne® A membrane is amphoteric and was used by many labs where radioactive detection of probe was desirable. This variety of membrane was validated for use in the Sedgwick County Regional Forensic Science Center (SCRFSC or RFSC) in Wichita, Kansas, USA, and the Kansas City Police Department Crime Laboratory, Kansas City, Missouri, USA. A portion of the archived membranes obtained from these casework laboratories were used herein and are of this variety. Most protocols developed for Biodyne® A applications included a cross-linking step to enhance binding of DNA to the membrane.

Pall Corporation's most versatile membrane, and widely used in forensic labs for DNA quantification, is the Biodyne® B membrane. This support bears a pore surface that is populated by a high density of quaternary ammonium groups and allows for multiple reprobings even if the UV irradiation step is omitted during the adsorption process. This membrane is commonly used for chemiluminescent assays and is the membrane of choice for RFLP testing conducted at Paternity Testing Corporation (PTC), a private laboratory in Columbia, Missouri, USA that also contributed archived membranes for the studies herein. It should be noted that these membranes, and other nylon membranes, are reportedly resistant to common solvents such as acetone, alcohol, chlorinated aliphatic hydrocarbons, formamide, NaOH, DMSO, and dimethylformamide, but are not compatible with concentrated acids [Pall Corporation, 2003a].

Due to availability, MagnaGraph® membranes were selected for optimization assays for studies conducted throughout this project. This membrane is often recommended for multiple probing applications and is "chemically optimized" from Micron, Separation, Inc. Since all optimization assays include a UV irradiation step, this membrane was selected for pilot studies to provide a good deal of insight regarding possible success with recovery from Biodyne® species that call for cross-linking. The later studies in this chapter included tests with Biodyne® B as well since some archived membranes used in terminal experiments (from PTC) were of this variety.

Interactions between macromolecules and supports are thought to occur in five steps. First, the macromolecule is transported to the surface of the support. This is followed by adsorption to the surface. The third step involves rearrangement of the adsorbed molecule. This is followed by potential desorption of the molecule, and finally, transport away from the support [Jones, 2001]. The fourth step is described as a possibility because the large number of binding sites present for any given molecule makes the binding interaction practically irreversible [Adamson, 1990]. While some binding sites may dissociate, it is unlikely that all will, and this is why there is generally a correlation between binding efficiency and nucleic acid size [Jones, 2001]. These interactions are all encouraged under conditions that are of high salt concentration and elevated pH and are furthered by baking or drying because all of these conditions are speculated to induce hydrophobic interactions. Specifications for the various membranes and binding conditions are described in Table 6.1.

	Transfer Buffer	**Transfer Method**	**Fixation**	**Fragments**	**Active Groups**
Nitrocellulose	20X SSC or SSPE	Capillary	1-2 hr 80°C	>300bp	~80 μg/cm^2 sites, Non specific, hydrophobic
- **Nylon**	10X SSC or 10X SSPE, or 0.01-0.4N NaOH	Capillary, alkaline, electroblotting	UV, microwave	All sizes, especially smaller	~450 μg/cm^2 sites, No modification, relies on UV link
+ Nylon	10X SSC or 10X SSPE, or 0.01-0.4N NaOH	Capillary, alkaline, electroblotting	1-2 hr 65-80°C, UV, microwave, alkaline	All sizes especially smaller	~450 μg/cm^2 sites, Surface amine enhances ionic/electrostatic interaction

Table 6.1: Membrane binding specifications. This table describes the buffering conditions that are optimal for the various membrane types, the transport mechanisms that are generally used, fixation parameters, macromolecule suitability, and surface modification chemistries [Pall Corporation, 2003b].

6.1.4 Disruption of the DNA:Nylon Complex and Other Considerations

The prospect of removing DNA from nylon membranes is a unique concept simply because it constitutes an endeavor historically avoided. With industrial efforts focused only on increased binding efficiency, and exact chemistries proprietary under many circumstances, the idea of recovering DNA after it is bound presents an interesting challenge.

The proposition of recovering membrane-bound DNA clearly requires a fundamental understanding of the process by which it is bound. While Nygaard and Hall reported techniques based upon the binding of DNA and RNA to a two-dimensional matrix of nitrocellulose [Nygaard and Hall, 1963], another monumental contribution occurred in 1975 when Southern reported methods for hybridization of sequence specific probes to membrane-bound DNA fragments [Southern, 1975]. These reports were foundational in molecular biology techniques involving immobilization of nucleic acids on static supports and are the basis for Southern/Northern transfer, dot and slot blotting techniques, and a variety of other applications that have provided a wealth of qualitative and quantitative information for the past fifty years. It is known that molecular weight, nucleic acid conformation, and ionic forces all play an important role in the binding of macromolecules to membrane supports, but the exact underlying mechanism of adsorption remains somewhat ambiguous [Jones, 2001]. Therefore, while recovery of DNA from a membrane support may seem inherently simple, approaches for reversing this interaction are difficult to determine given that the binding processes are not well described and/or have been optimized under proprietary endeavors.

Researchers affiliated with one membrane manufacturer, Pall Corporation, propose a binding model based primarily on hydrophobic interactions, where the surface chemistry (positively or negatively charged groups) plays a much smaller role in the binding process [Dubitsky and Perreault, 2007]. Like proteins coming in close contact with the membrane, nucleic acids maintain hydration associated with secondary structure. These layers of hydration are forced out upon contact with the membrane, allowing the biomolecules to flatten out and providing a stable system of increased entropy to drive the interaction [Wahlgren and Arnebrant, 1991; Plant *et. al*., 1991]. As stated previously, because nitrocellulose membranes are less flexible and not as conducive to repetitive probing, the nylon membranes were the static media of choice for labs validating RFLP techniques for forensic testing due to superior re-probing and chemiluminscent performance properties. While nylon polymer is mostly non-polar with terminal amino and carboxyl groups, the hydrophobic regions fold away

from the surface when cast into a membrane so that terminal polar groups are exposed for interactions with biomolecules [Dubitsky, 2007]. Furthermore, later nylon membranes were surface-modified so that charged groups present on the membrane can interact with the phosphate backbone of the nucleic acid; the linking chemistries most often employed are based on amine, carbonyl, carboxyl, or thiol and serve to enhance hybridization by affecting the orientation in which molecules bind [Plant *et. al*., 1991].

Preliminary assays tested for this project were based on mechanisms that failed to produce good blotting results. Of primary interest is the stripping process used during the multiprobing of membranes. Since stripping procedures that used detergents and heat were seemingly detrimental to the bound DNA, these agents were the first considered for recovering template that could be used for subsequent STR testing. Another approach is based on manufacturer literature and personal communication with Pall Corporation, which indicated nylon supports were incompatible with acids [Pall Corporation, 2003a]. Acid may be considered to destroy the membrane, but may also destroy the DNA. A variety of chemicals, many organic, will be considered to determine if the nylon can be dissolved leaving the DNA unaffected for recovery. And lastly, a variety of casework DNA extraction reagents and methods will be investigated for capability to remove DNA from membranes.

Physical removal techniques may also be an important avenue for consideration when attempting recovery. Membrane destruction caused by scraping, dicing, or surface damage could be useful in disturbing the DNA:nylon complexes. Another physical based approach is that of immobilization from the membrane by electrophoretic current. This idea is based upon electrophoretic transfer commonly used in Western blotting, which, if allowed to occur for extended periods of time, can cause transfer of macromolecules through the membrane [Ausubel, *et. al*., 1996]. While direct PCR amplification from DNA template covalently bound to an uncharged membrane has been reported [Sheikh and Lazarus, 1997], it is unknown if small quantities of restriction digested template can successfully be amplified using a multi-locus STR typing system. Finally, because of reports of using DNA covalently bound to nylon

membranes as a direct PCR template in the field of medical diagnostics, direct amplification approaches could potentially amplify template while bound [Sheikh and Lazarus, 1997]. Because most direct amplification tests published were conducted from uncharged substrates and employed at least 5 ng of localized template, it could not be directly determined from this literature whether the conformation changes imparted upon the degraded and widely dispersed macromolecule during charged membrane adsorption would allow PCR in the manner described by Sheikh and Lazarus. Nevertheless, this should be considered a possible approach for generating profiles and would have advantage over those involving lengthy chemical recovery processes.

6.1.5 Chapter Aims

The work described in this chapter involves a progressive approach to removing and/or multiplex typing archived RFLP membrane lanes. Amplification techniques will first be tested on restriction enzyme treated samples to ensure that multiplex testing is possible following digestion. Then, techniques for removal and/or direct amplification of bound samples will ensue. Some experimental approaches will involve principles tested throughout the course of the thesis so far; however, the primary progression involves pilot testing of chemical and buffered removal methods, followed by a more in-depth comparison of general extraction methods. Due to the limited availability of non-casework archived membranes, simulated bound samples must first be prepared by binding DNA, either high molecular weight (HMW) or restriction digested (RD), to localized segments of nylon membrane. Most of the pilot studies are aimed at HMW bound samples, then progress to RD bound samples. Techniques successful in removing restriction digested DNA bound to membranes can then be more specifically optimized and compared. The method identified to have best recovery will then be applied to true archived RFLP membrane lanes. In short, a variety of brief assays will be performed on HMW samples bound to localized areas of membranes to rapidly identify the most optimal procedure. The most promising procedures will be tested on samples that are restriction digested. Following the pilot studies, recovery techniques will be selected and performed in replicate on two membrane types for a more thorough comparison between

techniques. The method identified as best will be applied to archived RFLP membrane lanes in an attempt to fully address the overall project aim. The objectives toward the stated aim are:

1. Sample preparation and feasibility studies including *Hae*III restriction enzyme digestion, multiplex amplification of restriction digested samples and preparation of test fragments by fixing DNA to nylon membrane.
2. Recovery of high molecular weight membrane bound DNA using alkaline extraction, acidic extraction, organic chemicals, and buffered casework stain extraction.
3. Recovery of restriction digested membrane bound DNA using buffered casework stain extraction, preliminary studies involving the use of modified versions of casework extraction buffers with proteinase K and DTT additives, followed by a more extensive comparison of different casework stain extraction buffers and an automated paramagnetic capture method.
4. Additional novel recovery studies including direct STR amplification of digested membrane bound DNA, WGA of digested membrane bound DNA and DNA recovery using electrophoretic mobilization.
5. STR typing of DNA from archived RFLP membranes.

Due to the number of simulated fragment tests conducted using the various recovery approaches, a sample/method summary table, Table 6.3, is presented and serves as a useful point of reference when navigating through the progression of studies.

The basic principles of LCN analysis are applicable when attempting to type DNA samples recovered from or bound to nylon membranes, and thus directly apply to the ultimate goal of this project. The restriction digestion process produces samples very similar to fragmented template encountered in forensic casework samples, and even though relatively large quantities of DNA were used for RFLP, it is unlikely that generous quantities remain bound to the membrane or will likely be recovered from the membrane following the multi-probing process inherent to RFLP typing. Therefore, the challenges presented by the proposition of achieving STR profiles from membrane bound samples embodies the LCN topics explored throughout this thesis.

6.2 Membrane Recovery Methods and Materials

This section describes methods for assays which progressively address DNA recovery from archived membranes. The stepwise approach begins with determining if restricted DNA can be PCR amplified, then progresses to localization of HMW and restriction digested DNA samples to nylon membrane fragments to be used in preliminary recovery assays. Techniques that did not involve chemical extraction of DNA were also tested, including electrophoretic mobilization and direct amplification of membrane bound samples. The experimental design is divided into several major classifications. First, preparation of DNA samples, restriction digestion, feasibility studies for the amplification of restricted DNA with multiplex kits, and verification of membrane binding processes used to prepare localized bound samples will be presented. Next, acid/base, organic solvent and general DNA extraction buffer techniques will be described. A more thorough comparison of casework extraction techniques and buffer compositions will then be presented, followed by tests involving direct amplification and other novel approaches. Finally, the most promising recovery method will be applied to archived membranes.

6.2.1 Sample Preparation and Feasibility Studies

6.2.1.1 *Hae*III Restriction Digestion

High molecular weight DNA was incorporated into restriction enzyme (RE) reactions to produce digested DNA for subsequent binding/cross-linking to nylon membrane. Reactions were carried out as suggested by the manufacturer's product insert; Table 6.2 typifies the RE digest carried out prior to nylon binding [Promega, 2004b].

Restriction Digest Component	µL per well	µL total in master mix
NFW	16.5	214.5 (or 140.4)
Multicore RE 10X buffer	2.0	26.0
DNA-100 ng/µL (or 15 ng/µL)	1 (or 6.7)	13 (or 87.1)
*Hae*III enzyme - 10u/µL	0.5	6.5
TOTAL	*20*	*260*

Table 6.2: Recipe for *Hae*III digestion. Nuclease free water (NFW), restriction enzyme, multicore buffer, human DNA, and *Hae*III enzyme were mixed in these quantities and incubated at 37° C to achieve restriction cutting of the DNA. The components were combined in a master mix (column three) and then 20 µL of the mixture was placed into individual slot blot wells for membrane binding. Column two indicates component quantities expected on a single membrane test slot.

Where concentrations of DNA were less than 100 ng/μL, additional volume of DNA was used and the quantity of NFW adjusted accordingly; this is indicated parenthetically in Table 6.2. Likewise if BSA was added, each reaction received 0.2 μl of BSA (10 μg/μL) and the amount of NFW decreased by 2 μL. Digestion was carried out for 2-16 hours and checked for reaction completeness by visualization on agarose gels. Following qualitative/quantitative assessment, samples were heated to 65^{o} C for 10 minutes if temporary storage was necessary.

6.2.1.2 Multiplex Amplification of Restriction Digested Samples

For PowerPlex® 16 BIO/ FMBIO® II analysis, slot blot quantified DNA (QuantiBlot® Human DNA Quantitation) was digested as described in 6.2.1.1. Digests containing 100 and 50 ng of template DNA performed in 20 μL reactions were checked for completeness using a 1% agarose gel (data not shown). Post-digest sample was again quantified so that quantifications of pre- and post- digest samples could be compared and considered. Based on the QuantiBlot® Human DNA Quantitation data it was determined that 1 μL of the 100 ng reaction (~ 5 ng DNA based on pre-digest quantification and ~1.5 ng DNA based on post-digest quantification) and an equal volume of the negative restriction digestion control (containing no DNA) would be amplified using the PowerPlex® 16 BIO multi-plex PCR amplification system. The resulting profiles were analyzed at 1X and 0.5X loading volumes on the gel platform using parameters described in Chapter 2.

For the PowerPlex® 16/3100-series analysis, previously quantified (Quantifiler® Human) DNA was digested as described in 6.2.1.1. Digested DNA was then re-quantified using Quantifiler® Human so that quantifications of pre and post digest samples could be compared and considered. The restricted sample was amplified with PowerPlex® 16 in a range of quantities. Based on the post-digest quantifications, amplifications received 20 pg, 50 pg, 100 pg, and 200 pg of template; however these relate to approximately 40 pg, 100 pg, 200 pg, and 400 pg input DNA if pre-digest quantification were used to estimate input DNA. Each quantity was amplified in duplicate; positive and negative amplification controls were amplified alongside the

samples. Amplicons were separated using the ABI PRISM® 3100-series analyzers, data analysis was conducted with GeneMapper™ ID using parameters established in Chapter 2 (100 RFU cut-off).

6.2.1.3 Binding and Cross-linking of DNA to Nylon Membrane

Often in forensic casework, the quantity of sample is insufficient for exhaustive retesting and validation. When investigating specimens compromised by time and/or deleterious change, it is impossible to reconstruct exact scenarios and this is further complicated since many factors operate simultaneously across a range of severity levels. With this study, non-casework archived RFLP membranes suitable for research are rare. Therefore, an experimental strategy was devised allowing for evaluation of extraction techniques using simulated test fragments so that archived RFLP membranes could be reserved for final testing employing the most promising of the extraction techniques.

Defined quantities of high molecular weight (HMW) DNA (ranging from 10-50 ng) or restriction digest aliquots (containing approximately 100 ng) were prepared for membrane binding by denaturation in 250 mL 0.5M NaCl/0.5M NaOH solution. DNA extracts were prepared from blood drawn from a female (♀) or male (♂) volunteer; sources are specified in Table 6.3. Meanwhile, MagnaGraph® nylon membrane was soaked in 2X SSC for approximately 5 minutes. Samples were then transferred to the membrane using the Convertible® Filtration Manifold System in defined areas (slots) that could be excised for subsequent extraction from the static matrix. The Convertible® Filtration Manifold System was equipped with a top plate comprised of 48 loading slots, each providing a 0.75 x 7.5 mm fluid contact area to the membrane. With samples transferred, locations of DNA were marked and the membranes were placed in a volume 2X SSC-Tris HCl, pH 8.0 that covered the membrane. After a 5 minute soak, the membrane was drained, placed between filter paper, and then baked at 80^{o} C for 30 minutes. For Magnagraph® samples, the DNA was then UV cross-linked at an energy setting of 1200 (120,000 μJ/cm^{2}) on each side. The practice was based on established RFLP laboratory protocols [Davis, 1997b]. Binding efficiency using this process was initially verified by methylene blue staining

of a 10 ng HMW band. The membrane was stained for 30 seconds and then de-stained in type 1 water. Lower concentrations of DNA were verified using the QuantiBlot® Human DNA Quantitation method, beginning with membrane hybridization. Membranes were then stored frozen and areas of bound DNA excised as needed for extraction/recovery assays. Excised fragments were approximately 5x10 mm in size to fully encompass bound slots and to achieve a manageable fragment for handling with forceps without disruption of bound sample.

Biodyne® B membrane fragments were also prepared for studies involving unmodified casework extraction buffers (6.2.3.4). For these, Biodyne® B was soaked in prewetting solution for 15 minutes. Meanwhile, samples were denatured in 250 µL of spotting solution; samples were then loaded onto the membrane using the Convertible® Filtration Manifold System. The membrane was washed for 15 minutes in 0.2MTris:2XSSC and baked at 80^{o}C for 30 minutes to dry. Due to similarities between this procedure and current methods for slot blot, binding verification was not performed on the Biodyne® B membrane fragments.

6.2.2 Recovery of High Molecular Weight (HMW) Membrane-Bound DNA

High molecular weight (HMW) DNA extracted from blood was bound and cross-linked to localized areas of membrane fragments as described previously. The studies were performed first on HMW bound samples, then progressed to restriction digested (RD) bound samples. Various direct amplification approaches were also tested, as was removal via electrophoretic mobility. The progression of chemical and direct amplification tests are summarized in Table 6.3; electrophoretic mobilization assay is not included in this table.

Recovery Method	Sample Description	End-point Amplification Chemistry
heat/alkaline strip	50 ng bound K562 DNA	PowerPlex® 2.1
heat/alkaline strip	50 ng aqueous K562 DNA	PowerPlex® 2.1
heat/alkaline strip	50 ng aqueous K562 DNA + membrane cuttings	PowerPlex® 2.1
0.75M acid	50 ng bound K562 DNA	PowerPlex® 2.1
0.38M acid	50 ng bound K562 DNA	PowerPlex® 2.1
0.19M acid	50 ng bound K562 DNA	PowerPlex® 2.1
0.09M acid	50 ng bound K562 DNA	PowerPlex® 2.1
0.75M acid	50 ng aqueous K562 DNA	PowerPlex® 2.1
0.38M acid	50 ng aqueous K562 DNA	PowerPlex® 2.1
0.19M acid	50 ng aqueous K562 DNA	PowerPlex® 2.1
0.09M acid	50 ng aqueous K562 DNA	PowerPlex® 2.1
ethanol, methanol, acetone, DMSO, chloroform, phenol, PCI, formamide	50 ng bound K562 DNA	Amplification not attempted based on quantification
stain extraction buffer (SEB)	50 ng bound K562 DNA 30 ng bound K562 DNA 10 ng bound K562 DNA	PowerPlex® 16 BIO
unmodified/modified SEB	100 ng RD bound ♀DNA	PowerPlex® 16
modified sperm lysis buffer	100 ng RD bound ♀DNA	PowerPlex® 16
modified Differex™ lysis buffer	100 ng RD bound ♀DNA	PowerPlex® 16
modified Differex™ lysis buffer	100 ng RD bound ♀DNA, scraped and diced	PowerPlex® 16
unmodified sperm lysis buffer	100 ng RD bound ♂DNA *	PowerPlex® 16
unmodified SEB	100 ng RD bound ♂DNA *	PowerPlex® 16
unmodified Differex™ lysis buffer	100 ng RD bound ♂DNA *	PowerPlex® 16
Maxwell® 16 trace LEV protocol	100 ng RD bound ♂DNA *	PowerPlex® 16
direct STR amplification from membrane, AmpliTaq Gold® polymerase	100 ng RD bound ♀DNA	PowerPlex® 16
direct WGA from membrane	100 ng RD bound ♀DNA	PowerPlex® 16
electrophoretic mobilization	100 ng RD bound ♀DNA	not applicable

Table 6.3: Summary of recovery assays conducted on HMW and RD DNA samples localized and bound to membrane. A brief description of recovery methodology and the sample type to which each was applied are listed in the first two columns; all samples were quantified using slot blot and amplified with the chemistry indicated in the third column. PowerPlex® 16 BIO analyzed with the FMBIO® II; PowerPlex® 16 products analyzed on the ABI PRISM® 3100-*Avant*; Assays first evaluated recovery of HMW DNA, then advanced to recovery of restriction digested DNA. The progression from early studies to later studies coincides with advancements in STR chemistries available for use at the time of each study. All tests were performed from MagnaGraph® membrane fragments bound with K562 or female volunteer blood DNA extracts except those indicated with (*); which indicates tests were performed on both MagnaGraph® and Biodyne® B fragments bound to localized restriction digested DNA (prepared from male blood DNA extracts). Red methods are described in section 6.2.2.1. Orange methods are described in section 6.2.2.2. Yellow methods are described in section 6.2.2.3 and 6.2.2.4. Green methods are described in sections 6.2.3.1 and 6.2.3.2. Blue methods are described in section 6.2.3.4. Purple methods are described in section 6.2.4.

6.2.2.1 Recovery of HMW Membrane-Bound DNA using Alkaline Extraction

Single membrane cuttings bound to 10 ng HMW DNA were treated with 200 µL strong strip solution or 200 µL TE; 10 ng K562 DNA in aqueous solution was also treated with 200 µL strong strip solution or 200 µl TE. The tubes were each boiled for 30 minutes, cooled, centrifuged briefly, and purified/concentrated with Microcon® concentrators. Eluates were brought to a volume of 20 µL and quantified using the QuantiBlot® Human DNA Quantitation system. The product recovered from the membrane slot processed with TE was amplified in full (15 µl) using the PowerPlex® 16 BIO-FMBIO® II platform. The product recovered from the membrane slot processed with strong strip did not undergo amplification.

A dual step alkaline membrane strip process was also tested on the following samples described in Table 6.4.

Sample	Component description
DSS-A	50 ng HMW DNA (K562), UV cross-linked to membrane
DSS-B	50 ng HMW DNA (K562), in aqueous solution
DSS-C	50 ng HMW DNA (K562), in aqueous solution containing a small piece of unused nylon membrane (to ascertain if membrane interactions alone interfere with DNA recovery)

Table 6.4: Dual alkaline membrane strip assays. This table defines the nomenclature and description of samples that underwent dual step alkaline strip assays.

These three samples were treated with the alkaline membrane strip step one wash solution (100 µL) for 20 minutes in a boiling water bath; the wash was removed and reserved. The samples were then treated with the alkaline membrane strip step two wash solution (100 µL), soaked at room temperature for 10 minutes and subjected to a boiling water bath for five minutes. The second wash was then removed and combined with the reserved step 1 wash. Washes were brought to a volume of 400 µL with TE and DNA recovered by standard ethanol precipitation. Samples were then resuspended using 10 µL TE, and quantified using 2% agarose gel and the QuantiBlot® Human DNA Quantitation system. The samples were then amplified

using PowerPlex® 2.1 / FMBIO® II platform. The DSS-A eluate was amplified in full (6 μL remaining), while DSS-B and DSS-C were amplified in quantities based on slot blot quantification (1 and 2 μL, respectively).

6.2.2.2 Recovery of HMW Membrane-Bound DNA using Acidic Extraction

Concentrations of hydrochloric acid were prepared in the following seven molarities: 6.0, 3.0, 1.5, 0.75, 0.38, 0.19, and 0.09M. Small cuttings of MagnaGraph® nylon membrane, approximately 0.5 cm^2, were placed into aliquots of each acid concentration so that membrane property changes could be observed macroscopically. The 6.0M solution changed the membrane appearance slightly, rendering it thin in appearance, resembling that of a lint-free delicate task laboratory wipe (KimWipe). Other concentrations did not seem to cause a noticeable change in membrane morphology.

Single membrane cuttings bound to 50 ng HMW DNA were treated with the acid solutions (one membrane fragment was placed into 100 μL of each of the seven different concentrations). After 15 minutes, the acids were drawn off and placed into tubes containing 300 μl TE. To this, 500 μl of PCI was added. Samples were vortexed briefly and spun at 15,000 RPM for 2 minutes. Each aqueous phase was purified using Microcon® concentrations, employing a wash with water followed by a wash with TE. DNA was eluted from each into a final volume of 10 μL using TE.

Additionally, sample tubes containing 50 ng K562 DNA in aqueous solution were subjected to each of the acid treatments and processed as described above beyond the initial incubation and centrifugation steps.

Following initial acid removal assays employing Microcon® concentration devices, four unbound HMW 50 ng aqueous control samples were processed in a similar manner, using the four least concentrated acid solutions (0.09, 0.18, 0.38, and 0.75M) followed by DNA capture via ethanol precipitation. Sample pellets were resuspended using 10 μL TE and evaluated for product using a 2% agarose gel. Finally, membrane

recovery tests were repeated using ethanol precipitation to recover any DNA product. Bound and unbound samples treated with 0.09, 0.18, 0.38, and 0.75M acid solutions and concentrated with ethanol precipitation could then be directly compared with QuantiBlot® Human DNA Quantitation and PowerPlex® 2.1-FMBIO® II platform. Following quantification, bound recovery product samples were amplified in full (6 μL). Recovery products from unbound samples were amplified according to slot blot quantification; 0.75, 1.0, 6.0, and 6.0 μL of the unbound sample extracts were amplified from the 0.09, 0.18, 0.38, and 0.75M solution products, respectively.

6.2.2.3 Recovery of HMW Membrane-Bound DNA using Organic Chemicals

Single membrane cuttings bound to 50 ng HMW DNA were treated with 100 μL of ethanol, methanol, acetone, DMSO, chloroform, phenol, phenol/chloroform/isoamyl alcohol, or formamide and incubated 15 minutes at 56° C. Membrane fragments were all intact following the incubation. To each reaction, 400 μL of TE was added. Each sample was then treated with 400 μL PCI, vortexed briefly, and centrifuged for 5 minutes at 15,000 RPM. The aqueous phase from each then underwent ethanol precipitation and pellets were resolublized in 10 μL water. All resulting products were quantified and evaluated using 2% agarose gel and the QuantiBlot® Human DNA Quantitation system. PCR amplification was not performed.

6.2.2.4 Recovery of HMW Membrane-Bound DNA using Casework Stain Extraction

Single membrane slots bound to 50, 30, and 10 ng HMW DNA were treated with 400 μL forensic casework stain extraction buffer working solution (SEB) to which 10 μL of 20 mg/mL proteinase K had been added; tubes were incubated overnight at 56° C [Steadman, 2002a]. The extraction was completed with PCI/Microcon® purification and concentration. The samples were brought to a final volume of 16 μL and quantified using the QuantiBlot® Human DNA Quantitation system. The samples were then amplified using the PowerPlex® 16 BIO / FMBIO® II platform. For the amplifications, 2 μL of the 50 ng extract, 4 μL of the 30 ng extract, and 15 μL of the 10 ng extract were incorporated into the respective reactions.

6.2.3 Recovery of Restriction Digested (RD) Membrane-Bound DNA

*Hae*III restriction digestion (RD) was performed and aliquots (containing approximately 100 ng) were bound and cross-linked to membrane in localized slots as described previously and used for tests described herein.

6.2.3.1 Recovery of RD Membrane-Bound DNA using Casework Stain Extraction

Extraction was performed on a membrane slot bound with 100 ng digested DNA in the same manner as that described in for the fragment bound with HMW DNA. The sample was brought to a final volume of 11 μL NFW and quantified using the QuantiBlot® Human DNA Quantitation system. For the amplification, the remainder of the extract (10 μL) was incorporated into the reaction mixture and the sample was then amplified using the PowerPlex® 16 BIO / FMBIO® II platform.

6.2.3.2 Recovery of RD Membrane-Bound DNA using Modified Versions of Extraction Buffers

Given that casework stain extraction buffer proved most robust for removal of bound HMW DNA, it was determined that DTT and/or proteinase K may be key for disruption of DNA-membrane interactions. Therefore, multiple extraction buffers were prepared, each with increased levels of DTT and proteinase K for extraction comparison tests. Four extraction solutions were prepared and will be referred to as follows: modified sperm lysis buffer, modified stain extraction buffer working solution (SEB) prepared from extraction buffer stock solution (EBSS), modified Differex™ lysis buffer, and DTT+proK solution. The recipes are as follows [based on Laber, 1992; Steadman, 2002a; Promega, 2004a; Promega, 2008a]:

Modified Sperm Lysis Buffer
150 µL TNE
100 µL sarkosyl (from 20% or 200 mg/mL stock for 50 µg/µL final concentration)
80 µL DTT (from 0.39M or 60.12 mg/mL stock for 12 µg/µL final concentration)
60 µL NFW
10 µL proK (from 20 mg/mL stock for 0.5 µg/µL final concentration)

Modified SEB
300 µL EBSS
20 µL proK (1 µg/µL final concentration)
80 µL DTT (12 µg/µL final concentration)

Modified Differex™ Lysis Buffer
300 µL Promega Differex™ Lysis Buffer
20 µL proK (1 µg/µL final concentration)
80 µL DTT (12 µg/µL final concentration)

DTT + proK Solution
200 µL DTT (from 0.39M or 60.12 mg/mL stock for 40 µg/µL final concentration)
100 µL proK (from 20 mg/mL stock for 6.7µg/µL final concentration)

Extractions were performed on membrane slots bound with 100 ng digested DNA. One membrane slot was also scraped and diced prior to treatment with the modified Differex™ lysis buffer (referred to as scraping + Differex™ lysis buffer). All were incubated overnight at 56° C. The extractions were completed with PCI/Microcon® purification and concentration, brought to a final volume of 15 µL in NFW, and amplified in full using the PowerPlex® 16 / ABI PRISM® 3100-*Avant* platform. Electropherograms were analyzed down to 50 RFU.

6.2.3.3 Proteinase K and DTT Extraction Yield Study

Dried blood stains were prepared by placing 10 µL of liquid blood onto suspended cotton cloth swatches and allowed to dry. Spotting was performed in triplicate for each extraction method tested. Three stains were extracted with standard SEB; three stains were extracted with the following to achieve elevated levels of proteinase K and DTT during the overnight incubation step of the process:

300 µL EBSS
20 µL proK (20 mg/mL stock)
80 µL DTT (0.39M stock)

Each stain was extracted overnight at 56° C. Appropriate negative reagent controls were carried out for each buffer. Cuttings were suspended in filter baskets, centrifuged to dryness, and discarded. The extractions were completed with PCI/Microcon® purification and concentration, brought to a final volume of 25 μL in NFW, and quantified using Quantifiler® Human DNA quantification. Duplicate or quadruplicate quantifications were performed on each extract; yield averages were then compared between stains and extraction buffer methods.

6.2.3.4 Recovery of RD Membrane-Bound DNA using Casework Extraction Buffers

These tests were carried out in two parts. First, four extraction buffers were tested and compared (Four Buffer Comparison). From that work, a second study was performed to directly test the best two performing buffers identified among the four initially tested (Two Buffer Comparison).

6.2.3.4a Four Buffer Comparison

Unmodified extraction buffers were prepared for a more extensive extraction comparison test. These are referred to as "unmodified" because these reflect standard solutions used for routine extractions in the casework lab [Steadman, 2002a]. Sperm lysis buffer is used during the differential extraction process to prepare the sperm cell fraction, SEB is used for all standard sample extractions (non-differential), and the Differex™ buffer is used for cell lysis during differential extraction using Promega's Differex™ extraction kit. In accordance with those protocols, three extraction solutions were prepared and will be referred to as follows: sperm lysis buffer, SEB, and Differex™ lysis buffer. A fourth set of fragments were extracted using the Maxwell® 16 trace extraction method as described in 5.2.1. The extraction buffer recipes are as follows [based on Laber, 1992; Steadman, 2002a; Promega, 2004a; Promega, 2008a]:

Sperm Lysis Buffer (SP)
225 µL TNE;
75 µL sarkosyl (from 20% or 200 mg/mL stock for 25 µg/µL final concentration)
60 µL DTT (from 0.39M or 60.12 mg/mL stock for 6 µg/µL final concentration)
225 µL NFW
15 µL proK (from 20 mg/mL stock for 0.5 µg/µL final concentration)

Stain Extraction Buffer Working Solution (SEB)
600 µL EBSS (contains DTT at 6 µg/µL final concentration)
15 µL proK (from 20 mg/mL stock for ~0.5 µg/µL final concentration)

Differex™ Lysis Buffer (DFX)
576 µL Promega Differex™ Lysis Buffer
32 µL proK (from 20 mg/mL stock for ~1.1 µg/µL final concentration)

Maxwell® 16 Extraction Method (MAX)
Pre-incubation with 190 µL Incubation Buffer and 10 µL proK (1 hour)
Add 198 µL of Lysis Buffer and 2 µL of 1M DTT (5 min with vortex)

For the Maxwell® extraction, each sample was pre-incubated at 56° for 1 hour. Following incubation, 198 µL of Lysis Buffer and 2 µL of 1M DTT were added to each tube. Each sample was vortexed briefly and held at room temperature for 5 minutes. Following a brief centrifugal spin, the cuttings were removed from solution and suspended in a filter basket. The tubes were centrifuged at 15,000 RPM for 5 minutes; filter baskets containing substrates were discarded. Negative control consisted of DNA IQ™ Lysis Buffer/proK/DTT solution only. All preparations were then transferred to designated extraction cartridges on the Maxwell® 16 and processed in the standard forensic mode, with a 20 µL final volume of elution buffer in place for the final volume. All other extractions (Sperm Lysis, SEB, and Differex™ Lysis) were carried out at 56° overnight, followed by brief vortex, filter basket suspension and centrifugation, PCI/Microcon® concentration/purification, and elution into a 20 µL final volume, consistent with the elution volume achieved with the Maxwell® robotic extractions. Negative controls for the overnight extractions consisted of extraction buffer components only for each buffer tested.

Extractions were carried out on each of two membrane types in triplicate using each buffer/extraction system. These membrane types were MagnaGraph® and Biodyne®

B nylon fragments, prepared as described in section 6.2.1.4. Extracts were assigned the following nomenclature: M1-SEB, M2-SEB, M3-SEB, MRNC-SEB represent MagnaGraph® fragments 1 through 3 extracted with SEB, and the reagent negative control extract. B1-SEB, B2-SEB, B3-SEB, BRNC-SEB represent Biodyne® B fragments 1 through 3 extracted with SEB, and the reagent negative control extract. The suffix for the Sperm Lysis Buffer is SP, the suffix for Differex™ extractions is DFX, and the suffix denoting Maxwell® extractions is MAX. Each of four extraction methods (4 methods) was tested in triplicate with a negative control (4 fragments) using each of two membrane varieties (2 nylon types); therefore, 4 methods x 4 fragments x 2 membrane types=32 total extracts generated. Each extract was quantified in duplicate using Quantifiler® Human. An average value was determined for each fragment by averaging the duplicate quantification values. For ANalysis Of VAriance between groups testing (ANOVA), an average yield and standard deviation was determined for each extraction method/membrane type where n=3, since three fragments were extracted. Where method/membrane recovery quantities exceeded 3 ng/μL, equal portions of extracts were pooled, diluted, and amplified in triplicate using PowerPlex® 16 typing system. Where method/membrane recovery quantities were too low to require dilution, extracts of common method/membrane groups were pooled and divided in thirds for triplicate amplification in full. All negative control samples were amplified in full. All amplicons were separated using ABI PRISM® 3100-series analyzers, data analysis was conducted with GeneMapper™ ID using parameters established in Chapter 2 (100 RFU cut-off).

6.2.3.4b Two Buffer Comparison

A second study was conducted using the best two methods based on performance throughout the triplicate fragment study to more fully investigate recovery efficiency. During this study, post extraction pooling was not performed prior to amplification. These tests were conducted using only the SEB and Differex™ methods, based on the fact resources were limited and these clearly outperformed the Sperm Lysis and Maxwell® methods tested prior. These tests were carried out as described for previous unmodified extraction buffer comparison tests, however each buffer was tested on five fragments of each membrane type (plus a negative control), therefore 24

total extracts were obtained (denoted as extracts 4-8 for each method/membrane type). These were quantified in duplicate using the Quantifiler® Human kit, and the mean value was used to determine the amount of extract that would be incorporated into the PowerPlex® 16 reaction for each *individual* extract (no pooling prior). These averages between duplicate quantifications were also used to further analyze variance between these five and the previous three fragments, for both MagnaGraph® and Biodyne®, extracted with SEB and Differex™. All negative control extracts were amplified in the same manner as the least concentrated sample from that extraction group.

All amplicons were separated using ABI PRISM® 3100-series analyzers, data analysis was conducted with GeneMapper™ ID using parameters established in Chapter 2 (100 RFU cut-off).

6.2.4 Additional Novel Recovery Studies

This section describes methods tested at various points throughout the progressive membrane recovery work. These methods are a culmination of several novel recovery and amplification approaches and are drawn from points throughout the thesis bringing a variety of different topics together for simultaneous testing. Methods tested included direct PowerPlex® 16 amplification, Restorase® amplification, and WGA amplification. The section also describes an approach for electrophoretic removal of DNA from the membrane by application of electrical current to the membrane fragment embedded in an agarose gel.

6.2.4.1 Direct STR Amplification of RD Membrane-Bound DNA

A MagnaGraph® membrane slot bound with digested DNA was diced and placed in a standard PowerPlex® 16 BIO multiplex PCR amplification reaction. No profile resulted; therefore an intermediate assay was designed to determine if the physical binding properties inherent to the membrane interfered with the PCR. This test involved the amplification of two samples, each were provided 0.5 ng K562 DNA template. To one, clean diced MagnaGraph® membrane fragments were added (these membrane pieces were unprocessed and obtained directly from manufacturer's packaging); the other reaction was performed without membrane fragments. The

possibility of "pre-hybridizing" the free membrane with such components was also explored. MagnaGraph® membrane fragments bound with digested DNA were diced and pre-soaked in 20 µL of 10X Gold ST*R reaction buffer overnight at room temperature on an orbital shaker in an effort to prevent subsequent amplification components from interacting with free charges left on the membrane. Following the pre-soak, the fragments underwent three 100 µL NFW washes. PowerPlex® 16 BIO multi-plex PCR reaction components were then added and amplification carried out.

Following the above direct amplification attempts, an alternate amplification enzyme, Restorase® DNA polymerase, was tested for the ability to amplify digested DNA bound to membranes. For this study, a MagnaGraph® membrane fragment bound to digested DNA was treated with 19.2 µL nuclease-free water, 2.5 µL Gold ST*R 10X buffer, and 0.8 µL Restorase® DNA polymerase. The samples were incubated for 10 minutes at 37° C, followed by a secondary incubation at 72° C for 5 minutes. A volume of 2.5 µL primer mix (PowerPlex® 16) was added following the incubations. The 25 µL reaction then underwent 32 cycles of PCR using a modified version of the cycling parameters for the PowerPlex® 16 system (described section 4.2.2). The modification consisted of omitting the 11 minute hot start.

6.2.4.2 WGA Amplification of RD Membrane-Bound DNA

One of the final localized bound membrane tests consisted of application of whole genome amplification. One test was designed as an attempt to direct amplify the DNA bound to the membrane, and the other was designed to test the ability to whole genome amplify digested DNA recovered from MagnaGraph® membrane with modified SEB as described in section 6.3.3.2. Modified SEB extraction was performed on a MagnaGraph® membrane slot bound with digested DNA. The extraction was carried out overnight at 56° C and completed with PCI/Microcon® purification and concentration. The sample was eluted using 5 µL in NFW and then lyophilized.

Whole genome amplification was carried out on the lyophilized extract (denoted as WGA-sebws) and a MagnaGraph® membrane slot bound with digested DNA (denoted

as WGA-direct). A volume of 1 μL NFW was added to the membrane slot and lyophilized sample. A positive and negative control were also prepared, consisting of 1 μL 10 ng/μL 9947A (WGA-pos) and 1 μL TE, (WGA-neg) respectively. To each sample, 9 μL of sample buffer was added. Tubes were heated to 95° C for three minutes and snap-cooled on ice water. A reaction pre-mix, consisting of 1 μL ϕ29 enzyme and 9 μL of reaction buffer was added to each reaction tube while kept cold. Reaction tubes were placed at 30° C for 18 hours. Following amplification, the samples were heated to 65° C for 10 minutes and then stored at 4° C [Amersham, 2003]. Sample volumes were each increased using 100 μL TE prior to Microcon® concentration after which samples were eluted into a final volume of 20 μL NFW. A portion of each was further diluted for quantification using a 1% agarose gel and the QuantiBlot® Human DNA Quantitation system. Samples were amplified using the PowerPlex® 16 chemistry as follows:

WGA-pos: 1 μl undiluted, 1 μl 1:49 dilution
WGA-neg: 15 μL undiluted
WGA-sebws: 1 μl undiluted, 15 μL undiluted, 5 μl 1:49 dilution
WGA-direct: 15 μL undiluted

Positive and negative STR amplification controls (0.5 ng 9947A and NFW) were amplified and analyzed alongside the WGA samples. Amplicons were separated using a 3100-series analyzer, and data analysis was conducted with GeneMapper™ ID using parameters established in Chapter 2 (100 RFU cut-off).

6.2.4.3 Recovery of Restriction Digested Membrane-Bound DNA using Electrophoretic Mobilization

Removal of bound DNA was attempted using the application of electrophoretic force. A 2% agarose gel was prepared with capacity for an imbedded membrane slot; this was achieved by making an incision in the gel with a razor blade. A membrane slot with 100 ng restriction cut and membrane bound DNA was placed in the enlarged well. An electric potential was applied at constant voltage of 200 V for 15 minutes. The membrane fragment was removed and the gel stained briefly with ethidium bromide staining solution, after which an image was captured using the FMBIO® II. The membrane fragment was returned to the gel slot and a constant current was then

applied at 100 watts for 10 minutes, after which the membrane fragment was removed and a second image captured using the FMBIO® II. Images were captured using post-amp product quantification parameters.

6.2.5 STR Typing of DNA from Archived RFLP Membranes

Experiments outlined in this section describe the final culmination of method refinement and application of enhancement techniques to archived RFLP membranes. Tests conducted up to this point made use of laboratory test fragments of various types of nylon bound to either HMW or RD DNA prepared to simulate stored RFLP membranes. One main challenge which differentiates the simulated test fragments and true RFLP membranes is that the DNA is bound along a greater surface area, while the simulated fragments bound the DNA in a highly localized region. The test fragments were preferred for method comparison in that they presented ease of use and allowed for better extraction comparison due to the high degree of standardization between test fragments. Membrane lanes have much greater potential for variability between specimens and also require more manipulation (cutting/dicing) prior to extraction buffer treatment.

This section describes final application of the most optimal extraction procedure determined up to this point. The first section focuses on the information available for the historical sample membranes which were available for these tests followed by a description of how the membranes were processed for attempted STR profiling.

6.2.5.1 Archived Membrane Test Material

One archived RFLP membrane was obtained from Paternity Testing Corporation, Columbia, Missouri, USA. The membrane was prepared on June 4, 2003 and had been stored frozen in plastic wrap following the completion of six successive single-locus chemiluminescent probings. The PTC procedure relied upon fixation using Biodyne® B nylon membrane with baking; UV cross-linking was not employed. The membrane was accompanied by an autolume for orientation purposes, on which ten strong profile lanes were apparent [Beckwith, 2006]. A second archived RFLP membrane was obtained from Sedgwick County Regional Forensic Science Center,

Wichita, Kansas, USA. The membrane was prepared on January 21, 2000 and had been stored frozen between blotting pads following the completion of four multi-locus probings. The RFSC procedure relied upon fixation using Biodyne® A nylon membrane with baking and UV cross-linking. The membrane was also accompanied by an autolume. A third exhibit was obtained from the Kansas City Police Laboratory. The date of preparation was January 6, 1998. The exact membrane variety was unknown, however it was verified that *Hae*III was the restriction enzyme used, UV cross-linking was employed for binding, the membrane underwent four single locus p-32 labeled probings, and the samples present on the membrane were known volunteers with established STR profiles. This membrane had been stored frozen in plastic wrap and was supplied with an accompanying reprint of the autolume [Hummel, 2009]. These exhibits were considered typical of archived RFLP casework and were used for the archived membrane recovery tests herein. Availability of these test membranes is extremely limited, therefore lane excision was conservative in order to preserve samples for possible future testing.

6.2.5.2 Processing of Archived RFLP Membranes

Two lanes with visible profiles present on the autolume were excised from the KCPD and PTC membranes with a clean razor, while a single lane was excised from the RFSC membrane. In general, RFSC and KCPD archived membrane lanes were approximately 5 x 150 mm and PTC lanes were approximately 5 x 180 mm. Cuttings were rehydrated for 5 minutes in 2.0M Tris:2XSSC, followed by two brief rinses with NFW. Lane cuttings were then dried, diced, placed in microcentrifuge tubes, and treated with the SEB according to methods described in section 6.2.3.4. A reagent negative control was extracted alongside each membrane type (KCPD-RNC, RFSC-RNC, and PTC-RNC). Extraction volumes were increased to account for substrate volume (600 µL of SEB with 15 µL 20 mg/µL proK per sample). All were incubated overnight at 56^{o} C. Cuttings were not removed prior to addition of PCI, which was added to each sample in a volume of 750 µL. Tubes were vortexed briefly and centrifuged to achieve phase separation (5 minutes at 15,000 RPM). Aqueous phases then underwent purification and concentration with Microcon®. Samples were brought to a final volume of 15 µL in NFW, and membrane lane samples and all

reagent negative controls were amplified in full using the PowerPlex® 16 and fragments were separated and detected using the ABI PRISM® 3100-*Avant* platform. Appropriate positive and negative amplification controls (PAC and NAC) accompanied amplification of the membrane samples and reagent negative controls.

6.3 Membrane Recovery Results and Discussion

The results and discussion for this chapter are presented in the same order as the description of methods and has been numerically organized to coordinate with the Methods outlined in this chapter (section 6.2).

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6.3.1 Sample Preparation and Feasibility Studies

While preparatory in nature, results presented in this section are critical in assuring that downstream membrane recovery tests are based on sound experimental techniques. The extraction, digestion, and membrane fixation of DNA samples were key in the preparation of experimental components used to test the various recovery methods. Therefore, performance checkpoints for these assay components must first be presented.

6.3.1.1 *Hae*III Restriction Digestion

Dried blood stains underwent standard organic extraction followed by Microcon® purification and concentration. Extracts were then evaluated and quantified using agarose gel assessment and the QuantiBlot® Human DNA Quantitation Kit. Extracts of standard cuttings (single drops dried on cotton cloth) generally resulted in yields of approximately 15 ng/µl. The agarose gel and blot obtained from a standard blood stain extraction are presented in Figures 6.5 and 6.6.

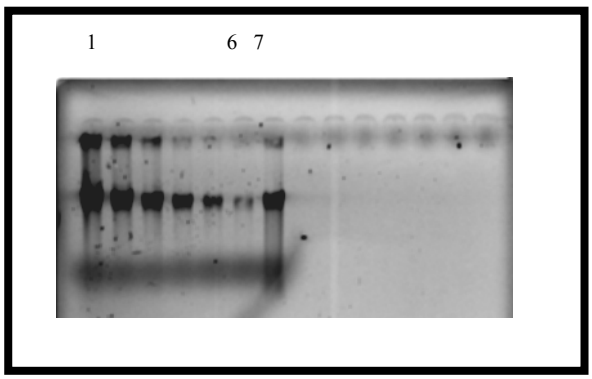

Figure 6.5: Product gel (1%) of Microcon®-purified products of DNA from dried blood. Lanes 1-6 contain the quantification standard series in the nanogram quantities of 200, 100, 50, 25, 10, and 5 ng of DNA. Lane 7 contains 1 µL of the female volunteer's DNA extract.

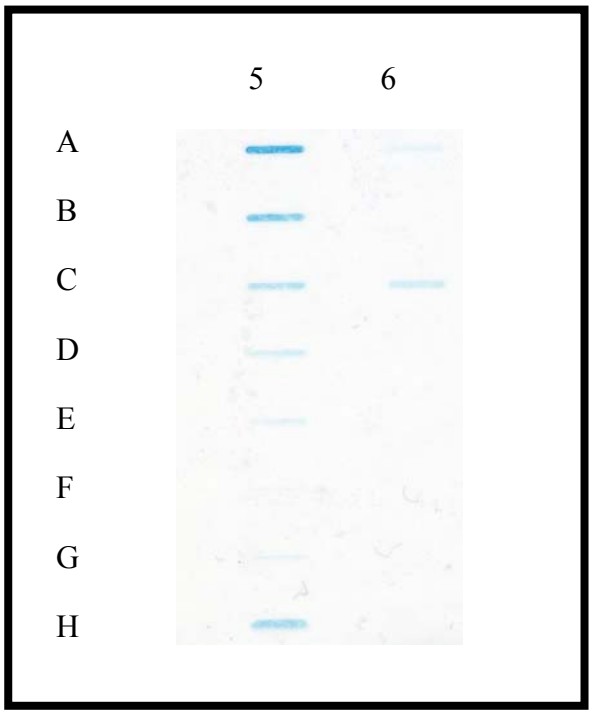

Figure 6.6: Colorimetric detection of Microcon®-purified products of DNA from dried blood. Column 5 (A through G) contains the quantification standard series in the following nanogram quantities (top to bottom): 10, 5, 2.5, 1.25, 0.63, 0.32, and 0.15 ng. Well 6D contains a 1:49 dilution of the female volunteer's DNA extract; calibrators representing 3.5 and 0.5 ng of DNA were loaded in wells 5H and 6A, respectively. Sample wells 6B and 6C are not related to this study.

High molecular weight DNA was incorporated into restriction enzyme (RE) reactions to produce digested DNA for subsequent amplification binding/cross-linking to nylon

membrane. Digestion was carried out as described in section 6.2.1.1 and digests containing 100 and 50 ng of template DNA performed in 20 μL reactions were checked for completeness using a 1% agarose gel; see Figure 6.7 for the gel image. Results obtained in Figure 6.7 verified that restriction digestion had taken place.

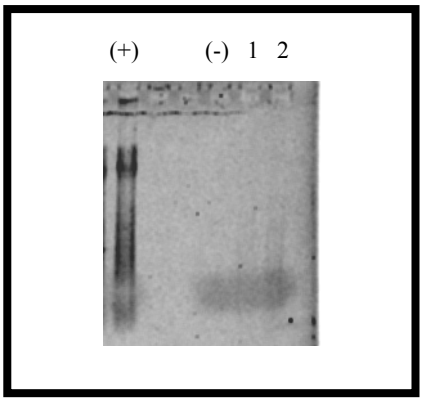

Figure 6.7: Product gel (1%) verification of restriction digestion reactions. Lane (+) contains HMW DNA extracted from a known reference sample (buccal swab). (-) indicates the negative restriction digest control (no template DNA); lanes marked 1 and 2 contain digest products of the 50 and 100 ng restriction digest reactions, respectively. A volume of 2 μL (~10% of each reaction) was loaded for all restriction digest samples.

6.3.1.2 Multiplex Amplification of Restriction Digested Sample

While the amount of DNA included in the restriction digest was established, QuantiBlot® Human DNA Quantitation was conducted for the samples following the restriction digestion in an effort to gain some understanding about how the quantification method performs for the detection of degraded DNA. Based on the HMW input DNA quantification data, 5% of the 100 ng sample analyzed on slot blot would contain 5 ng DNA. However, the band intensity on the blot conducted post-digestion was consistent with approximately 1.5 ng of DNA, indicating that some quantity of degraded DNA was likely not detected by this method. As a conservative measure, 1.5 ng of DNA was incorporated into the PCR reaction, as quantified by slot blot. The profile, comprising Figure 6.8, was analyzed at a 1X and 0.5X loading volume.

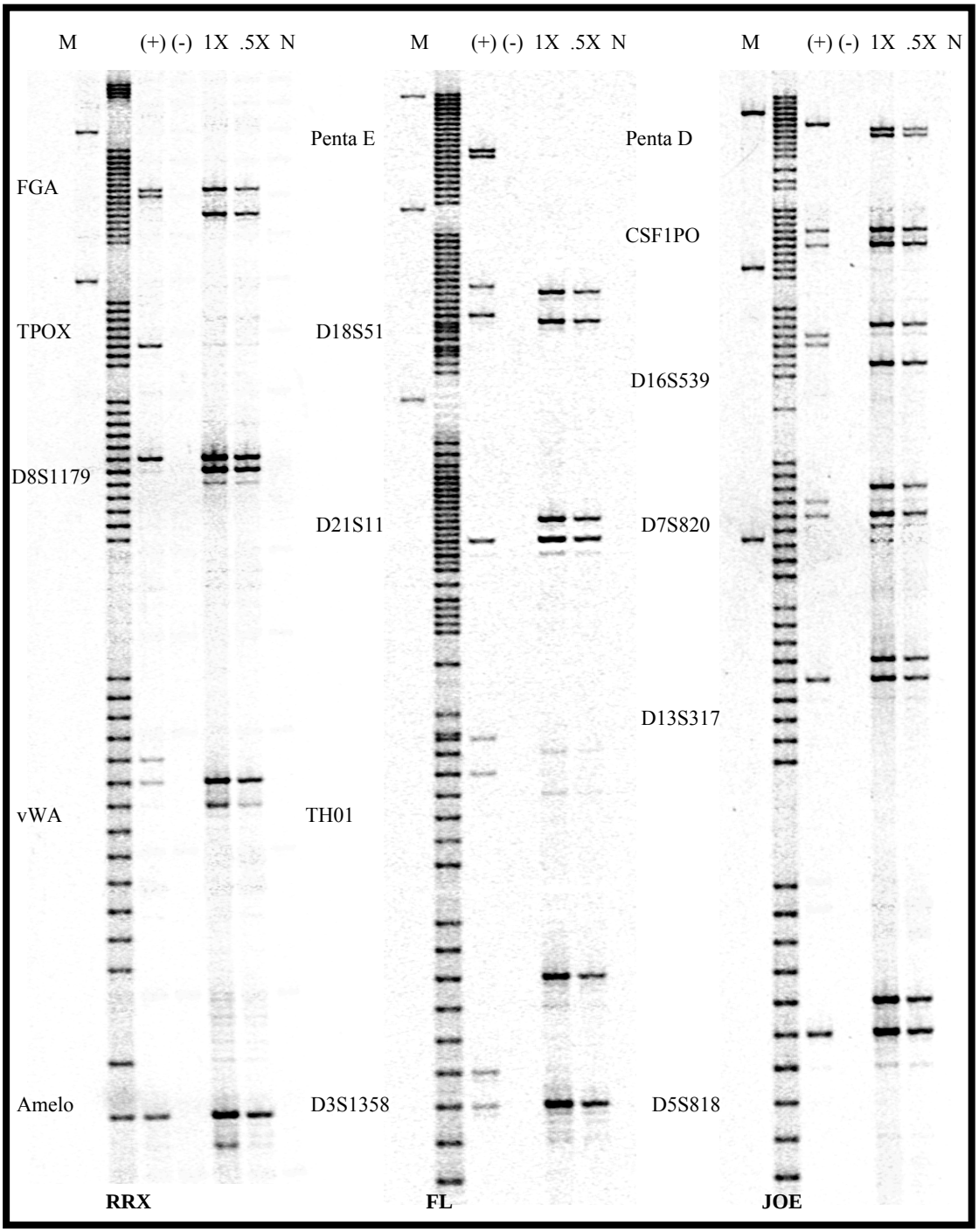

Figure 6.8: PowerPlex® 16 BIO amplification of *Hae*III restriction digested DNA. Image color separation was optimized for detection of Rhodamine red ™ (RRX), fluorescein (FL), and 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE)-labeled products, with locus designations indicated to the left of the corresponding ladders. The amplified product (digested female volunteer DNA extract serving as template) was examined at 1X and 0.5X loading volumes, as indicated above the corresponding lanes. Negative controls were analyzed at 1X loading volume. (+)=positive amplification control (9947A); (-)=negative amplification control; N=negative restriction digest control; M=color separation matrix.

The 0.5X loading volume was more suitable for analysis giving a full profile at all loci except Penta E and TPOX. This can be accounted for if there is a *Hae*III cut site present in the primer binding sites or in the sequences between the priming sites and the repeat stretches for these loci. Profile intensity was also severely diminished at TH01, which supports the presence of a restriction recognition site; minor amplification may have been possible due to incomplete restriction enzyme cutting of template DNA. Nevertheless, these tests indicate that restriction-cut DNA can successfully be analyzed using STR technology. While cut sites may interfere with the ability to type all loci, a sufficient quantity of loci were still highly detectable, and typing results may be expected from as many as 13 or 14 loci using this amplification typing kit. Moreover, these results support predicted product loss based on sequence analysis of target amplification regions for these primers.

As for the PowerPlex® 16 products analyzed using capillary electrophoresis, results were very similar and encouraging. Each quantity of DNA was amplified in duplicate to gain a better understanding of any stochastic effects that may take place in limited template amplifications. The resulting allele calls for the 20, 50, 100, and 200 pg amplification reactions are summarized in Table 6.5 Amplification controls performed to expectation; a negative digest control was not included with the capillary studies since these reagents were quality checked using the gel platform.

	20pg-1	20pg-2	50pg-1/2 100 pg-1/2 200pg-2	200pg-1	Expected DNA Profile
D3S1358			16,18	16,18	16,18
TH01					
D21S11	28,30	30	28,30	28,30	28,30
D18S51	12,15		12,15	12,15	12,15
Penta E					
D5S818	9,11	9,11	9,11	9,11	9,11
D13S317	9		9,10	9,10	9,10
D7S820	8,11	11	8,11	8,11	8,11
D16S539	11,12	11	11,12	11,12	11,12
CSF1PO	10,12	10	10,12	10,12	10,12
Penta D	10,12	10,12	10,12	10,12	10,12
Amelo	X,Y	X,Y	X,Y	X,Y	X,Y
vWA	16	16	16	16	16
D8S1179	13,14	14	13,14	13,14	13,14
TPOX				8	
FGA	19	19,22	19,22	19,22	19,22
Total Alleles Detected	21	14	25	26	25
Percent Alleles Detected	84%	56%	100%	104%	100%

Table 6.5: PowerPlex® 16 profiles obtained from different concentrations of restriction cut template. Allele calls are reflected for various concentrations of DNA that were restriction digested prior to STR multiplex amplification. Post-digest quantification indicated that reactions received 20, 50, 100, and 200 pg of DNA, each template quantity was amplified in duplicate (denoted as -1 and -2 for each quantity indicated). An 8 was detected at TPOX for one of the 200 pg samples; the allele is expected to drop-out due to *Hae*III restriction site in amplicon region; however the allele is consistent with the DNA source, indicating partial digestion likely took place.

Again, while cut sites may interfere with the ability to type all loci, a sufficient quantity of loci were still highly detectable, and typing results may be expected from as many as 13 or 14 loci using this amplification typing kit. While it is expected that TH01, TPOX, and PentaE will not amplify, partial digestion by *Hae*III may leave intact template for STR typing. Restriction enzyme efficiency can be affected by many factors, including buffer used during the digestion reaction. While inhibitors and other components in the reaction can also have a detrimental affect on any enzyme efficiency, the problems associated with partial digestion by *Hae*III, especially as the issue relates to RFLP analysis, has been well documented [Benzinger, *et. al.*, 1997; Duewer and Benzinger, 1997]. Therefore detection of alleles at these loci may be observed, but profile results from both platforms did

confirm complete loss or severe intensity decreases at these loci. For example, the 8 detected at TPOX from the 200-1 sample was 104 RFU, while other loci in this panel (Amelogenin, vWA, D8S1179, and FGA) exhibited heterozygote alleles from 1192-2338 RFU and a homozygotic peak height of 2836 at vWA. Therefore, these results do support predicted product loss based on sequence analysis of target amplification regions for these primers.

6.3.1.3 Binding and Cross-linking of DNA to Nylon Membrane

Future studies were to be designed such that DNA samples, either HMW or restriction digested, would be transferred to localized regions of a membrane using the Convertible® Filtration Manifold System in defined areas (slots) that could be excised for subsequent extraction from the static matrix. This binding and fixation procedure was verified prior to fragment/extraction comparison studies and was conducted as described in section 6.2.1.4, with fixation by baking and UV irradiation. Binding efficiency using this process was initially verified by methylene blue staining of a 10 ng HMW band which is illustrated in Figure 6.9. Lower concentrations of DNA were verified using the QuantiBlot® Human DNA Quantitation method, beginning with membrane hybridization; results are illustrated in Figure 6.10.

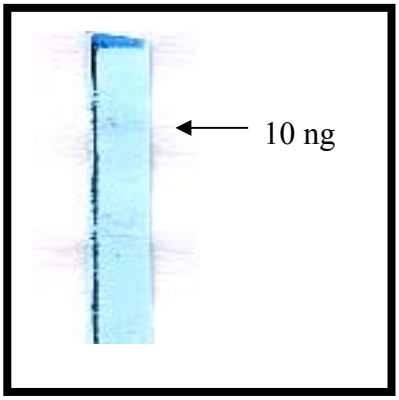

Figure 6.9: Methylene blue staining verification of membrane-bound DNA. High molecular weight DNA was bound and cross-linked to nylon membrane using the Convertible® Filtration Manifold System fitted with the slotted gasket and cover. A section was stained with methylene blue to verify the presence of bound DNA. The 10 ng band indicated with the arrow was visible following destaining in water.

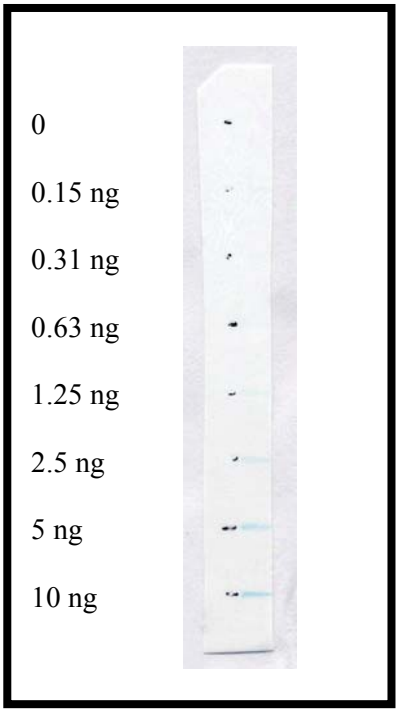

Figure 6.10: QuantiBlot® Human DNA Quantitation verification of membrane-bound DNA. High molecular weight DNA was bound and cross-linked to nylon membrane using the Convertible® Filtration Manifold System fitted with the slotted gasket and cover. A section underwent QuantiBlot® Human DNA Quantitation to verify the presence of bound DNA. Nanogram quantities of bound HMW DNA are indicated to the left of each loading slot; bands were visible at a sensitivity range of approximately 0.5 ng.

Methylene blue staining was first conducted and did result in the clear visualization of the 10 ng HMW band; the 5 ng band was also visible immediately following destain, but was less detectable upon digital documentation of the test results as is apparent by Figure 6.9. Therefore the QuantiBlot® Human DNA Quantitation method was employed in an attempt to document lower DNA concentrations. As shown in Figure 6.10, banding was detectable in the 0.5 ng range, indicating that DNA was sufficiently cross-linked to the membrane. Since recovery tests were generally conducted on membrane slots bound to 50 ng of DNA, achieving greater blot sensitivity was not pursued. These binding verification tests show that localized DNA binding could be achieved. Since the membrane binding procedure used was adopted from the RFLP protocol previously used by the laboratory, membrane slots of localized binding were considered valid test subjects for recovery experiments. Should a recovery method for bound HMW membrane slots be feasible, slots could then be prepared with bound restriction digested DNA for additional recovery protocol optimization. This

approach conserves precious RFLP membranes archived in storage and standardizes the recovery sample and substrate between tests; such standardization could not be expected from RFLP membranes where information regarding the quantity/quality of DNA present or long term storage conditions may not be readily available.

6.3.2 Recovery of HMW Membrane-Bound DNA

The results presented and discussed in this section reflect the DNA recovery approaches based on alkaline, acid, organic, and buffered extraction methods which were tested and compared on HMW samples of DNA bound to localized areas of nylon fragments.

6.3.2.1 Recovery of HMW Membrane-Bound DNA using Alkaline Extraction

The first alkaline recovery method tested was one that employed heat and a strong stripping solution. The stripping method was compared to control assays performed with heat and TE. Single membrane cuttings bound to 10 ng HMW DNA were treated with 200 μL strong strip solution or 200 μL TE; aliquots of 10 ng K562 DNA in aqueous solution were also treated with 200 μL strong strip solution or 200 μL TE. Samples were processed as described in section 6.2.2.1, and quantified using the QuantiBlot® Human DNA Quantitation system. Quantification results are illustrated in Figure 6.11.

Figure 6.11: Colorimetric detection of heat recovered products using strong strip and TE. Column 2 (A through G) contains the quantification standard series in the following nanogram quantities (top to bottom): 10, 5, 2.5, 1.25, 0.63, 0.32, and 0.15 ng. Column 6 contains 4 µL each of the alkaline recovered products. Well 6A contains product from membrane slot processed with strong strip solution; well 6B contains product from membrane slot processed with TE; wells 6C and 6D contain products from aqueous DNA samples processed with strong strip solution and TE, respectively. Product was recovered only from the aqueous DNA sample processed with TE (6D). Calibrators representing 3.5 and 0.5 ng of DNA were loaded in 2H and 3A, respectively. Other samples on the blot are unrelated to this study.

Results presented in Figure 6.11 represent data obtained following recovery attempts made to membrane slots bound to 10 ng HMW DNA when treated with strong strip solution or TE. These samples were also further compared to a positive control consisting of 10 ng K562 DNA aqueous solution aliquots that underwent treatment and processing in strong strip solution or TE. Product was detected only from the aqueous DNA sample processed with TE, and the band on the blot was estimated at 0.5 ng. Since 4 µL (20%) of the eluate was loaded, the sample was quantified at 0.125 ng/µL.

No product was observed from the aqueous positive control sample treated with strong strip, indicating that either capture or recovery were not effective when using this method. This may be expected given that the Microcon® devices used to

concentrate the samples are dependent upon membrane capture. Given that the control sample (aqueous K562 aliquot) treated with the strip solution did not result in detectable recovery, the eluate captured from the membrane slot processed with strong strip solution was not analyzed further. The eluate obtained from the membrane slot processed in TE using heat was amplified in full to determine if heat alone could serve to remove a sufficient quantity of typable DNA. Only two alleles were detected upon scanning with the FMBIO®II, as indicated in Figure 6.12. The markers detected consisted of a 13 at D8S1179 and a 17 at vWA, both of which are consistent with the profile of the source DNA.

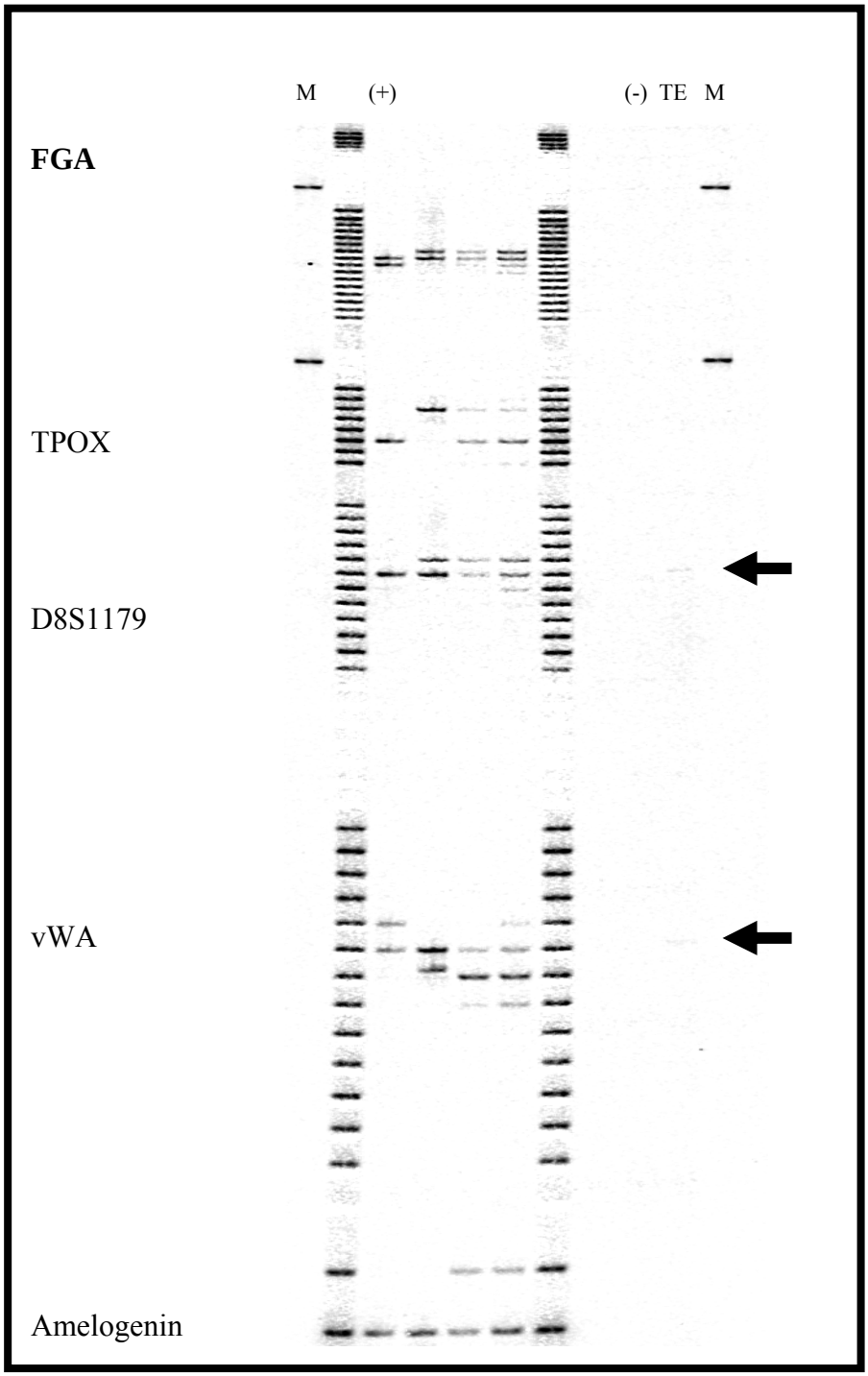

Figure 6.12: FMBIO® II detection of heat+TE recovered product using strong strip and TE following amplification with PowerPlex® 16 BIO. Rhodamine red™-X loci are illustrated, with locus designations indicated next to the corresponding ladders. Arrows indicate markers at D8S1179 (13) and vWA (17) detected following amplification of the sample recovered with heat in TE (lane denoted "TE"). Other samples present on this gel are unrelated to this study. (+)=positive amplification control (9947A); (-)=negative amplification control; M=color separation matrix.

These results indicated that heat may serve to impart some dissociation between the DNA and membrane, but alone could not serve to recover sufficient DNA to be amplified. Considering that the heat-treated aqueous control sample resulted in a recovery of 0.125 ng/μL when 20% of the recovered product was quantified, this means that 0.625 ng of the 10 ng of DNA initially introduced to the experiment was recovered. Therefore, the process itself resulted in a product loss exceeding 93%. Even if the strip treated sample had been recoverable using Microcon® capture, it is likely that product loss would have been even greater than that observed with the application of heat alone. Therefore, it was determined that purification/concentration following processes involving high alkalinity and/or an excess concentration of detergent may be better achieved with ethanol precipitation and that the application of excessive heat alone may not result in efficient removal of bound DNA.

A second alkaline stripping, a dual step alkaline stripping process (dual step strip or "DSS"), was also tested on 50 ng samples of HMW K562 DNA, one sample was UV cross-linked to membrane (DSS-A), one was in aqueous solution (DSS-B), one was in aqueous solution containing a small piece of nylon membrane (DSS-C). These three samples were treated with the dual step strip solution as described in 6.3.2.1. The combined washes underwent DNA isolation, were concentrated to a volume of 10 μL in TE, and quantified using 2% agarose gel and the QuantiBlot® Human DNA Quantitation system. Results obtained from the product gel quantification are presented in Figure 6.13.

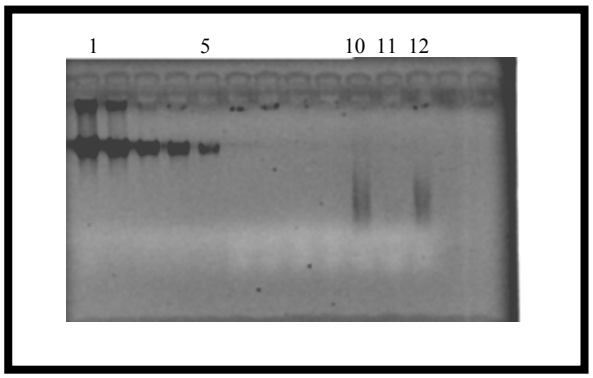

Figure 6.13: Product gel (2%) following dual strip solution (DSS) assays. Lanes 1-5 contain the quantification standard series in the nanogram quantities of 50, 25, 10, 5, and 2.5 ng of DNA. Lane 10 contains 4 μL of DSS-B (DNA in aqueous solution), lane 11 contains 4 μL DSS-A (cross-linked), and lane 12 contains 4 μL of DSS-C (DNA in aqueous solution with membrane fragment).

Samples designated B and C did yield detectable recovery products, as indicated by smears in wells 10 and 12 of Figure 6.13. However, the sample that was membrane bound did not (A); as no stained product was observed in well 11 of Figure 6.13. While both aqueous samples tested did indicate recovered product, it appeared degraded with little signal in the HMW range.

An absence of signal was also noted upon human quantification of DSS-A (slot blot data not shown), therefore all the remaining eluate was amplified. DSS-B and DSS-C were amplified in quantities based on slot blot quantification (1 and 2 μL, respectively). Based on profiles presented in Figures 6.14 and 6.15 (lanes A-C), partial or full profiles resulted from each sample. The sample containing aqueous K562 DNA without membrane cuttings gave a complete profile, although sister alleles did exhibit noticeable heterozygote imbalance at FGA (7.8%), TPOX (48.3%), and Penta E (32.8%). The sample of aqueous K562 with membrane cuttings performed similarly, except that one allele at Penta E was not fully detected, and imbalance at FGA (2.8%) and TPOX (40.2%) was slightly more exaggerated. The membrane bound sample, DSS-A, exhibited total drop-out at five loci (TPOX, PentaE, D18S51, D21S11, and TH01) and only one allele was detected at FGA. K562 exhibits a homozygotic profile at the remaining loci (D8S1179, vWA, and D3S1358).

The DSS assays, therefore, did result in apparent removal of some bound DNA. However, the amount of information obtained was limited to markers for four out of the nine system loci. While the presence of membrane fragments did not seem to have a detrimental affect on the process overall, the process itself may cause some damage to longer fragments of DNA as indicated by allelic imbalance or drop-out of more lengthy system amplicons. It was determined that further removal systems would be tested prior to the application of the DSS process to archived RFLP membranes.

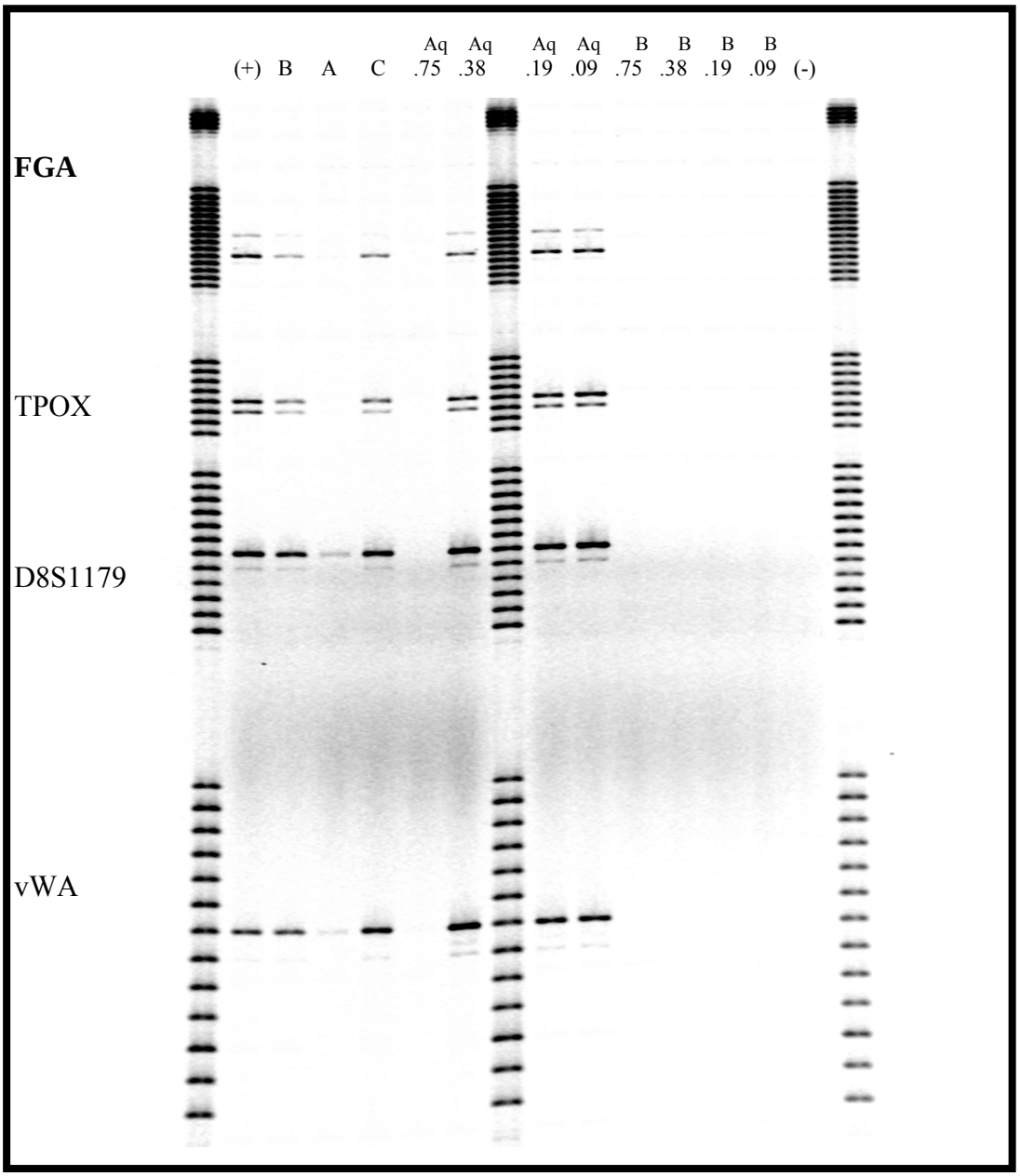

Figure 6.14: PowerPlex® 2.1 amplified dual strip solution and acid recovered products-carboxy-tetramethylrhodamine™(TMR) loci. Image color separation was optimized for TMR-labeled products, with locus designations indicated next to the corresponding ladders. Lanes containing DSS recovered products are labeled B, A, and C. Lanes containing acid-recovered products from aqueous DNA samples are denoted with "Aq" and the corresponding molarity of acid used during processing. Lanes containing acid-recovered products from membrane-bound DNA samples are denoted with "B" and the corresponding molarity of acid used during processing. (-)=negative amplification control with no bands detected, (+)=positive amplification control (K562).

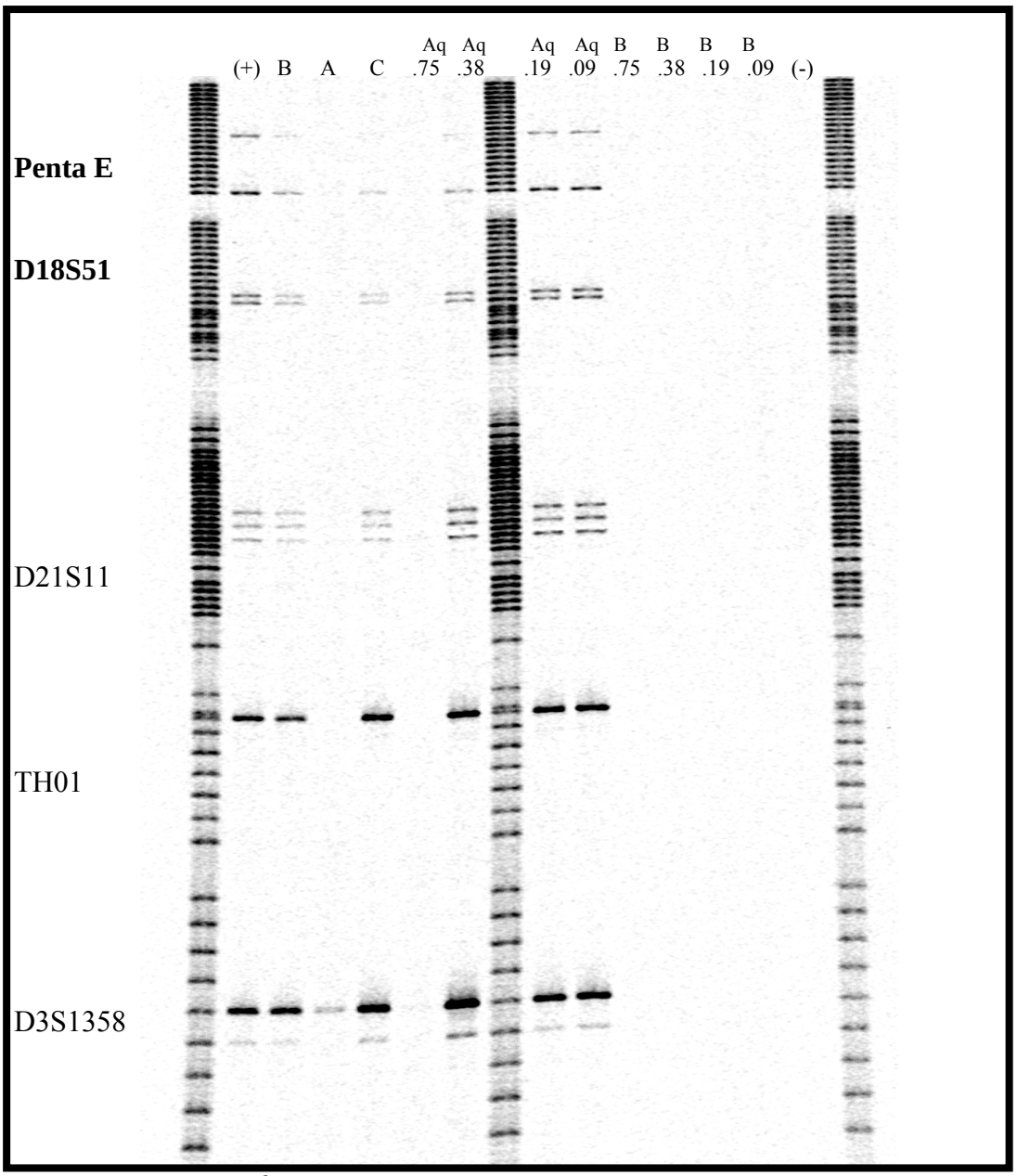

Figure 6.15: PowerPlex® 2.1 amplified dual strip solution and acid recovered products-fluorescein (FL) loci. Image color separation was optimized for FL-labeled products, with locus designations indicated next to the corresponding ladders. Lanes containing DSS recovered products are labeled B, A, and C. Lanes containing acid-recovered products from aqueous DNA samples are denoted with "Aq" and the corresponding molarity of acid used during processing. Lanes containing acid-recovered products from membrane-bound DNA samples are denoted with "B" and the corresponding molarity of acid used during processing. (-)=negative amplification control with no bands detected, (+)=positive amplification control (K562).

6.3.2.2 Recovery of HMW Membrane-Bound DNA using Acidic Extraction

The technical support group at Pall Corporation advised for the use of acid to disrupt the properties of the membrane itself (the cast nylon). This was meant to induce dissociation of interactions by detriment to the membrane properties even though it would also damage the DNA. Nevertheless, acid treatment was tested for disruption of the 6,6 polymer at the advice of the manufacturer. Experiments were set forth in an attempt to achieve an acid concentration suitable for disrupting the nylon but dilute enough such that damage to the DNA would be minimal. Initial acid experimentation consisted of bound and unbound HMW DNA samples (50 ng HMW K562), each treated with 100 μL of one of seven different concentrations of acid (6.0, 3.0, 1.5, 0.75, 0.38, 0.19, 0.09M); this method is described in section 6.2.2.2. These were purified/concentrated with Microcon® centrifugal devices and a portion of each 10 μL eluate was placed on the product gel presented in lanes 6-12 of Figure 6.16. Sample tubes containing 50 ng K562 DNA in aqueous solution were also subjected to each of the acid treatments and processed alongside the membrane assays following the initial incubation and centrifugation steps. These initial assays were purified/concentrated with Microcon® concentrators and DNA was eluted from each device with TE into a final volume of 10 μL. The samples were visualized on a product gel, presented in lanes 13-19 of Figure 6.16.

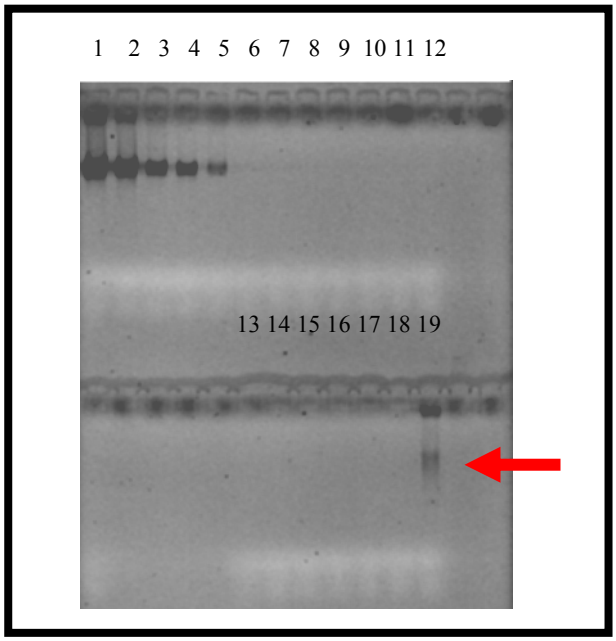

Figure 6.16: Product gel (2%) containing Microcon® concentrated products following acid-recovery of membrane bound and aqueous HMW DNA. Lanes 1 through 5 contain HMW quantification standards in the nanogram quantities of 50, 25, 10, 5, and 2.5 ng. Lanes 6 through 12 each contain 4 µl of the acid-treated membrane bound eluates (6.0M, 3.0M, 1.5M, 0.75M, 0.38M, 0.19M, 0.09M, respectively). Lanes 13 through 19 each contain 4 µl of the 50 ng K562 sample eluates that were treated with the same acid concentrations as the blot samples. Only the 0.09M positive control sample yielded any indication of product, indicated with the arrow.

Only the 0.09M acid-treated aqueous sample control resulted in detectable recovery. Following initial acid removal assays, it was noted that Microcon® concentration of acid-treated samples may not have resulted in efficient recovery, given that the Microcon® itself relies upon membrane/DNA interaction for product capture. Inadequate removal of acid was noted with greater DNA recovery in the lower acid concentration as well as the fact that the samples imparted a color change to the bromophenol blue loading dye used when loading product gel samples. Therefore, acid recovery tests were repeated for unbound HMW 50 ng aqueous control samples processed with the least concentrated acid solutions (0.09, 0.18, 0.38, and 0.75M). Upon repeating the assay, DNA capture was achieved by ethanol precipitation. Sample pellets were resuspended using 10 µL TE and evaluated for product using a 2% agarose gel (presented as Figure 6.17).

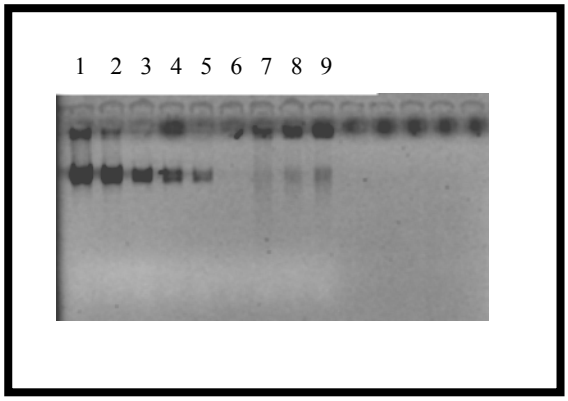

Figure 6.17: Product gel (2%) containing ethanol precipitated products following acid-recovery of aqueous HMW DNA. Lanes 1 through 5 contain the HMW standard series in nanogram quantities of 50, 25, 10, 5, and 2.5 ng. Lanes 6 through 9 each contain 4 µl of the acid-treated aqueous sample eluates (0.75M, 0.38M, 0.19M, 0.09M, respectively).

Based on the fact that agarose gel indicated product yield only in the 0.09M reaction following Microcon® purification, but ethanol precipitated products were apparent at 0.09, 0.18, 0.38, and 0.75M concentration, it was confirmed that acid likely interfered with the binding capacity of the Microcon® resulting in low DNA yield from unbound HMW samples. Therefore membrane-bound tests were repeated with 0.09, 0.18, 0.38, and 0.75M acid solutions using ethanol precipitation to recover any DNA product. Bound and unbound samples concentrated with ethanol precipitation could then be directly compared with QuantiBlot® Human DNA Quantitation and PowerPlex® 2.1 multi-plex PCR amplification. Following quantification, bound recovery product samples were amplified in full (6 µL). Recovery products from unbound samples were amplified according to slot blot quantification (data not shown); 0.75, 1.0, 6.0, and 6.0 µL of the unbound sample extracts were amplified from the 0.09, 0.18, 0.38, and 0.75M solution products, respectively. Amplified products are presented in Figures 6.14 and 6.15 (previously presented).

Figures 6.14 and 6.15 indicate that the three aqueous samples treated with the weakest acid dilutions did yield full STR profiles. Similar to the profile results of base-treated samples, heterozygote imbalance resulted at FGA (18.5-32.3%), TPOX (42.6-56.4%), and Penta E (24.0-43.4%) within the three resulting profiles. The aqueous sample treated with 0.75M acid resulted in no profile. Overall, quantification and STR typing

tests conducted following recovery of acid treated samples demonstrated the known principle that acid is damaging to DNA (Horton *et. al*., 2002). Higher concentrations of acid seemed to result in reduced yield from aqueous control samples. As for membrane-bound samples, STR profiles were not detected in any of the sample tests. This indicates that acid treatments in ranges that allow for DNA recovery are not useful for the dissociation of bound DNA from membrane slots. Therefore, further testing involving acid removal of bound DNA was not pursued.

6.3.2.3 Recovery of HMW Membrane-Bound DNA using Organic Chemicals

Given that acid/base tests conducted thus far had not resulted in effective removal of bound DNA from nylon membranes, it was determined that organic chemicals and solvents would be tested for their capability of dissolving or damaging the nylon matrix itself, allowing free DNA to then be recovered and typed. Membrane cuttings bound to 50 ng HMW K562 DNA were processed using ethanol, methanol, acetone, DMSO, chloroform, phenol, phenol:chloroform:isoamyl alcohol (PCI), or formamide as described in section 6.2.2.3. Following ethanol precipitation, all resulting products were quantified and evaluated using 2% agarose gel and the QuantiBlot® Human DNA Quantitation system. No product was observed for any of the samples on the blot, as indicated by the absence of signal in wells 4A through 4H in Figure 6.18. While it was noted that STR typing provides sensitivity superior to that achieved with slot blot, these results were obtained concurrently with those from similar assays using casework stain extraction buffer (later described). Since the alternative extraction buffer resulted in signal on the blot, the solvent-recovered samples were not examined further for STR typing suitability. Refer to Figure 6.18 for blot data obtained from the organic chemical recoveries.

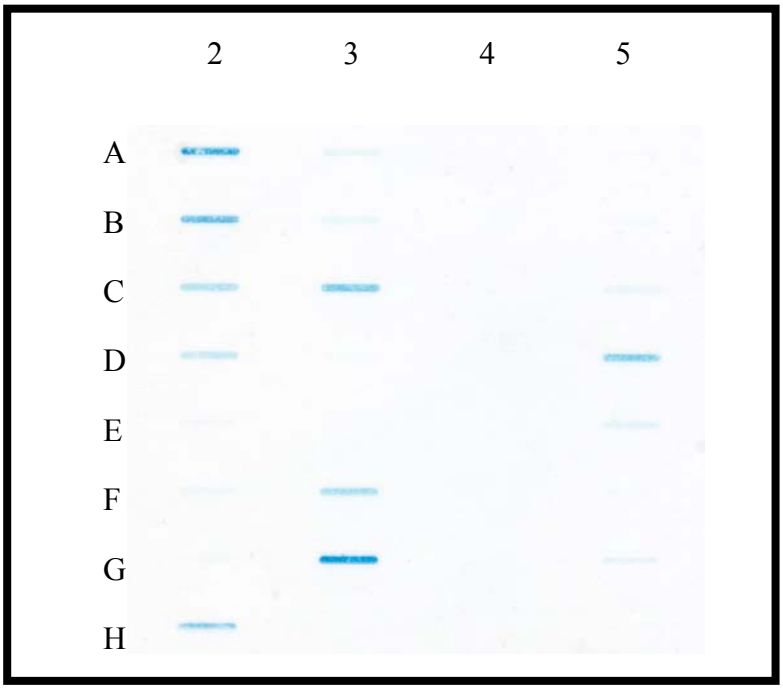

Figure 6.18: Colorimetric detection of membrane bound HMW DNA products using organic chemicals and forensic casework stain extraction buffer working solution (SEB). Column 2 (A through G) contains the quantification standard series in the following nanogram quantities (top to bottom): 10, 5, 2.5, 1.25, 0.63, 0.32, and 0.15 ng. Column 4 contains 1 µL each of the organic chemical recovered products (ethanol, methanol, acetone, DMSO, chloroform, phenol, PCI, and formamide extraction products were loaded in well 4A through 4H, respectively). Calibrators representing 3.5 and 0.5 ng of DNA were loaded in 2H and 3A, respectively. Other samples on the blot are unrelated to this study.

6.3.2.4 Recovery of HMW Membrane-Bound DNA using Casework Stain Extraction

Membrane cuttings bound to 50, 30, and 10 ng HMW DNA (K562) were processed with forensic casework stain extraction buffer working solution (SEB) as described in section 6.2.2.4. Following PCI/Microcon® purification and concentration, the samples were brought to a final volume of 16 µL and quantified using the QuantiBlot® Human DNA Quantitation system (data not shown). An advantage to this method is that it is conducive to the use of Microcon® devices for purification and concentration. All recovery products except that from the 10 ng sample were visible by colorimetric detection (data not shown). Samples were then amplified using the PowerPlex® 16 BIO multi -plex PCR amplification. For the amplifications, 2 µL of 50 ng extract, 4 µL of 30 ng extract, and 15 µL of 10 ng extract were incorporated into respective reactions. Resulting profiles are presented in Figure 6.19.

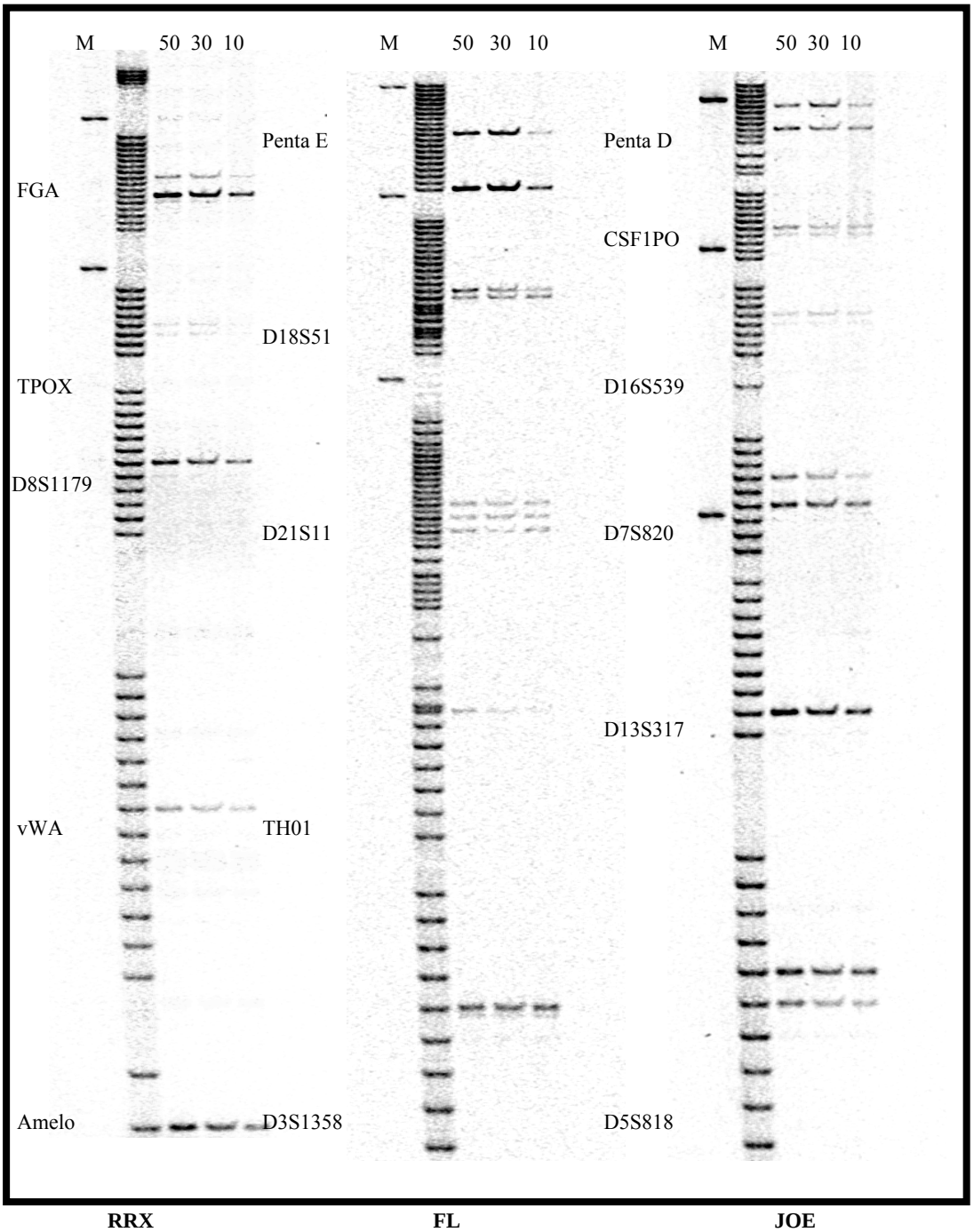

Figure 6.19: SEB-recovered HMW DNA products following amplification using PowerPlex® 16 BIO. Image color separation was optimized for detection of rhodamine red™ (RRX), fluorescein (FL), and 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE)-labeled products, with locus designations indicated to the left of the corresponding ladders. Nanogram quantities of membrane bound DNA extracted are indicated above respective lanes; all samples were loaded at 2X volume. Positive and negative amplification controls were run separately and performed to expectation (data not shown). M=color separation matrix.

Upon evaluation of profiles presented in Figure 6.19, it became apparent that this extraction method was the most successful of those attempted so far. Full profiles were detected from the 50 and 30 ng samples, and two loci dropped out within the profile of the 10 ng sample. While imbalance was noted at many of the loci detected, this extraction process marked the first to yield appreciable recovery of quantifiable and amplifiable product. Mean percent heterozygote ratios were calculated for the 50 and 30 ng samples (homozygotic and tri-allelic loci were not considered for the calculation), and the mean ratio across the nine heterozygote loci was 52.1% and 49.1%, respectively for the 50 ng and 30 ng samples. The mean imbalance for the 10 ng sample across the eight heterozygotic loci (TPOX dropped out), was 38.7%. While imbalance did occur, all markers detected were fully attributable to the source profile. It was also noted that some level of imbalance may be attributable to the cell line nature of the source inherently, as tissue culture lines are known to exhibit allele imbalance and other anomalies from generation to generation [Roy, *et. al.*, 2001].

Given this successful recovery, it was determined that casework extraction buffer methods would be more fully evaluated. The next step in the evaluation process would be to test this system and/or others similar for the ability to remove restriction digested samples bound to localized area of membrane.

6.3.3 Recovery of Restriction Digested Membrane-Bound DNA

6.3.3.1 Recovery of Restriction Digested Membrane-Bound DNA using Casework Stain Extraction

Extraction was performed on a membrane slot bound with restriction digested DNA as described in section 6.2.3.1 and brought to a final volume of 11 µL in NFW. No signal was observed on the QuantiBlot® Human DNA Quantitation blot (data not shown); therefore, the remainder of the sample (10 µL) was amplified using PowerPlex® 16 BIO multi-plex PCR amplification. The amplified sample and corresponding negative control are presented as Figure 6.20; the positive control was verified alongside casework (data not shown).

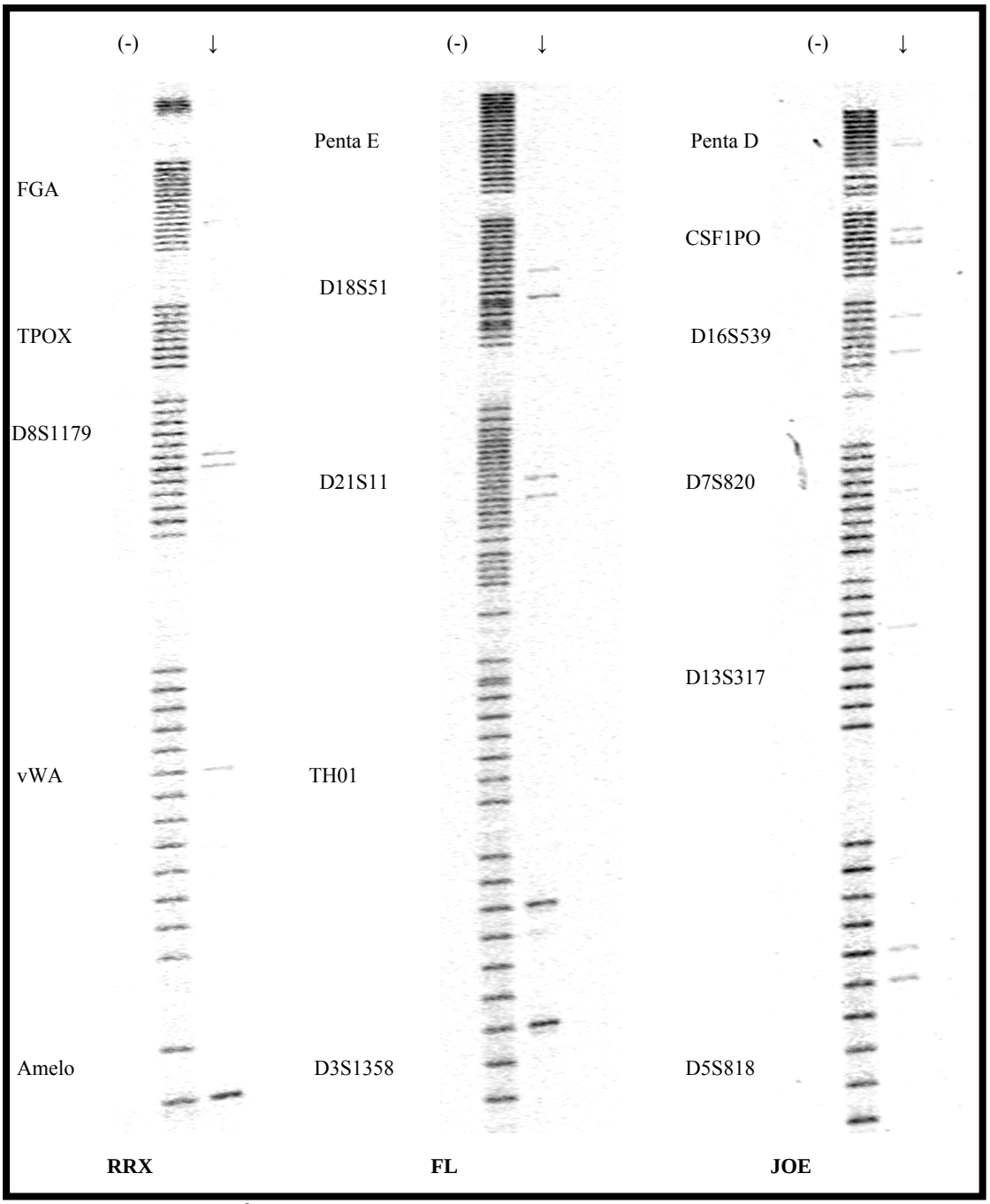

Figure 6.20: PowerPlex® 16 BIO amplification of *Hae*III restriction digested DNA bound to nylon membrane slot and recovered with SEB. Image color separation was optimized for detection of rhodamine red™ (RRX), fluorescein (FL), and 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE)-labeled products, with locus designations indicated to the left of the corresponding ladders. The amplified product (↓) was examined at 2X loading volume, as was the negative amplification control. (-)=negative amplification control.

Although no signal was observed on the slot blot, the remainder of the eluate did yield a partial profile when amplified with PowerPlex® 16 BIO. As shown in Figure 6.20, a substantial quantity of profile information resulted from this process. As expected from previous amplification of restricted DNA from this source, no profile was obtained from Penta E, TPOX, or TH01. Furthermore, no markers were detected at FGA. The inability to generate a profile at FGA may be attributable to a combination of restriction digestion and membrane recovery, resulting in poor amplification of these lengthy amplicons. Additionally, one marker at D13S317 was not sized due to the misshapen nature of the band. While the signal is weak in comparison to the heterozygote sister fully detected at this locus, the inability to fully designate this as a true allele was likely attributable to electrophoretic artifact inhibiting the distribution of label evenly across the lane throughout this portion of the gel. This occurrence was not considered drop-out since some signal was detectable. Regardless, the application of this method for recovery of membrane-bound DNA seemed promising since profile information was obtained across 12 of the 16 loci targeted with this kit. The profile information detected represented 21 of the possible 30 expected alleles, or 70% of the total genetic markers. D13S317 and the homozygotic loci for this DNA source were not considered for heterozygote imbalance calculations, however, the remaining nine loci were evaluated and resulted in a profile mean imbalance of 68.2%. This value exceeds that achieved with HMW recovery tests conducted on 50, 30, and 10 ng DNA samples. While this sample does represent recovery from a 100 ng quantity of bound DNA, this superior profile quality was somewhat surprising given the fact the bound DNA was restriction digested prior. It should be noted that 50-500 ng of DNA were generally bound throughout each RFLP sample lane, so the ability to type 12 loci from 100 ng of localized bound DNA did seem promising.

Upon review of SEB components, it was determined that this buffer consisted of two key components that were otherwise absent during previous assays: DTT and proteinase K. Therefore, it was determined that other common extraction buffers and solutions containing these two components in elevated quantities would be explored for recovery capability.

6.3.3.2 Recovery of Restriction Digested Membrane-Bound DNA using Modified Versions of Extraction Buffers

Modified sperm lysis buffer, modified SEB, modified Differex™ lysis buffer, and DTT+proK solution were each tested for the ability to remove restriction cut DNA bound to localized areas of membrane slots as described in section 6.2.3.2. One membrane slot was also scraped and diced prior to treatment with the modified Differex™ lysis buffer (referred to as scraping + Differex™ lysis buffer). Following PCI/Microcon® purification and concentration, eluates were brought to a final volume of 15 µL in NFW, and amplified in full using the PowerPlex®16 multi-plex PCR amplification system; appropriate amplification controls were in place. Alleles detected above a 50 RFU threshold for each extraction solution are reported in Table 6.6.

	modified sperm lysis buffer	modified SEB	modified Differex™ lysis buffer	DTT + proK	scraping+ modified Differex™ lysis buffer	Source DNA Profile
D3S1358	14,18	14,18	14,18		14,18	14,18
TH01		9	7,9		7,9	7,9
D21S11	30,31.2	30,31.2	30,31.2		30,31.2	30,31.2
D18S51						14,18
Penta E						12,23
D5S818	12	11,12	11,12		11,12	11,12
D13S317		11,12	11,12		11,12	11,12
D7S820		10	10,12		10,12	10,12
D16S539		9,13	9,13		9	9,13
CSF1PO	12	10,12	10,12		10,12	10,12
Penta D		10				10,11
Amelo	X	X	X		X	X
vWA	17	17	17		17	17
D8S1179	12,13	12,13	12,13		12,13	12,13
TPOX						8,11
FGA		24	20			20,24
Total Alleles Detected	10	20	21	0	19	30
Percent Alleles Detected	33.3%	66.7%	70%	0%	63.3%	100%

Table 6.6: PowerPlex® 16 profiles obtained from restriction cut/membrane bound slots subjected to extraction with modified casework extraction buffers. Profiles recovered from membrane slots extracted using modified sperm lysis buffer, modified SEB, modified Differex™ lysis buffer, DTT+proK solution, and scraping+Differex™ lysis buffer are reported here. The right column lists full profile information for the DNA source and the bottom two rows indicate total alleles detected and percent total alleles detected for each extraction system. Positive and negative amplification controls performed to specification.

Modified extraction buffers consisted of those commonly employed on casework stains, but with excessive amounts of proteinase K and DTT. This approach was taken since these chemicals are generally added to extractions to induce dissociations between DNA and proteins (i.e. interactions between membrane surface moieties and bound macromolecules). It was speculated that DTT may serve to release DNA from protamine-like interactions with the membrane surface chemistry, while the proteinase K may serve to digest protamines. The components of these buffers and extraction systems are routinely used to extract forensic samples, it has been established that these extraction processes are conducive to follow-up with

PCI/Microcon® purification, which generally produces higher yield than ethanol precipitation.

Based on the data summary presented in Table 6.6, the modified SEB and modified Differex™ lysis buffer resulted in recovery of DNA that provided the most allelic designations, with detection of 20 and 21 of the possible 30 markers, respectively. These results are comparable to those achieved using the SEB with standard amounts of DTT and proK, however, mean RFU values (profile intensity) and mean imbalance (profile quality) calculations cannot be directly compared between assays due to the fact the samples were run on different platforms; slab gel OD units cannot be compared to capillary RFU values and these two platforms exhibit different expected ranges for sister allelic imbalance. Also, modified buffers were tested using restricted samples, while the unmodified version was applied to HMW test strips. Nevertheless, the SEB gave the strongest overall profile, based on RFU values detected, and also exhibited a great deal of sister allelic balance, based on profile mean ratio calculations. Interestingly, the scraped sample did not yield more information than that extracted intact, indicating that this procedure is likely not conducive to better recovery. One must also consider the scraping and manipulation as potential opportunities for contamination entry and additional product loss. Therefore, scraping will not be employed when processing archived membranes. While the modified sperm lysis buffer resulted in a strong profile with respect to peak height, it recovered about half as much genetic information as the modified SEB and Differex™ lysis solutions.

It was noted that the data collected with this assay represent one sample for each extraction test and that further information may have been available had triplicate extractions been conducted for each modified system or solution tested. However, the data from these pilot studies does suggest that the modified SEB, and modified Differex™ lysis buffer perform similarly for recovery of bound DNA. Even though samples were typed using different platforms, modified SEB and unmodified SEB tests yielded very similar results, indicating some level of reproducibility and reliability for this recovery method overall.

6.3.3.3 Proteinase K and DTT Additive Extraction Yield Study

Quantification results obtained from blood stains extracted using conventional SEB and the DTT/proK-enhanced method (experimental method), were compiled. For the conventional extracts, the average values of each of the quadruplicate quantifications (duplicate loadings on each of two plates) for each sample are reported in Table 6.7. For the experimental extracts, quantifications were carried out in duplicate on one plate and are reported in Table 6.8. Quantifications of the negative extraction controls for each buffer resulted in undetermined quantities, as expected.

Quant Identifier	Stain 1-conventional (ng/uL)	Stain 2-conventional (ng/uL)	Stain 3-conventional (ng/uL)
081027ss1	9.35	12.56	12.11
081027ss1	10.26	12.58	11.68
081027ss2	9.12	13.02	11.86
081027ss2	9.36	13.45	12.37
sample yield mean	9.52	12.90	12.01

Table 6.7: Quadruplicate quantification values for stains extracted with conventional SEB. Quantification data obtained from 10 μL blood stains extracted using standard organic method is recorded. Average recovery for each sample, calculated from the four replicate quant wells, is expressed in the bottom row of the table. Quantifications of the negative extraction control for this buffer resulted in undetermined quantities, as expected.

Quant Identifier	Stain 1-experimental (ng/uL)	Stain 2-experimental (ng/uL)	Stain 3-experimental (ng/uL)
081031ss1	3.93	6.73	3.96
081031ss1	4.15	6.18	4.13
sample yield mean	4.04	6.45	4.05

Table 6.8: Duplicate quantification values for stains extracted with excess DTT and proK. Quantification data obtained from 10 μL blood stains extracted using standard organic method is recorded. Average recovery for each sample, calculated from the replicate quant wells, is expressed in the bottom row of the table. Quantifications of the negative extraction control for this buffer resulted in undetermined quantities, as expected.

Results from each study were then combined into a single table for direct method yield comparison. Table 6.9 expresses the overall method averages from triplicate samplings as well as the standard deviation between quantifications. For conventional measurements n=12; for the experimental group, n=6.

Method	Method Mean Yield (ng/uL)	Yield Standard Deviation (ng/uL)
Conventional (SEB)	11.5	1.54
Experimental (excess DTT+proK)	4.85	1.26

Table 6.9: Overall yields of SEB vs. Excess DTT/proK extractions. This table reports the average concentration of extracts resulting form extraction of stains using the conventional SEB and an experimental buffer with excess DTT and proK.

Based on this straightforward study, there does not seem to be an apparent advantage to the addition of excess DTT and proK to the extraction buffer prior to overnight extraction. In fact, the resulting quantifications were about 50% the concentration of those determined from conventional extracts. Therefore, increased levels of proK and DTT will not be used in subsequent recovery assays.

While DTT is important for disruption of cellular complexes via reduction of disulfide bonds in proteins, it is possible that at higher levels, the agent actually increases levels of DNA damage. It has been demonstrated that in the presence of metals, DTT induces oxidative damage to nucleic acids [Netto and Stadtman, 1996]. This has been shown in the presence of Ni (II) and Fe (III), lending the biomolecule open to free radical damage [Oikawa, *et. al*., 2002; Woldemariam and Mandal, 2008]. Therefore, higher levels of DTT in the extraction process may, depending on extract composition, be detrimental to DNA recovery. The test described herein certainly does not indicate that any advantage is gained, and while further testing may have been useful to test DTT and proK separately, the extraction buffer long used by the lab was initially optimized with specified levels of these components and will be used in the manner originally validated for casework for downstream recovery applications.

6.3.3.4 Recovery of Restriction Digested Membrane-Bound DNA using Casework Extraction Buffers

Results of this study are presented in two parts. The first study reflects a broad comparison of four major extraction buffers. From this, the best two were identified and subsequent comparison of those two comprise the second part of the study.

6.3.3.4a Four Buffer Comparison

MagnaGraph® and Biodyne® B nylon fragments extracted with casework stain extraction buffer working solution (SEB), sperm lysis buffer (SP), Differex™ extraction buffer (DFX), and the Maxwell® 16 LEV (MAX) were quantified in duplicate and each extract quantification was considered the average of the duplicate real time quantitation values obtained. The values for triplicate MagnaGraph® and Biodyne® extracts were compiled and subjected to analysis of variance; two extraction processes were selected for further testing (SEB and DFX) on five additional fragments of each nylon variety. Resulting extracts (fragments #4-8) were also quantified in duplicate and the resulting average values are compiled in Table 6.10, along with the initial triplicate fragment studies involving all four casework buffers.

Ext #	SEB-M (ng/µL)	DFX-M (ng/µL)	SP-M (ng/µL)	MAX-M (ng/µL)	SEB-B (ng/µL)	DFX-B (ng/µL)	SP-B (ng/µL)	MAX-B (ng/µL)
1	0.00185	0.00132	0.00062	ND	3.55	3.52	0.15700	0.00359
2	0.00668	0.00369	0.01685	ND	3.57	3.15	0.07075	0.00453
3	0.00305	0.00660	0.00090	ND	3.54	3.37	0.07480	0.00564
4	0.01970	0.03070			4.36	4.55		
5	0.00281	0.04190			4.28	4.12		
6	0.01030	0.00709			3.86	4.18		
7	0.12800	0.00154			4.20	4.32		
8	0.01230	0.04130			4.41	4.60		
Mean (x) 1-3	0.00386	0.00387	0.00612	ND	3.55	3.35	0.10085	0.00459
SD (σ) 1-3	0.00252	0.00265	0.00929	ND	0.0153	0.186	0.0487	0.00103
Mean (x) 1-8	0.0231	0.0167			3.972	3.976		
SD (σ) 1-8	0.0428	0.0180			0.382	0.555		

Table 6.10: Quantification data obtained from casework extraction buffers on MagnaGraph® and Biodyne® B membrane fragments bound with restriction-digested DNA. The table describes quantification results when restriction cut DNA was bound to MagnaGraph® (M) and Biodyne® (B) nylon membrane fragments and then extracted with stain extraction buffer working solution (SEB), sperm lysis buffer (SP), Differex™ extraction buffer (DFX), or using the Maxwell® 16 LEV (MAX). The first three fragments (blue) represent initial triplicate buffer studies. The most robust buffers, SEB and DFX were further tested on five fragments of each membrane type (bound with restriction-cut DNA), therefore duplicate quant averages are also reported for each of five additional fragments of each membrane type for these two buffer solutions. The mean and standard deviation for the triplicate study (indicated in blue where n=3) and all studies (indicated in black where n=8) are reported at the bottom of the table. The SP system reagent negative control (RNC) resulted in a minute quantification result of 0.00372 ng/µL, however amplification of this extract in full did not result in an STR profile. No quantification data was obtained from other negative control extracts therefore all negative controls were determined to perform to specification. ND=not determined.

The Maxwell® 16 LEV (MAX) groups clearly underperformed the other methods and, for the MagnaGraph® extracts, did not result in detectable quantification data. Because this method failed to recover detectable quantities of DNA from MagnaGraph® fragments, the method could not be included in statistical comparisons for that substrate. Regarding MagnaGraph® extracts, quantification data from the

other three buffers underwent analysis of variance (ANOVA) testing. Regarding Biodyne® B extracts, quantification data from all four methods were subjected to variance testing. From the extractions conducted from MagnaGraph® fragments using SEB, DFX and SP methods, results indicated that the various extraction methods employed did not impart significant differences in the amount of DNA recovered from membrane fragments, $F(2,6)=0.154$, $p>0.05$. However, from the extractions conducted from Biodyne® B fragments, results indicated that the amount of DNA recovered was significantly affected by the extraction method employed, $F(3,8)=1245.2$, $p<0.05$. Since there was no significant difference between methods when extracting from MagnaGraph®, further statistical comparisons of the triplicate extracts were not pursued for this substrate. However, for the Biodyne® B extraction quantitations, Tukey post hoc testing was carried out to compare all different combinations of the Biodyne® B group. Results indicated that significant differences did not exist between SEB and DFX buffer recovery (at 0.109, $p>0.05$) or between the MAX and SP procedures (at 0.631, $p>0.05$). However, there was a significant difference between SEB when compared to MAX /SP systems and DFX when compared to MAX /SP systems (at 0.000, $p<0.05$ upon all comparisons). All statistical evaluation was conducted using the Statistical Package for Social Sciences (SPSS), re-branded in 2009 as Predictive Analytics SoftWare, PASW [Field, 2005]. This data indicates that the sperm lysis buffer method (SP), while seemingly as effective on MagnaGraph® extractions, performed significantly worse than the SEB and DFX methods when tested on Biodyne® B-bound samples. Therefore, in an effort to isolate the best recovery method overall, the SP system was disregarded as a possible best method based on the failure to recover ample quantities of DNA from Biodyne® B membrane fragment. Statistically, no apparent difference was observed between the SEB to DFX regardless of nylon substrate. While the mean for the Biodyne® B membrane recovery was slightly higher using the DFX buffer, the opposite was true from the MagnaGraph® samples where a slightly elevated average was achieved with SEB. Since it is apparent that Biodyne® B extracts will present far less challenge for recovery, the limiting system seems to be recovery from MagnaGraph® nylon fragments. Therefore, if choosing a method, one may be inclined to draw focus to that which may optimize MagnaGraph® recovery, given

Biodyne® B recovery is likely to yield greater quantities of template overall. Post hoc analysis of the triplicate samplings from SEB and DFX did not indicate a significant difference, so additional sampling was pursued in an effort to conduct a thorough comparison of these two specific extraction methods. In Table 6.10, extracts #4-8 for each method indicate recovery quantities from either membrane type, and constitute the addition of five samples to each extraction method/substrate set. Based on a comparison of quantities of DNA recovered from MagnaGraph® with the SEB or DFX or the Biodyne® B using either SEB or DFX, t-test results indicate that no significant difference was observed when SEB was compared to DFX for each substrate type. In other words, observed recovery from Biodyne® B fragments was not significantly greater with the DFX buffer (M=3.976, SE=0.196) than with the SEB (M=3.972, SE=0.135), $t(8)= -0.018$, and $p>0.05$ at 0.986. Likewise for the MagnaGraph® comparison, recovery from was not significantly greater with the DFX buffer (M=0.0167, SE=0.00636) than with the SEB (M=0.0231, SE=0.0151) $t(8)=0.385$, $p>0.05$ at 0.706. All tests were conducted using SPSS software where equal variances were not assumed [Field, 2005].

The SP system reagent negative control (RNC) resulted in a minute quantification result of 0.00372 ng/μL, however amplification of this extract in full did not result in an STR profile. No quantification data or STR profile was obtained from other negative control extracts; therefore all negative controls were determined to perform to specification with respect to the triplicate fragment study.

Following the quantification, initial triplicate extracts were pooled for each extraction method. For MagnaGraph® extracts, the pooled retentates were divided in thirds and each portion consumed for amplification. For Biodyne® B extracts, the pooled retentate was diluted based on the average quantitation value of the three components, and an equal amount of the dilution was amplified three times. In order to further illustrate similarity between the SEB and DFX methods, the profiles obtained from the pooled extracts were analyzed for number of alleles detected and the peak heights within the resulting profiles. For Biodyne® B extracts, this analysis was not performed since both factors are related to the quantification from which dilutions

were prepared for pooled amplifications. However, for MagnaGraph® fragment groups, the pooled profile results should reflect extraction method comparison only with less variability due to template or amplification reaction differences. The results of the allele numbers and heights are summarized in Table 6.11.

Method-Fragment #	Number Alleles >50 RFU
SEB—1	3
SEB—2	3
SEB—3	2
Mean/SD (x/σ) alleles per sample for SEB (n=3)	2.7/0.58
Mean/SD (x/σ) peak height (RFU) for SEB (n=3)	67.3/17.6
DFX—1	4
DFX—2	3
DFX—3	4
Mean/SD (x/σ) alleles per sample for DFX (n=3)	3.7/0.58
Mean/SD (x/σ) peak height (RFU) for DFX (n=3)	71.4/28.8

Table 6.11: STR profile summary of restriction digested DNA from triplicate MagnaGraph® fragment membrane extracts pooled following recovery with SEB and DFX buffer systems. The number of alleles detected within each profile are indicated using a 50 RFU threshold. The table also reports the average values and standard deviation for number of alleles detected and average peak height and standard deviation of allele height for each method/fragment combination; raw data for peak heights is not included. Three fragments were extracted with each buffer type, separately quantified, and then pooled and consumed across three amplification reactions. n=3 for each group.

While 100 RFU analysis is generally preferable, the SEB extraction amplifications did not produce any peaks above 100 RFU. The DFX system yielded three peaks just slightly exceeding 100 RFU in two of the amplifications. Therefore, a 50 RFU threshold value was used in an effort to generate some data following these tests. At 100 RFU threshold, all Biodyne® B amplifications for both SEB and DFX pooled extracts performed as expected when amplified in triplicate. Data was detected at all loci except drop-out did occur at loci with *Hae*III restriction sites within the amplicon region. Where data was not lost, peak heights were severely reduced at the TH01, TPOX, and Penta E loci. When Biodyne® B pooled extract amplifications were analyzed at 50 RFU, one of the SEB amplifications resulted in the detection of a n+4 peak at D13S317. For Biodyne® B pooled extracts recovered using DFX, when profiles were analyzed down to 50 RFU, one amplification exhibited artifacts related to pull-up and shouldering. At all threshold levels, the positive and negative amplification controls performed to specification. Although the 50 RFU detection level can result in detection of artifacts, it was useful to gain an understanding of the recovery potential for these two buffers.

6.3.3.4b Two Buffer Comparison

Additional extractions were conducted on both nylon types using the SEB and DFX buffers, resulting in five more quantification data sets and amplified profiles for each method/membrane combination. For the last set of extractions (#4 through 8), each extraction was not only quantified individually, but was also amplified individually. In contrast to the triplicate fragment study initially conducted with all four extraction test methods, the latter comparison focusing on SEB vs. DFX was carried out with no pooling prior to amplification for either MagnaGraph® or Biodyne® B derived extracts. The five extracts from the two membrane types using SEB and DFX buffers were amplified individually in an effort to simulate archived membrane lanes, and number of alleles detected at 100 and 50 RFU from MagnaGraph® recoveries are reported in Table 6.12. All individually amplified extracts (non-pooled from the 5X replicate testing) Biodyne® B extracts resulting from either buffer system resulted in full profiles, with no data or severely diminished data at TPOX, TH01, and Penta E (as would be expected due to restriction enzyme cut sites within these amplicon

regions). Because peak height may be reliant on the quantification accuracy/precision and dilutions prepared for amplification, peak heights from Biodyne® B extract profiles were not considered critical for buffer selection. Rather, peak presence and intensity were very critical when comparing MagnaGraph® extract profiles and were more carefully considered. One fragment extracted using SEB (#4) indicated a quantity of DNA present upon real-time quant that did not require consumption; however this resulted in a partial profile; therefore the remainder of the extract (9.1 of the initial 15 µL total extract) was re-amplified in an effort to obtain additional STR types for the source. The re-amplification data was used for final comparison since more peaks resulted when additional template was incorporated into the PCR reaction. All other MagnaGraph® extracts were consumed for the initial amplification; this was true for both extraction buffers tested.

Method-Fragment #	Number Alleles >100 RFU	Number Alleles >50 RFU
SEB--4	1	6
SEB--5	0	0
SEB--6	2	4
SEB--7	14	21
SEB--8	2	4
Mean/SD (x/σ) alleles per sample for SEB (n=5)	3.8/5.8	7.0/8.1
Mean/SD (x/σ) peak height (RFU) for SEB (n=5)	306.5/233.8	198.8/207.9
DFX--4	1	6
DFX--5	7	8
DFX--6	0	1
DFX--7	0	7
DFX--8	4	10
Mean/SD (x/σ) alleles per sample for DFX (n=5)	2.4/3.0	6.4/3.4
Mean/SD (x/σ) peak height (RFU) for DFX (n=5)	224.3/66.9	112.4/85.6

Table 6.12: STR profile summary of restriction digested DNA recovered from MagnaGraph® membrane using SEB and DFX buffer systems. The number of alleles detected within each profile are indicated at both 100 and 50 RFU cut-offs. The table also reports the average values and standard deviation for number of alleles detected and average peak height and standard deviation of allele height for each method/fragment combination; raw data for peak heights is not included. Five fragments were extracted with each buffer type, therefore n=5 for each group.

For the secondary extractions on five fragments of each nylon type with SEB or DFX, all controls resulted in undetermined quantifications and no profile was obtained upon consumption of the extracts for STR amplification. Therefore, all negative controls related to the secondary extraction were determined to perform to specification.

Because quantification data from each fragment was conducted individually and prior to any pooling, the quantification data from the triplicate extractions (ext #1-3) and the secondary extractions (ext #4-8) was derived under the same experimental design and could be grouped and used together to compare the SEB and DFX buffers for recovery potential via the t-test. However, because triplicate extracts were pooled prior to amplification and quintuplicate extracts were not, post amplification data (number of alleles detected and profile intensity) could not be combined for each method and compared in any meaningful way. Nevertheless, the raw data was considered in this discussion since it does illustrate some interesting points. First of all, the amount of DNA indicated by real-time quantification was not always predictive of the number of STR alleles that may result. For example, for SEB extract #8 from MagnaGraph® membrane, the amplification contained approximately 41 pg of DNA and resulted in the detection of four peaks above 50 RFU. Yet, for SEB extract #4 from MagnaGraph® membrane, the amplification received approximately 20 pg of DNA in the amplification and resulted in the detection of six peaks in the STR profile using the 50 RFU threshold. This is important to note, first because the relative amount of profile information achieved from HMW DNA at these template quantities (as established by minimum template studies conducted in section 2.3.7.3) is substantially better than that observed in this chapter when amplifying restricted and recovered template. It was already demonstrated that quantification data from restricted templates behaves differently than that of HMW samples, and the results herein exemplify this fact even further. Also, quantification data at these extremely low levels, even using real-time technology, may not always accurately reflect one's ability to obtain an STR profile. Therefore, while useful to make general comparisons between buffer types (especially from Biodyne® B fragments where yields were in a range of more reliable quantification determination), real-time quantification should not be used as endpoint analysis in determining whether a STR profile could potentially result. For the system finally applied to archived RFLP membranes, this does not negate the importance of real-time quantification prior to STR amplification. However, when those results indicate extremely low DNA recoveries, consumption of the extract is prudent for the best chance of generating an STR profile since the STR result is truly the endpoint experiment.

6.3.3.4c Buffer Comparison Overview

In summary, the initial studies involving triplicate fragment extracts from both membrane types using four different buffers proved useful in identifying two extraction methods that outperformed the others. Subsequent studies designed to more fully investigate and compare these two methods in a manner most applicable to the manner of intended use (archived membrane lane extraction), further indicated no significant difference existed between recoveries achieved by these two approaches. Therefore, given the SEB is prepared in house and has been fully validated for extraction using this method on casework, it was determined that sample lane studies involving actual archived RFLP membranes would be extracted using the standard SEB extraction protocol.

6.3.4 Additional Novel Recovery Studies

This section reflects results obtained throughout the course of several novel recovery and amplification approaches. Methods tested included direct PowerPlex® 16 amplification, Restorase® amplification, and WGA amplification. The section also describes results obtained from attempted electrophoretic removal of DNA from the membrane by application of electrical current to the membrane fragment embedded in an agarose gel.

6.3.4.1 Direct STR Amplification of Restriction Digested Membrane-Bound DNA

A membrane fragment bound with digested DNA was diced and placed in a standard PowerPlex® 16 BIO multi-plex PCR amplification reaction. No profile resulted and controls performed to specification (data not shown). Figure 6.21 consists of results obtained from the intermediate assay which was designed to determine if the physical binding properties inherent to the membrane interfered with the PCR amplification of K562 DNA, as described in section 6.3.4.1. Results indicated that decreased amplified product was obtained from the reaction containing diced membrane fragments in comparison to product obtained from samples that did not contain membrane, indicating that the membrane in the reaction may compete for reaction components needed for amplification of template.

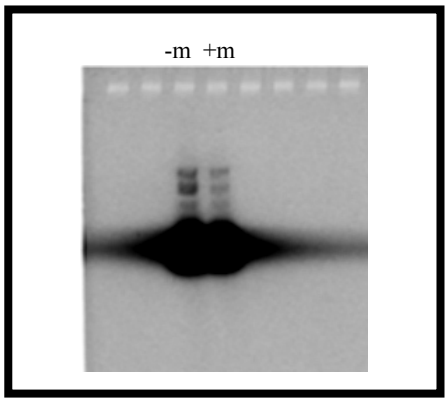

Figure 6.21: Post-amplification product gel (3%) containing amplification products in the absence and presence of nylon membrane. A volume of 2 µL of amplified product obtained from PowerPlex® 16 BIO multi -plex PCR positive amplification reactions (0.5 ng template K562) run in the absence and presence of nylon membrane fragments was loaded in each lane. (–m)=membrane absent; (+m)=membrane present

Therefore, the possibility of "pre-hybridizing" the membrane slot with 10X Gold ST*R buffer was explored as described in 6.2.4.1. Following pre-hybridization, amplification was carried out using the PowerPlex® 16 multi-plex PCR reaction kit. Controls performed to specification, however no profile was generated from the pre-hybridized slot (data not shown).

Given that no amplified product resulted from either of the above direct amplification attempts, an alternate amplification enzyme, Restorase® DNA polymerase, underwent testing for the ability to amplify digested DNA bound to membranes as described in section 6.2.4.1. Controls performed to specification, however no profile was generated from the pre-hybridized slot (data not shown).

Therefore, initial attempts for direct amplification of DNA bound to membrane using PowerPlex® 16 BIO multiplex PCR amplification system were unsuccessful. As indicated by Figure 6.21, the properties of the nylon membrane alone may interfere with the reaction, possibly by competitive binding for amplification components. While this may be true, blocking of the membrane with these very components (present in 10X Gold ST*R buffer) prior to amplification did not prove to be a successful approach for overcoming issues related to membrane interference.

Given that some polymerase activity is dependent upon proper DNA conformation, another explanation for the inability to carry out amplification may be due to damage in DNA imparted during the cross-linking process. However, tests designed using an alternative polymerase, Restorase® DNA polymerase, were also unsuccessful in amplification of bound template. The method tested involved substitution of this polymerase for AmpliTaq® Gold, using the standard 10X Gold ST*R buffer provided in the kit since it also contains other necessary PCR reaction components. While this buffer does not provide the same pH range as the buffer supplied by the manufacturer for Restorase®, the substitution was quality control tested on a positive control sample, 5 ng of 9947A, prior to attempting membrane-bound template amplification (Chapter 4). Since the manufacturer of this product boasts the systems ability to "repair" and successfully amplify damaged template, it seemed appropriate for testing the restricted and membrane-bound samples. Based on these studies, additional approaches involving direct amplification of bound DNA samples will not be pursued at this time.

6.3.4.2 WGA Amplification of Restriction Digested Membrane-Bound DNA

Another process tested was pre-amplification of bound or recovered template using WGA. The GenomiPhi™ Kit (Amersham Biosciences) for WGA employs Phi29 (ϕ29) DNA polymerase and is fully described in Chapter 3. This kit was chosen for membrane recovery studies in order to explore the ϕ29 DNA polymerase's ability to amplify bound or otherwise structurally modified templates. While proper template conformation is extremely important to achieving amplification with conventional thermostable enzymes, the ϕ29 DNA polymerase operates with a rolling-circle strand displacement that could potentially be capable of amplifying templates where Taq polymerase fails.

To review methods described in 6.2.4.2, one membrane fragment bound with digested DNA underwent DNA extraction using the modified SEB (section 6.2.3.2) and recovered DNA was then lyophilized. This sample was denoted as "WGA-sebws". A second membrane fragment bound with digested DNA was diced and placed in a

standard amplification tube so that direct amplification of template bound to membrane could be attempted. This sample was denoted as "WGA-direct". Both samples, along with the appropriate 9947A positive (WGA-pos) and NFW negative (WGA-neg) controls, then underwent whole genome amplification as described in section 2.3.1. Following amplification, the amplification products were assessed with a 1% agarose gel and the QuantiBlot® Human DNA Quantitation system. Results of these quantification assays comprise Figure 6.22 and 6.23, respectively.

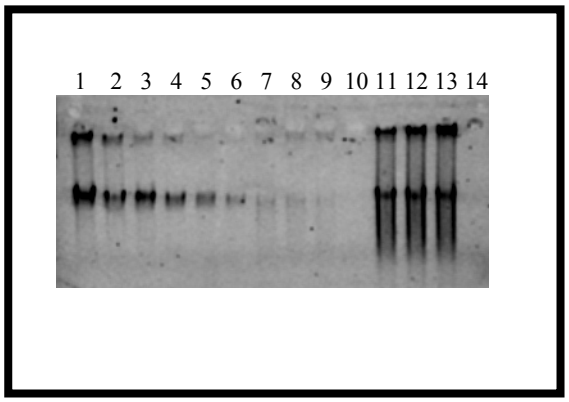

Figure 6.22: Product gel (1%) containing WGA products following membrane-bound template amplification (WGA-direct) and SEB-recovered template amplification (WGA-sebws). Lanes 1 through 6 contain the HMW standard series in nanogram quantities of 200, 100, 50, 25, 10, and 5 ng. Lanes 7 through 10 each contain 1 μL of the 1:49 dilution of WGA products in the following order: WGA-pos, WGA-neg, WGA-sebws, and WGA-direct. Lanes 11 through 14 contain 1 μL of the undiluted retentate of each WGA product in the following order: WGA-pos, WGA-neg, WGA-sebws, and WGA-direct.

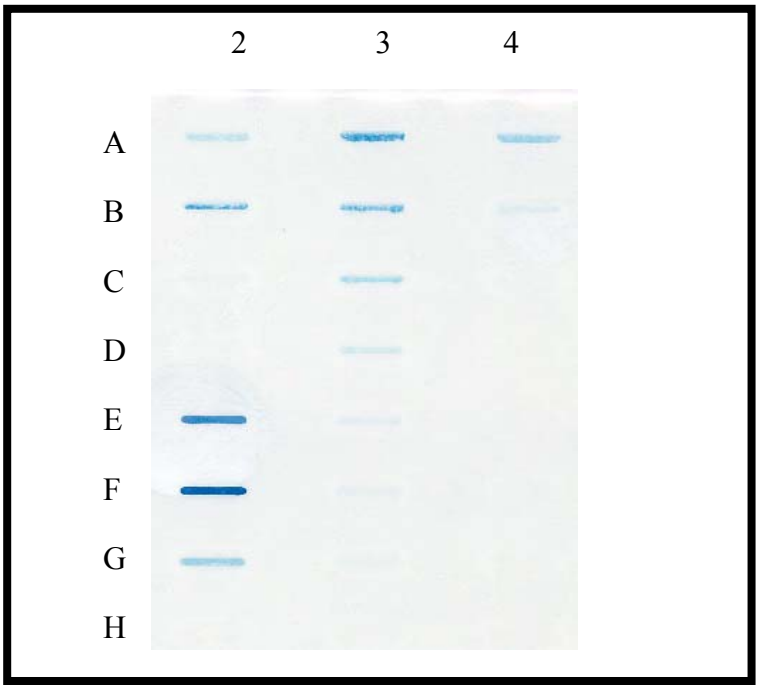

Figure 6.23: Colorimetric detection of WGA products following membrane-bound template amplification (WGA-direct) and SEB-recovered template amplification (WGA-sebws). Column 3 (A through G) contains the quantification standard series in the following nanogram quantities (top to bottom): 10, 5, 2.5, 1.25, 0.63, 0.32, and 0.15 ng. Wells 2A through 2D contain 1 µL of the 1:49 dilution of WGA products in the following order: WGA-pos, WGA-neg, WGA-sebws, and WGA-direct. Wells 2E through 2H represent 1 µL of the undiluted retentate of each WGA product in the following order: WGA-pos, WGA-neg, WGA-sebws, and WGA-direct. Calibrators representing 3.5 and 0.5 ng of DNA were loaded in 4A and 4B, respectively.

The limitations of agarose gel and slot blot quantitation of WGA product was fully discussed in section 3.1.1, and were taken into account when interpreting membrane recovery/amplification data. WGA product obtained was readily apparent by agarose gel when analyzed at a 1 µL quantity. However, unlike previous WGA quantitations where a 50-fold dilution produced optimal results, the dilutions of recovered or directly amplified products were scarcely visible (lanes 7-9). Both the diluted and undiluted sample obtained following the direct amplification assay were undetectable by agarose gel visualization (lanes 10 and 14). Slot blot quantitation confirmed trends observed on the gel; diluted and undiluted samples were apparent for the controls and sample "WGA-sebws" with diminished signal for the more diluted samples. Also, colorimetric detection did not result from either of the dilutions of sample "WGA-direct". Ramifications involving detection of signal in the negative control

(discussed in Chapter 3) were apparent on this blot as well and signal actually exceeded that detected for the positive control. Nevertheless, appropriate dilutions for the controls were selected and dilutions for the samples estimated in order to multiplex type the samples.

Samples were amplified using the PowerPlex® 16 chemistry as follows:

WGA-pos: 1 µl undiluted, 1 µl 1:49 dilution
WGA-neg: 15 µL undiluted
WGA-sebws: 1 µl undiluted, 15 µL undiluted, 5 µl 1:49 dilution
WGA-direct: 15 µL undiluted

The results obtained from the WGA post-recovery assays are summarized in Table 6.13, which also contains information regarding the number of artifacts observed in profiles generated following WGA. Positive and negative STR amplification controls (0.5 ng 9947A and NFW) were amplified and analyzed alongside the WGA samples, both of which performed to specification. Table 6.13 summarizes the remaining profile results where appreciable genotype information was obtained.

	WGA-pos 1 μL (1:49)	WGA-sebws 1 μL (undiluted)	WGA-sebws 15 μL (undiluted)	9947A-pos STR amp control	Membrane Bound DNA Profile
D3S1358	14,15	(1)	14 (1)	14,15	14,18
TH01	8,9.3 (2*)			8,9.3	7,9
D21S11	30	30	30	30	30,31.2
D18S51	15,19			15,19	14,18
Penta E	12,13			12,13	12,23
D5S818	11 (6)	12 (2*)	12 (3)	11	11,12
D13S317	11 (8)			11	11,12
D7S820	10,11 (2*)	10,12	10,12	10,11	10,12
D16S539	11,12			11,12	9,13
CSF1PO	10,12			10,12	10,12
Penta D	12			12	10,11
Amelo	X	X	X	X	X
vWA	17,18 (4)	(1)	17 (1)	17,18	17
D8S1179	13		12,13	13	12,13
TPOX	8			8	8,11
FGA	23,24			23,24	20,24
Total Alleles Detected	25	5	9	25	30
Percent Alleles Detected	100%	16.7%	30%	100%	100%

Table 6.13: PowerPlex® 16 STR results of amplified WGA products obtained from restricted, membrane-bound samples. Alleles detected for each sample are indicated at each locus, with the number of artifacts observed parenthetically indicated at each locus. * indicates one of the observed artifacts binned as an allele, however RFU value for such artifacts was significantly lower than the true alleles detected at the locus. Pull-up, off-ladder peaks, and (n-10) peaks were considered artifacts for the purpose of this summary, however spikes and elevated stutter were not considered as such. The total number of alleles expected for each template is tabulated in the shaded columns, as are the number of detected alleles for each WGA sample. The bottom row indicates the percent overall profile detected for each WGA sample out of the 100% possible for each template.

WGA-sebws (5 μL of a 1:49 dilution) yielded only a 12 at D5S818, with no peaks or artifacts otherwise detected. The WGA-neg control produced a total of five off-ladder artifacts in the D5S818, D13S317, D16S539, and vWA regions of the electropherogram. The WGA-direct amplification resulted only in the detection of an X at Amelogenin (113 RFU). The STR negative amplification control produced no profile, as expected.

As noted in Table 6.13, the WGA applications tested here resulted in numerous artifacts, even in positive control samples. Most of the artifacts could be classified as raised baseline or were otherwise commonly observed by the lab using this typing system (n-10 and pull-up) and were therefore fully explainable. These artifacts are probably due to the complex nature of the multiplex typing reaction, which would include random hexamer primers from the WGA itself. While these primers are not fluorescently labeled, random binding to labeled fragments and other random pairing events would occur during the conventional STR typing. Given the artifacts were of far less intensity than the true alleles, the overall interpretation was relatively undisturbed.

Regarding the ability to recover profile information, the sample that represented 15 μL of template in the PCR reaction yielded the most genotype information with 9 of 30 possible alleles detected. Not surprisingly, the 1 μL template sample resulted in the detection of only 5 alleles, and the 1:49 dilution resulted in the detection of only one single allele. While some peaks exceeded the interpretation threshold in the negative control, none binned as alleles and therefore do not represent contamination in the reaction (this is likely background from the complex reaction). Finally, the direct amplification resulted in only a weak X at Amelogenin, marking much less success than samples removed from the membrane prior to amplification.

While some genotypic information was gained from multiplex amplification of WGA products, the overall results were not substantially successful. Given that there was not an appreciable amount of information gained and these assays were more prone to detection of artifacts, it was determined that there was no advantage to WGA of recovered samples prior to multiplex typing. Regarding the "WGA-direct" sample, there is justified concern that the amplification was diminished by interaction of WGA components with the membrane itself. While blocking mechanisms have been shown to increase success in amplifications containing membrane fragments, it was determined that further tests would not be pursued given that the assay indicated no marginal advantage for the ability to amplify extraction buffer recovered sample. Other factors surrounding the decision to not test WGA further on membrane

recovery efforts included the fact that the manufacturer does not suggest the system for use on degraded samples [Amersham, 2002]. It would be extremely time-consuming to determine an appropriate blocking system for further direct amplification tests, the tests are not likely to result in greater recovery or profile information due to the systems inability to amplify digested template, and WGA has been shown to produce profiles that are imbalanced or otherwise highly artifactual even when provided a range of template conditions (Section 3.4).

6.3.4.3 Recovery of RD Membrane-Bound DNA Using Electrophoretic Mobilization

Removal of bound DNA was attempted using the application of eletrophoretic force as described in Section 6.2.4.3. Following the administration of an electric potential of 200 V for 15 minute (constant voltage), the gel was stained with ethidium bromide and the FMBIO® II was used to capture the image represented by Figure 6.24A. Then, constant current was applied at 100 watts for 10 minutes, after which a second image, Figure 6.24B, was captured using the FMBIO® II. Figure 6.24 indicates that DNA was not effectively removed from the membrane by electrical current. Standard electroblotting employs specialized vertical gel transfer apparatuses to achieve transfer. A vertical unit was not used since the mechanics do not allow for capture of macromolecules upon removal from the support, as the collection chamber would be large and filled with buffer. Therefore, a vertical system was designed for these tests since mobilized DNA could be excised from a localized area of the gel following electrophoresis. Nevertheless, this system proved unsuccessful for removal of DNA from the membrane. Whether due to limitations in the level of electrical current applied or because the DNA was immobile due to cross-linking, this method demonstrated little promise and was not pursued further.

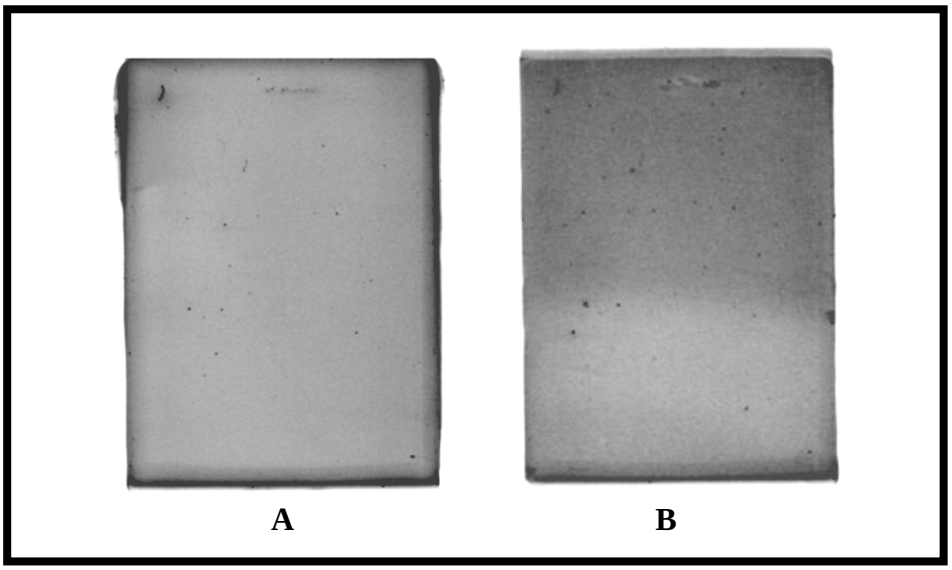

Figure 6.24: Agarose gel (2%) images following electrophoretic mobility testing. The center three lanes were adjoined to accommodate a membrane fragment cutting bound to restricted DNA. A) captured following application of 200 V for 15 minute (constant voltage); B) captured following application of additional current (100 watts for 10 minutes).

6.3.5 STR Typing of DNA from Archived RFLP Membranes

The results from the experiments in this section are comprised of data obtained from samples recovered from archived membrane lanes and are discussed from the perspective of quantification comparisons between membrane types and lanes as well as comparison of end-point STR typing of recovered DNA extracts.

6.3.5.1 Archived Membrane Test Material

Membranes tested were accompanied by reprints of the autolumes, which were produced either by radioactive or chemiluminscent detection of signal using standard photographic film exposure. While all membranes underwent multiple probings, the probe locus was of no significance and autolume records were needed only for lane placement about the actual nylon membrane removed from storage. The images for each membrane from which lanes were excised are presented in figures 6.25 through 6.27; these originated from Kansas City Police Department Crime Laboratory (KCPD), Sedgwick County Regional Forensic Science Center (RFSC), and Paternity Testing Corporation (PTC), respectively.

Figure 6.25: KCPD membrane autolume. The KCPD film produced following detection using a P-32 single locus probe. This image was used to prepare an overlay for the membrane so that exact lane location could be determined for excision of single lanes. Lanes 5 and 14 were cut for analysis as indicated by the red arrows. Lanes 1-3 were indicated as "empty" and were not transferred to membrane. Actual membrane dimensions = 102x162mm.

Lanes 5 and 14 were used for analysis from this membrane. Fairly standard quantities of DNA seem to have been loaded with little variation in signal intensity from lane to lane. This membrane had not been processed prior to this testing and was returned to frozen storage in plastic following lane excision.

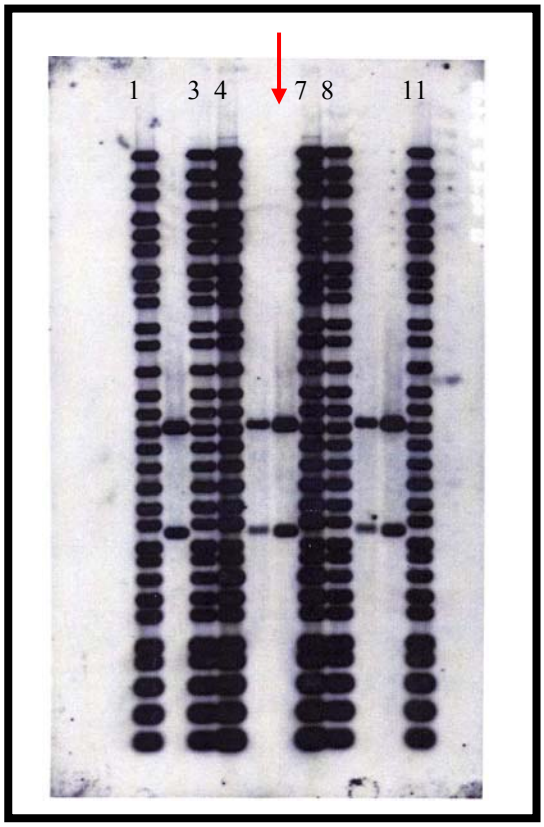

Figure 6.26: RFSC membrane autolume. The RFSC film produced following detection using a chemiluminescent single locus probe. This image was used to prepare an overlay for the membrane so that exact lane location could be determined for excision of single lanes. Lane 6 was cut for analysis as indicated by the red arrow. Actual membrane dimensions = 110x188mm.

The RFSC membrane was the only one of the three that was not stored directly in a plastic film wrap; instead it had been dried and frozen between filter paper/pads prior to plastic bag storage. Also, intermittent periods may have occurred where the membrane was kept at room temperature; therefore subject to fluctuating environmental conditions. Neighboring lanes from this membrane had been previously processed for testing conducted outside the scope of experiments described herein. The membrane was returned to original long term storage in the freezer bag between filter paper. Lane 6, which appears to have appreciable quantities of DNA loaded by comparison to neighboring lanes, was the only lane cut from this membrane for this extraction attempt.

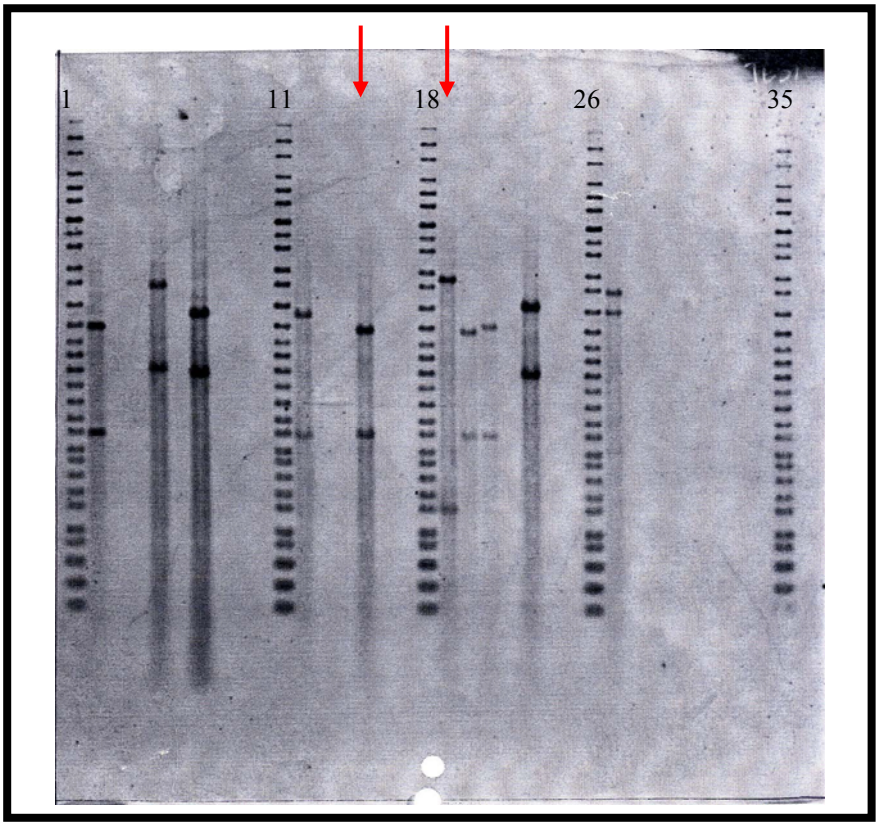

Figure 6.27: PTC membrane autolume. The PTC film produced following detection using a chemiluminescent single locus probe. This image was used to prepare an overlay for the membrane so that exact lane location could be determined for excision of single lanes. Lanes 15 and 19 were cut for analysis as indicated by the red arrows. Actual membrane dimensions = 200x197mm.

The PTC membrane was the only of the three that consisted of Biodyne® B material. Lanes 15 and 19, each with moderate quantities of signal intensity were excised for this extraction attempt. Other neighboring lanes from this membrane had been previously sampled for similar testing; after all processing the membrane was returned to frozen storage with intact plastic wrap. Fragments removed from the PTC membrane extended from the top of the membrane to the region where visible background ends within lane 7 (see figure 6.27); KCPD and RFSC membrane lanes were cut completely from top of membrane to bottom.

6.3.5.2 Processing of Archived RFLP Membranes

The DNA extracted from each membrane underwent Quantifiler® Human DNA quantification and resulted in the quantities indicated in Table 6.14.

Membane-Lane #	Human DNA Quantity (ng/μL)	Estimated template to PCR reaction (ng)
KCPD-5	0.104	1.560
KCPD-14	0.0718	1.077
RFSC-6	Undetermined	Unknown
PTC-15	0.016	0.240
PTC-19	0.00732	0.110

Table 6.14: Quantitation of excised membrane lane recovered DNA. The table reports Quantifiler® Human results obtained from single samplings of each extract. Values obtained were multiplied by 15 to calculate the estimated amount of template provided each PCR reaction upon consumption for amplification. All RNCs resulted in undetermined quantification results as expected and were also amplified in full.

The quantities obtained from the real-time quantification indicated that each lane of the KCPD membrane yielded over 1 ng of potential template if the entire extract were consumed for amplification. No quantification was obtained from the RFSC membrane lane. Some DNA was detected from the PTC lanes, although the real-time method indicated the recovery was ten times less than that from the KCPD lanes. Nevertheless, projected template quantity supplied by PTC reactions was in the range one may expect full or partial profiles, based on sensitivity studies presented in section 2.3.7.

Following amplification and electrophoresis, resulting electropherograms from five and nine second injection durations were analyzed. Figures 6.28 through 6.31 present the electropherogram results from the KCPD and PTC membrane lanes obtained from nine second injections. Alleles detected above 100 RFU are noted. STR results from PTC membrane Lane 19 showing D3S1358, D5S818, and vWA loci under the five second injection duration comprise Figure 6.32 since saturation, pull-up, and associated artifacts at these loci occurred with the 9 second injection. Using the 9 second parameters, the KCPD lane 5 resulted in the detection of 12 peaks, one of which is an artifact (18 at vWA). The triplicate pattern detected at D21S11 is characteristic of the K562 template bound in this lane. The peak at vWA is obvious artifact given the relative intensity compared to the 16 is less than 3%; furthermore,

the true allele is above 6000 RFU which is generally not considered an optimal target peak height. The KCPD lane 14 resulted in the detection of 8 peaks, all of which were attributable to the source DNA [verified by Hummel following blind submission of results via e-mail, 2009]. Only one peak was detected at D21S11 where the source is heterozygous. The sister peak (29) can be visualized at the locus, but is below the 100 RFU cut-off and was listed as a possible allele candidate upon blind submission. Although the genotype for the locus could not be definitively determined from this electropherogram, it is important to note that validation-based training and experience with the typing system resulted in careful interpretation and awareness that drop-out was probable for this particular locus.

The PTC membrane lanes resulted in greater amounts of genotype information even though real-time quantification indicated that less template was afforded these reactions. Lane 15 resulted in detection of 18 peaks across 10 loci. One peak, an 18 at vWA, is an artifact and would clearly be interpreted as such given the fact the peak is less than 3% relative intensity to others at this loci, each of which are in the 7000 RFU range and indicated by the software as offscale peaks. It is also noted that drop-out may be occurring at FGA due to a visible peak below the 100 RFU cut-off. While the shorter injection duration served to better optimize saturated loci, the 20 at FGA was not detected using those analytical parameters. Finally, PTC membrane lane 19 resulted in the detection of 17 true alleles across 10 loci. Four other peaks above 100 RFU also binned at vWA and D5S818. Of these, one is stutter just slightly exceeding the cut-off (13%) at vWA. Others are likely due to saturation at these loci, the offscale data is identified by the analysis software. Therefore, the five second injection duration data for these loci are presented in Figure 6.32. Although some peaks are still saturated at short amplicon length loci, the artifacts in allelic bins previously observed are no longer identified by the software at this duration.

No profile was detected for the RFSC membrane lane (data not shown); no profiles resulted from RNCs related to this study. While multiple lanes could be excised from the RFSC membrane for further testing, other lanes previously tested did not result in the detection of profiles using previously tested methods that were found successful

for recovery/amplification from the PTC membrane (data not included). Because this result was consistent with previously experienced lack of recovery from this membrane type, and the relative amount of genotype data obtained from the PTC membrane using the SEB extraction method was consistent with the amount of data obtained during the unreported preliminary testing, profiling the RFSC membrane was not further pursued.

These results indicate that an appreciable amount of genotype information can potentially be obtained from archived membranes. While real-time PCR quantification may not accurately reflect the STR potential of this sample type (restriction digested and nylon bound), end-point analysis using multi-plex PCR should be carried out to fully ascertain the template viability for typing.

It is unknown whether the differences observed between membranes is due to the nature of the matrix or storage conditions under which the membranes were kept for the past decade. Regardless, these tests, as well as preliminary studies not presented in this thesis, suggest that Biodyne® B membranes result in much greater recovery with respect to typable template. This is undoubtedly due to the fact that this nylon does not require cross-linking of DNA prior to probe/strip cycles and relies upon charged surface modification reactions to bind the DNA. Unfortunately, it was not possible to test a broad variety of Biodyne® A membrane lanes due to their rarity. Not fully addressed by these studies, it is also possible that storage conditions could affect the ability to recovery template from nylon membranes. Related to these studies, the membranes stored directly in plastic wrap did yield genotype information while the dried membrane did not. As reported with respect to downstream re-probing viability, the plastic wrap conditions appear to be more conducive to downstream typing, whether re-probing for RFLP loci or recovery for STR analysis.

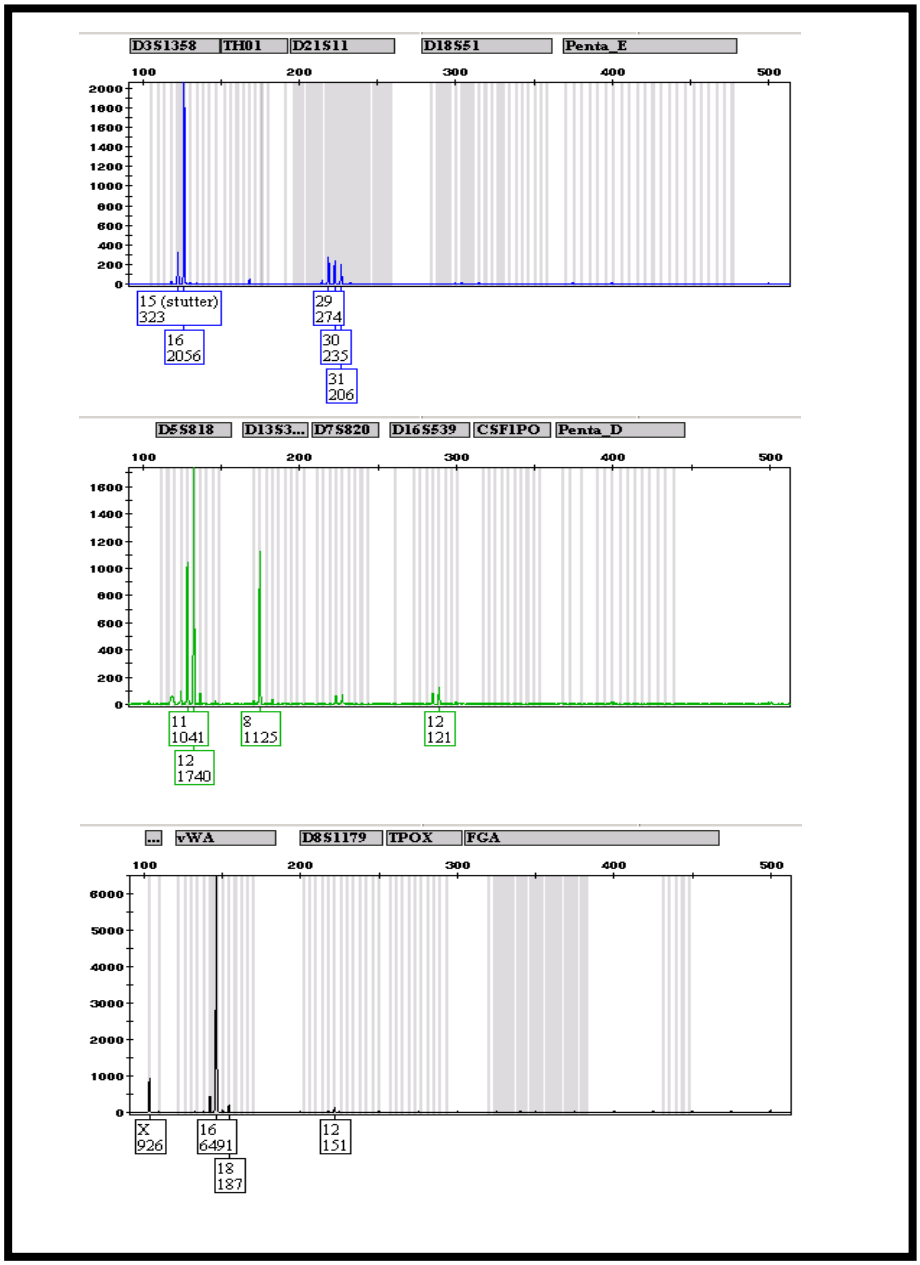

Figure 6.28: STR results from KCPD membrane Lane 5. The electropherogram from Sample Lane 5 excised from the KCPD archived membrane. The amplified product was injected for a 9 second duration and analyzed using a 100 RFU threshold. Peaks were assigned by the GeneMapper™ software based on parameters described in Figure 2.5. The data was formatted to indicate allele designation and peak height (RFU). A stutter peak exceeding the 15% cut-off is indicated at D3S1358. An artifact at vWA is present in the 18 bin.

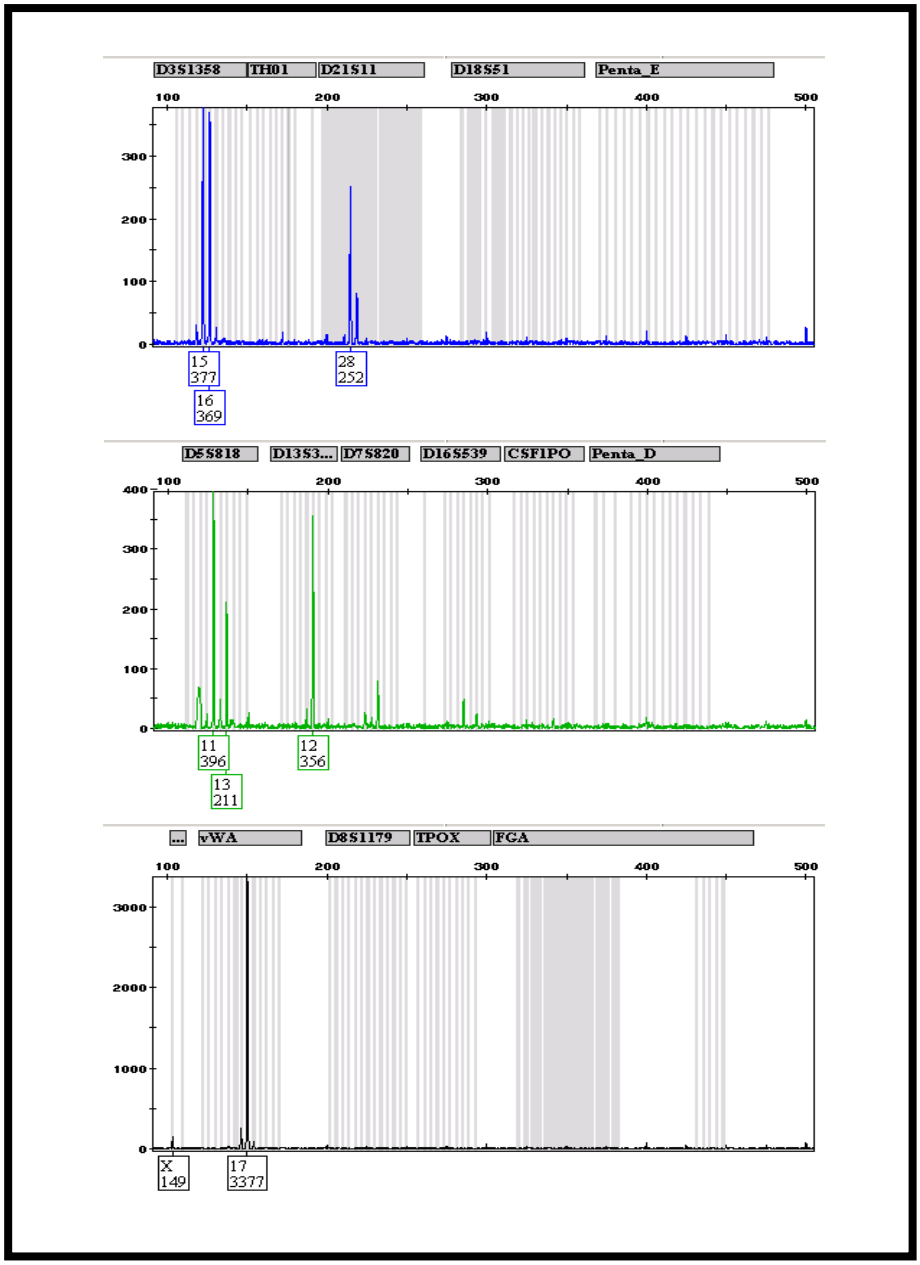

Figure 6.29: STR results from KCPD membrane Lane 14. The electropherogram from Sample Lane 14 excised from the KCPD archived membrane. The amplified product was injected for a 9 second duration and analyzed using a 100 RFU threshold. Peaks were assigned by the GeneMapper™ software based on parameters described in Figure 2.5. The data was formatted to indicate allele designation and peak height (RFU).

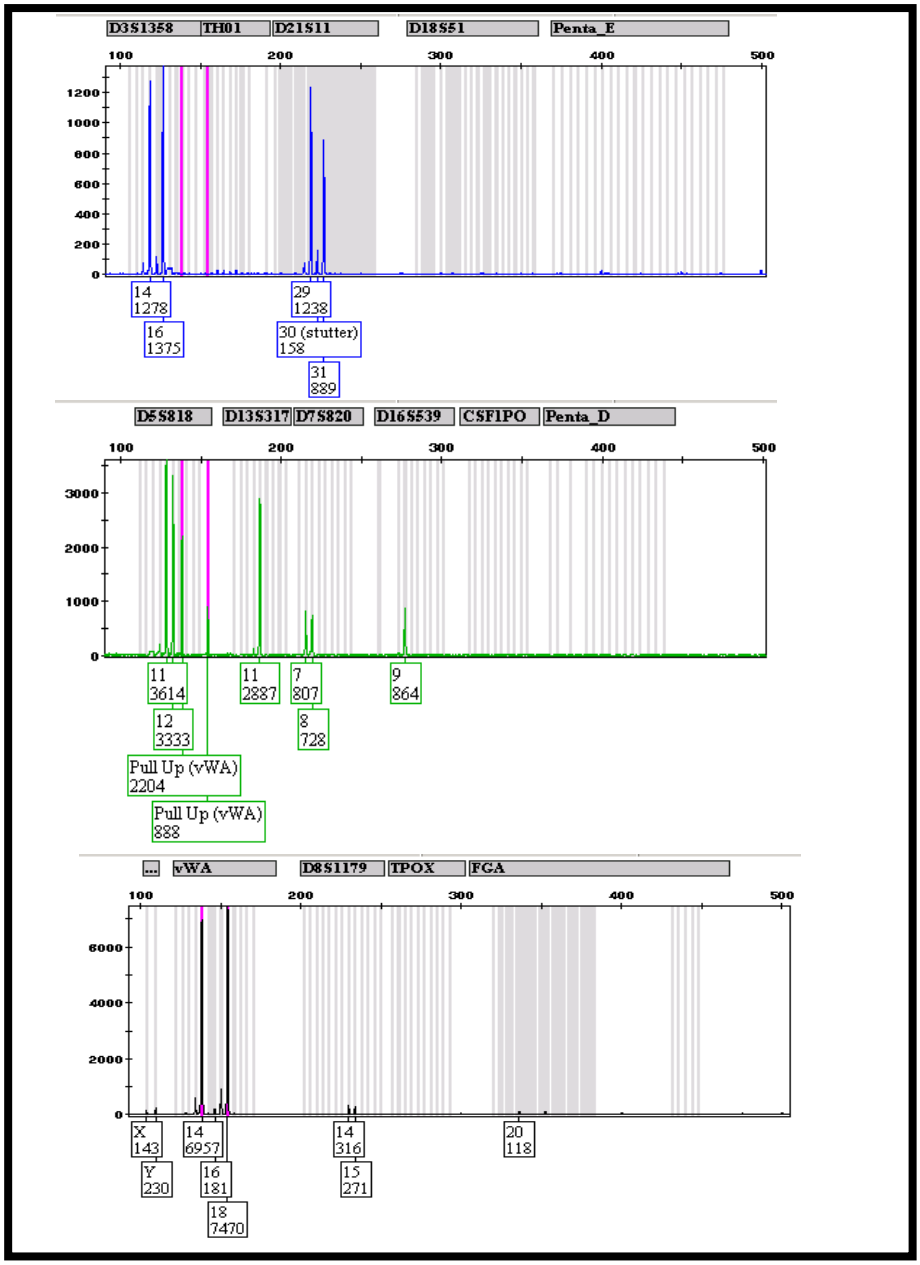

Figure 6.30: STR results from PTC membrane Lane 15. The electropherogram from Sample Lane 15 excised from the PTC archived membrane. The amplified product was injected for a 9 second duration and analyzed using a 100 RFU threshold. Peaks were assigned by the GeneMapper™ software based on parameters described in Figure 2.5. The data was formatted to indicate allele designation and peak height (RFU). Pull-up peaks are designated as such; pink highlighting indicates offscale peaks that exceed the dynamic range of the collection instrument. A stutter peak exceeding the 14% cut-off is indicated at D21S11. An artifact at vWA is present in the 16 bin.

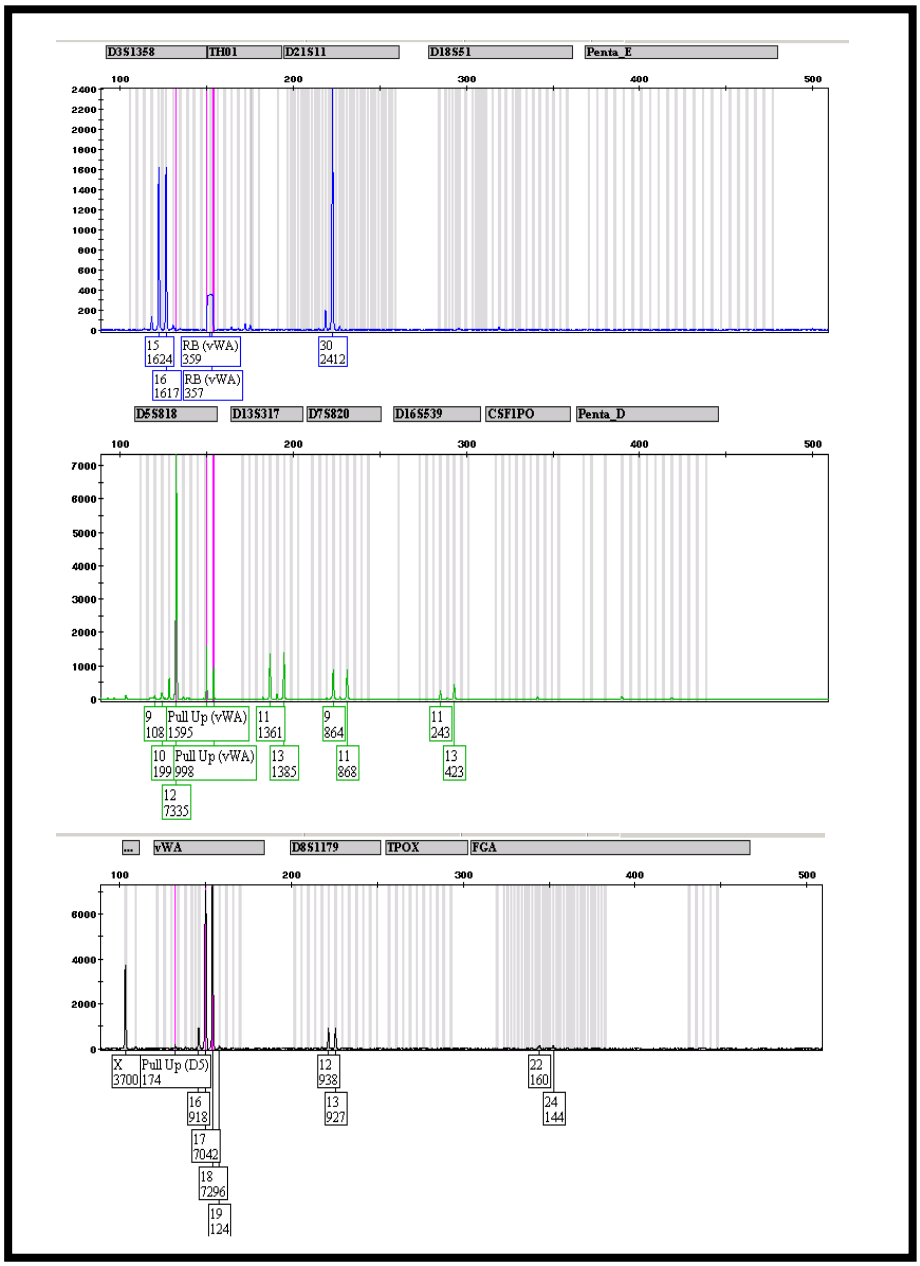

Figure 6.31: STR results from PTC membrane Lane 19. The electropherogram from Sample Lane 19 excised from the PTC archived membrane. The amplified product was injected for a 9 second duration and analyzed using a 100 RFU threshold. Peaks were assigned by the GeneMapper™ software based on parameters described in Figure 2.5. The data was formatted to indicate allele designation and peak height (RFU). Pull-up peaks and raised baseline (RB) are designated as such; pink highlighting indicates offscale peaks that exceed the dynamic range of the collection instrument. Offscale data at D5S818 and vWA caused excessive artifacts; a shorter injection duration achieved more optimal peak heights within this region of the electropherogram.

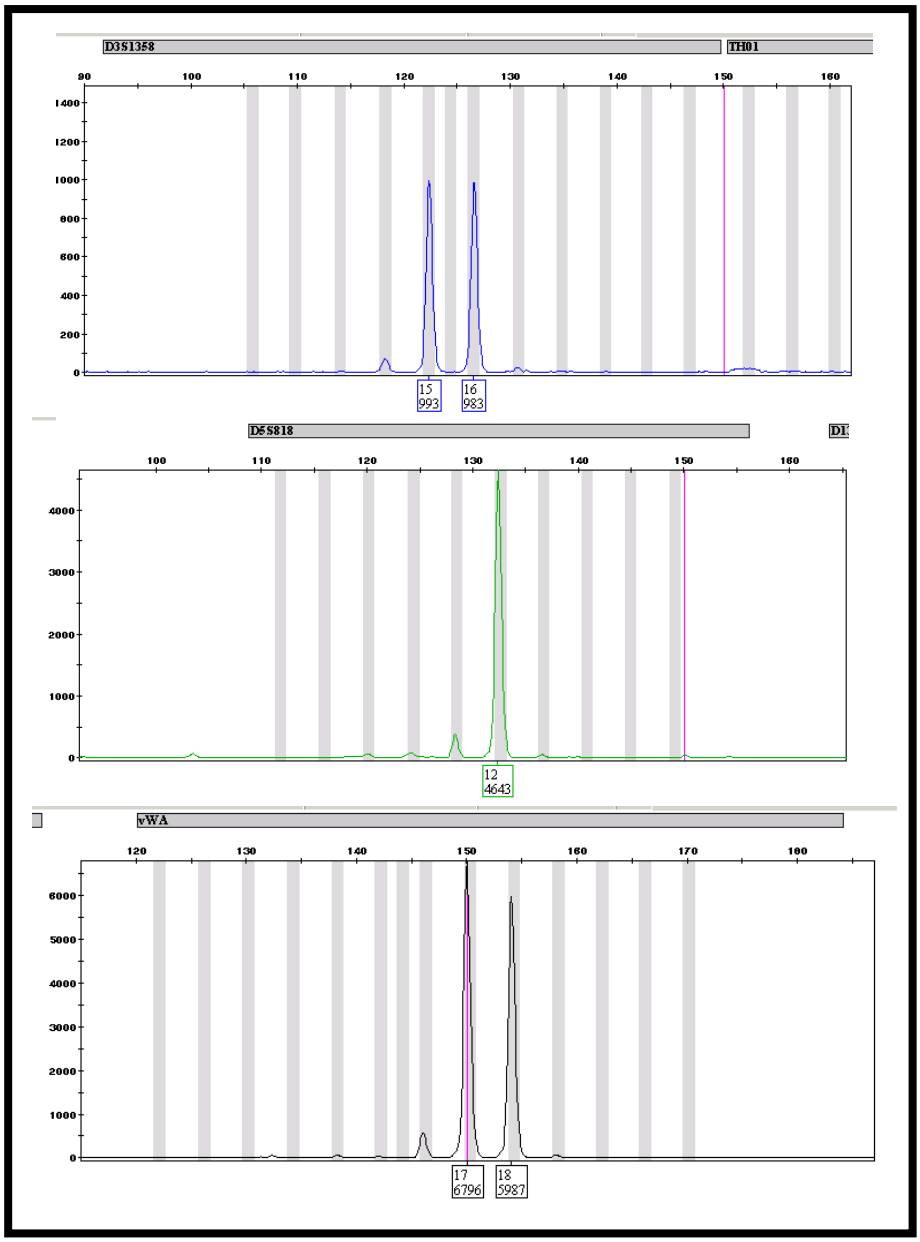

Figure 6.32: STR results from PTC membrane Lane 19, optimized for D3S1358, D5S818, and vWA. The electropherogram from Sample Lane 19 excised from the PTC archived membrane. The amplified product was injected for a 5 second duration and analyzed using a 100 RFU threshold. Peaks were assigned by the GeneMapper™ software based on parameters described in Figure 2.5. The data was formatted to indicate allele designation and peak height (RFU) at the D3S1358, D5S818, and vWA loci previously found to be saturated under 9 second injection duration conditions. Raised baseline between D3S1358 and TH01, pull-up peaks at D5S818 and vWA, and other artifacts were not sized using standard analysis parameters at this injection duration. Still present are pink highlighting indicating one offscale peak at vWA. The offscale mark does not align between panels since panel images were scaled for each locus rather than a standard data point range.

6.4 Conclusions of Membrane Recovery Assays

This chapter of the thesis represents a step-by-step evaluation of methods directed at recovering amplifiable DNA from archived membrane. The experimental approach sought to first recover HMW DNA bound to localized areas of nylon membrane. Then, procedures were tested on RD DNA samples bound to localized areas of nylon membrane. After ruling out the usefulness of acid, base/detergent, and organic chemical removal of the DNA, as well as other mechanical or direct amplification methods, standard extraction buffers were compared for recovery capabilities and a best method identified for testing on rare archived specimens. While little difference was noted between the Differex™ and SEB methods tested throughout the course of this chapter, the SEB method was ultimately selected for application on archived membrane. This was largely due to the non-proprietary nature of this in-house buffer. Due to the extreme unavailability of archived membranes, it was necessary to design the experiments around laboratory simulated test fragments in order to conserve lanes of actual archived membranes.

An appreciable amount of effort went into verification of the experimental design and theory behind the basic idea of archived membrane recovery of DNA for STR typing. A progressive approach was taken to verify the multiplex amplification of *Hae*III-cut template by both sequence and in practice. This confirmed that the basis for the research was sound and that typing digested DNA would theoretically be possible. Simulated fragments bound to HMW and RD DNA were then prepared and binding procedures were verified by present day chemiluminescent detection. Testing of acid, base, and organic chemistry to remove the DNA was pursued but found to underperform classic casework extraction methods. Therefore, several casework buffers and techniques, along with paramagnetic extraction, were attempted and compared.

Preliminary studies previously conducted using similar extraction components with excess DTT and proK indicated that the PTC membranes would yield amplifiable DNA. However, because of experiments presented in section 6.3.3.3, it was determined that final evaluation and comparison of buffers should be performed using

casework validated levels of these components. Nevertheless, pilot studies yielded similar results with respect to STR results from the PTC membrane and no results from the RFSC membrane, indicating that the results presented herein regarding recovery from archived specimens are consistent with preliminary findings under slightly modified conditions.

Even though the real time quantification detected more DNA from the KCPD membrane, the PTC membrane extracts produced more genotype information. The quantification system does have limitations when estimating the amount of highly degraded template; degraded template is quite similar to restriction digested samples. The difference in quantification may also be due to the region of membrane cut for each lane. For example, the KCPD membrane lane was cut from top to bottom. The PTC membrane lanes were cut from the top to a region where background signal was no longer visible. Therefore, a greater relative amount of shorter DNA fragments may have been excised from the KCPD lane than was for the PTC membrane. This may have resulted in elevated quantification levels, but the additional fragments present may not have been conducive to STR multiplex amplification.

One issue for discussion is the inability to recovery sample from the RFSC membrane, while the KCPD and PTC membrane lanes produced typable extracts. Several basic differences between the archived samples should be addressed. First, PTC employs Biodyne® B membrane, which does not require UV cross-linking prior to multi-locus probing. It was initially thought that this cross-linking imparts damage to the DNA that inhibits the ability of polymerase to interact with and/or assemble the complimentary DNA strands. However since the KCPD membrane was cross-linked, it is evident that this alone does not impart the inability to STR amplify recovered template and this suggests that relative recovery is dependent on storage conditions. Secondly, the age of the membrane alone is not predictive of viability. While the PTC membrane was the "youngest", the others originated during a similar period and the KCPD membrane performed far better than the RFSC membrane. Finally, a third difference between the laboratories' membranes samples were the conditions of storage. The KCPD and PTC membranes had been wrapped airtight in plastic wrap

prior to frozen storage. The RFSC membrane was dried and stored between blotting paper prior to long-term storage, which may have fluctuated greatly over the course of six years. It has been documented in the literature that plastic wrapped and frozen storage conditions were more conducive to re-probing [Giusti and Budowle, 1992; Keane *et. al*., 2000], and it seems that these conditions could also be more conducive to recovery of sample based on testing conducted herein. However, long term storage conditions have not been investigated and the work was not directed at DNA recovery by extraction; this would constitute a separate set of studies. Indeed, such studies may be conducted to compare the ability to recover DNA from membranes that were prepared using different methodologies and stored under different conditions. Unfortunately, because most archived samples contain potentially probative evidence, additional membranes for a broad study of this nature are not often available for experimental applications. Therefore, it would be most productive for the laboratory interested in recovery of membrane bound sample to test the particular membrane matrix/storage condition combination historically employed to determine how successful this procedure may be for the given set of conditions surrounding particular specimens. Moreover, some available membranes were hybridized with radioactive probes, and this introduces a separate set of considerations for processing beyond the scope of this project.

One last question surrounding the efficiency of the removal is whether other enhancement techniques presented throughout the course of the thesis could be used to improve recovery from archived membranes. While repair systems, like Restorase®, may successfully overcome damage due to depurination, conformational changes in the double helix caused by disulfide bonds may prove to be irreversible, either due to DNA-to-DNA interactions or bonds formed during this process between the DNA and compounds present on modified membrane surfaces. The repair system, when used in conjunction with NDIS approved primers, produced profiles of compromised quality in that peaks were imbalanced between and within loci and some artifacts (n-1) were apparent above the detection threshold. Because the make-up of the repair system is proprietary, it is noted that many repair enzymes require double stranded DNA so that the complimentary strand sequence can be used to direct

the repair of the lesion. Since bound and recovered DNA will be single stranded, these exhibits will be far less receptive to conventional enzymatic repair. Therefore, it is highly unlikely that the system would improve typability of restriction digested single stranded DNA fragments. With respect to WGA, this approach did not improve the ability to STR type low copy number samples and the manufacturer's literature specifically indicates that the system is not recommended for highly degraded samples (such as restriction digested samples). Both Restorase® and WGA methods involve pre-processing of template to enhance the final STR amplification. While neither of these systems proved useful for enhancing low copy number samples throughout the course of these studies, they were considered for testing related to recovery because they employ alterative polymerases in the reactions that may overcome problems specific to restriction digested and cross-linked, or otherwise bound template, related specifically to polymerase functionality.

Preliminary sequence and typing studies in this chapter verified that a full (16 locus) profile would never be achievable because some of the primer binding sites would be cleaved by the digestion process inherent to RFLP analysis. However, in the context of a full profile representing markers across 16 loci, even a partial profile can be quite probative for comparison purposes. The national CODIS database requires 13 loci be attempted for amplification and the national database accepts profiles with as few as 10 loci. Therefore, if 10 loci profiles are not producing excessive random matches when conducting searches between millions of profiles, it is reasonable to conclude that 7-10 locus profiles would be suitable for direct comparisons. Statistically, such profiles would have random match probabilities in the magnitude of millions (9 locus) and would, therefore, seem relevant from a statistical evaluation perspective. Given the extraction process developed herein successfully recovered a 13 locus profile from a test slot and a 9 locus profile from an actual archived membrane, it is reasonable to claim this work constitutes substantial recovery of genotypic information.

This chapter served to present the final culmination of testing conducted throughout the thesis for applicability to remove of DNA from RFLP membranes and type extracted template. The tests in this chapter explore the use of acids, bases, and

organic chemicals to extract DNA bound to nylon. Furthermore, some enhancement techniques conducted throughout the course of the thesis that constitute a unique approach to recovery were also tested in this chapter. For example, it was established that extraction using paramagnetic chemistry did not achieve recovery from forensic samples greater than that of organic extraction, however, it was unknown if the extraction chemicals, heated processing, and bead affinity could offer a unique approach to membrane bound sample recovery. Likewise, various polymerase and enzyme repair systems did not improve typing success compared to the current STR PCR techniques; however, it was unknown if these could achieve amplification from damaged or bound template by unique mechanism. Other enhancement techniques, such as PCR enhancement additives, were not tested on membrane bound samples since the mechanism surrounding the enhancement would not be likely to achieve improved recovery or typing success from bound specimens. Throughout the testing, it was determined that general casework extraction buffers outperformed other approaches tested and a series of experiments were designed to identify the best extraction buffer and technique using simulated test fragments comprised of two membrane types bound to restriction digested samples. Following this comparison, the best technique was applied to archived membranes.

The buffer that is commonly used to extract forensic stains in the casework laboratory proved successful in recovery of DNA from archived membrane lanes from two different labs. A third lab's membrane offered no recovery. The results herein indicate that this may be a useful technique for extraction of bound membrane lanes, even after more than a decade of storage. Although studies investigating recovery as it related to storage conditions were not possible, results indicate that recovery is possible from samples even if cross-linking was performed and suggest that success rates may be highly dependent on whether the membranes were exposed to consistent long term storage conditions.

Finally, any lab embarking upon recovery of DNA from archived membranes for STR typing of cold cases should consider a variety of key points prior to attempting recovery of samples from archived membranes. These include preparation,

processing, and storage techniques. Also, due to the magnitude of difference in sensitivity of STR technology over RFLP testing, profile interpretation should be carefully performed and persons involved in processing the membrane (either RFLP or STR analysis) should be excluded as contributors to any profile obtained or considered valid for forensic comparison. While some peaks were detected above threshold from membrane bound samples that were not attributable to the source of the bound sample, these were either highly characteristic of documented system artifacts (elevated stutter, pull-up, etc.), or so weak relative to the major contributor at offscale loci that interpretation for comparison purposes would be highly unlikely by an experienced analyst skilled in low copy profile interpretation. Furthermore, locus intensity optimization served to clearly resolve the correct source genotypes and this was evident by successful blind submission of STR typing results to collaborating labs that provided the membranes for the purposes of this study.

While additional considerations surrounding the scientific proposition of STR typing membrane samples will be discussed in the final chapter of this thesis, the work herein demonstrates that it is possible to achieve a reasonable and fairly discriminating amount of genotype information from samples bound to membrane that have been archived for periods of 6-12 years.

7.0
Discussion

7.1 Project Overview

The comparability of different test platforms and the sensitivities of the tests used have been established in the course of this project. Performance verifications of both the gel and capillary detection systems reveal limitations of each platform and provide a foundation for interpretation of low template samples. Chapter 2 describes this platform comparison, the outcome of which was detailed in Chapter 2 and briefly summarized in section 7.1.1. Using established multiplex chemistry, the work went forward to investigate the usefulness of typing enhancement techniques, most of which focused on improving the ability to type challenging or limited quantity samples. An overview of results for the whole genome amplification tests is presented in section 7.1.2. Summaries of the work related to PCR enhancement techniques (Restorase® and PCRboost™ systems) are presented in section 7.1.3. Finally, assays aimed at increasing extraction yield are addressed in section 7.1.4; this overview revisits the results of automated extraction tests and the use of a freezer mill to grind samples prior to extraction. All of these approaches apply to the final goal of recovery of DNA from an archived RFLP membrane since all of the systems tested throughout previous chapters address issues encompassing the challenge of membrane recovery. This is because the membrane bound sample embodies the very aspects of a challenging forensic specimen in that the DNA is degraded (digested), baked and often UV irradiated (exposed to environmental insult), and since membrane binding was intended to be irreversible, recoverable quantities are low (low copy number). An overview addressing the ultimate scientific challenge of removing template DNA from a nylon matrix comprises section 7.1.5, and discusses the usefulness of methods presented in sections prior towards completing this ultimate goal. In short, the thesis establishes the current standard for STR detection platforms, investigates enhancement techniques that are employed at various stages of sample processing, and culminates with membrane recovery studies that make use of previously tested enhancement systems and other extraction techniques specifically evaluated for typing of bound template. Each chapter aim will now be addressed and summarized as it applies to the overall goal of this thesis.

7.1.1 Platform Performance Verification

The platform verification aspect of the project clearly established the current technological capacity for typing low quantity samples by two prevalent multiplex system platforms, PowerPlex® 16 BIO/FMBIO® II and the PowerPlex® 16 /ABI PRISM® 3100-*Avant*. In addition to standard validation studies, two broader studies were executed to fully explore the sensitivity of these systems. The first involved determination of target template amount, the second was the determination of minimum amplifiable template quantity. Both aspects of the study focused on allele detection, genotype determination, signal intensity and uniformity, and relative heterozygote ratio balance. Overall, the two platforms were found highly comparable. Of utmost concern related to this project was the level of sensitivity achieved by the two different platforms. The gel and capillary systems performed comparably from a sensitivity standpoint in that both will achieve full profiles in the 60-125 pg range of template (see section 2.3.7 and figures 2.7 and 2.8a-d). On a more critical level, the quality of the profiles obtained with respect to signal intensity, uniformity, and relative heterozygote ratio balance were also similar. Having been established, these criteria could be used to evaluate and compare enhancement techniques tested in later chapters. While the detection systems are quite different, the end-point result variation in results between the platforms was minimal and both were acceptable for the low template applications conducted in this project.

7.1.2 Whole Genome Amplification

Having established detection limitations, section 3.1 covers studies designed to determine if whole genome amplification would offer an advantage over the current STR- PCR testing that is standard in most forensic labs. Two processes were investigated for the evaluation of the commercially available GenomiPhi™ kit; WGA conducted on purified DNA dilutions (section 3.2.1), and WGA conducted immediately after cell lysis, prior to purification and concentration of DNA (section 3.2.2). Initial test results obtained from the commercially available GenomiPhi™ kit proved to offer little or no advantage to conducting WGA on template prior to conventional STR PCR amplification. While profiles were generally obtained, many contained artifacts and/or high baseline and background. There was no direct

relationship between the amount of DNA template in the WGA reaction and the quality of the STR profile obtained upon typing the WGA products. Allelic imbalance was prevalent which would complicate interpretation of mixtures and, in many cases, made it difficult to determine if the profile could soundly be considered single source. However, most troubling was the detection of alleles not attributable to the template source, referred to as "drop-in", that were observed in figures 3.17-3.19 and summarized in table 3.4. The inability of WGA to enhance forensic typing capability was true for assays conducted on purified samples and WGA-amplified lysis products generated prior to DNA capture and purification. The current STR PCR methods produced profiles from similar limited quantities of DNA and the profiles were of far better quality than those resulting from WGA products. While the initial premise for investigating WGA in a pre- STR PCR sense did not yield seemingly rewarding results, it was apparent that the reaction is more forgiving than conventional multiplexing based on the fact that amplification takes place in crude cell lysate conditions. Due to this possibility that the enzyme may demonstrate processivity on bound template uncharacteristic of thermostable polymerases, studies with WGA were revisited in the final chapter of the thesis, but did not offer an advantage when included as a pre-amplification treatment of bound samples. Because the manufacturer's literature specifies the system is not designed for highly degraded template, an exhaustive study pertaining to WGA of restriction digested samples was not pursued.

7.1.3 Amplification Enhancement Techniques

Amplification enhancement techniques were explored in chapter 4. These included pilot studies with the Restorase® and PCRboost™ systems described and discussed in sections 4.2.1 and 4.3.1. The experiments described in section 4.2.2 with the Restorase® repair enzyme did produce a DNA profile on amplification of ample levels of template, however the profiles presented and discussed in sections 4.3.2 were not as balanced between, or within, loci as the conventional typing system. The experimental amplification also resulted in the detection of shouldering artifacts (see figures 4.6a-c and 4.7), perhaps due to inadequate addition of non-tempated adenine by the Restorase® polymerase (lack of A-overhangs). Nevertheless, it was

demonstrated that the system had potential for use in conjunction with the validated primer system and was later considered as a candidate for direct amplification from nylon-bound template in an effort to explore the potential of the alternate polymerase to amplify structurally impaired template deemed characteristic of irradiated samples. These breadth of these studies were somewhat limited by resources. Therefore, the amount of template provided the reaction was elevated as a conservative measure since the main goal was to obtain a profile and extensive sensitivity studies were not performed. The remainder of the enzyme mix was reserved to investigate it's use for direct amplification of bound template and preliminary work with recovered template, both of which will be later discussed.

The PCRboost™ product was initially tested for the ability to improve profile quality by a sensitivity study comparison. Replicate samplings of the same template amounts were amplified with, and without, the additive. This demonstrated that it had no apparent affect on profile intensity or balance, as discussed in section 4.3.1, and that the additive did not improve the genotype information gained from limited quantity samples. This being one of the primary claims of the manufacturer, the additive was not further pursued for applications related to the studies herein.

7.1.4 Extraction Enhancement Techniques

In an effort to improve extraction yield, automated paramagnetic extraction and freezer mill pulverization of specimens prior to extraction were explored in chapter 5. Experiments in section 5.2 describe the verification of two instruments for use in the forensic setting. While conducting performance verifications, the intricacies of the systems were evaluated for usefulness related to recovery of DNA from challenging samples.

One method tested was the potential of an automated process based on paramagnetic extraction of DNA. Samples prepared in the lab were processed using the trace extraction protocol with the Maxwell® instrument and recovery was compared to that obtained from same samples with organic extraction (see discussion in section 5.3.1). While table 5.6 indicates that the recovery using the robot was far less than standard

extraction, it was recognized that the different mechanism by which this procedure captures DNA might offer a unique approach to membrane bound samples and a full performance verification was carried out so that paramagnetic extraction could later be applied to membrane recovery assays.

The other key focus of this chapter was the possible use of cryogenic pulverization to pre-process samples for standard organic extraction using a freezer mill (section 5.1.3). While the instrument performed to specification and was highly useful for skeletal remains, the mechanics of the instrument somewhat limit the ability to process tiny specimens. Although the pulverization is quite effective for samples where the DNA is structurally bound in the sample matrix (bones) or otherwise harbored within a non permeable surface (teeth), it does not offer an efficient way to capture DNA from small specimens. As discussed in section 5.3.2, this is because the large size of the sample vial becomes coated with the specimen itself and a great deal of product loss occurs due to vial retention. The system was able to process swabs and nylon fragments, but data presented in table 5.9 from progressive sampling of swabs in the same vial showed that sample recovery increased as the walls became coated by previously processed specimens, confirming the product retention problem suspected upon initial testing. Given that pulverization is not capable of disrupting chemical bonds between molecules (DNA to nylon surface moieties), it was determined that this approach was not optimal for small samples and the mill was not further evaluated as a possible pre-processing step in membrane bound recovery experiments.

7.1.5 STR Recovery and Typing of DNA from Nylon Membrane

In many ways, the membrane-bound template exemplifies a challenging forensic sample given it is digested, intended to be irreversibly fixed to a substrate that actually competes for and binds STR typing components, stored for long periods of time, and found to result in little, if any, recovery upon extraction.

The work so far reported has included many aspects that are highly relevant to the forensic community for profile enhancement. While preliminary assays using WGA,

Restorase®, PCRboost™, cryogenic disruption, and paramagnetic extraction did not prove to offer a direct benefit to STR typing, a broad understanding of the mechanisms upon which these systems were based, and verification of system performance, provided the foundation necessary to evaluate each for potential application to the final scientific question of whether DNA could be recovered from archived membranes.

Regarding membrane recovery, preliminary studies exploring the typability of restriction digested template verified that some loci would not be amplified due to the presence of *Hae*III restriction sites in the primer binding regions of the DNA (the theory of which is substantiated in figures 6.1-6.3). Therefore, it was known that complete profiles would not be obtainable unless high levels of partially restricted sample had been placed on the membrane. Having conducted exploratory experiments with membrane bound template, it was quickly determined that an appreciable amount of DNA was not going to be easily recovered from archived membranes and that direct amplification was problematic.

It was known that there are case-related membranes maintained for storage in numerous forensic labs, but few have been kept that can be used for experimental purposes. Therefore, to conserve the limited amount of archived samples available, simulated test fragments were prepared so that extraction techniques could be tested and compared on high molecular weight and digested samples bound to common membrane types. Acidic/alkaline/detergent solutions, organic chemicals, and standard forensic casework buffers were all tested to identify the best method for use on archived membranes. A standard casework extraction buffer was finally selected, and having recognized the benefit of the proK and DTT components of this buffer, elevated levels of these components was investigated for possible use on archived membrane lanes. A brief study indicated that increasing levels of these together did not achieve greater yield, and it was determined that standard amounts would be employed for the final testing on membrane lanes.

Three membranes from three different agencies (Kansas City Police Department-KCPD, Paternity Testing Corporation-PTC, and the Sedgwick County Regional Forensic Science Center-RFSC) were processed using the optimized extraction procedure. Results were obtained from both excised lanes from two of the three membranes, those obtained from KCPD and PTC. The RFSC and KCPD samples were bound to Biodyne® A nylon and PTC employed Biodyne® B, therefore correlation between recovery and membrane type was not readily apparent. However, the storage conditions of the PTC and KCPD membranes were the same, so this factor might be very important.

While the differences observed were discussed in Chapter 6, recovery was successful and genotype information was achievable and verified correct upon blind submission of STR results to collaborating laboratories. In some cases, the amount of genotype information was appreciable indicating that, depending on membrane type and storage conditions, it is possible to recover a profile suitable for CODIS entry. These results may well encourage DNA laboratories housing archived membranes to explore the potential of analyzing bound DNA in cold cases where DNA was consumed for RFLP typing. At the very least, it beckons the careful review of these membranes prior to disposal.

7.2 Ancillary Studies and Considerations

Throughout the course of any long-term research investigation, studies are conducted that may be outside the prescribed progression of experimental design. Pilot tests can become an entire branch of experimentation that ultimately provide little insight to the ultimate forensic question. Nevertheless, these studies can prove to offer insight to the scientist as it is often the studies that fail whereby the greatest intellect is gained. This thesis was no exception, and the overview of the work would stand incomplete without mention of several such experiments.

7.2.1 Supplemental Archived Membrane Lane Extractions

Results of intermediate studies presented in section 6.3.3.2 were performed with buffers containing an excess of proK and DTT and resulted in recoverable and amplifiable template comparable to studies described herein. Some preliminary cuttings were taken from the RFSC membrane to be used for a direct amplification test and for some other assays. While these did not yield usable results, the experiments demonstrated the need to use simulated fragments in order to refine a technique and conserve the limited stocks of membrane lanes. Furthermore, when extracts were made from early fragment tests using excess proK and DTT and amplified using the Restorase® product, the results contained numerous artifacts that caused sizing difficult using PowerPlex® 16 sizing bins and much of the genotype information was not present. In the end, there were many experiments conducted that did not give a result that could be used to properly genotype the sample and these have not been presented. It is noted that the RFSC membrane only underwent a single lane sampling in Chapter 6. This is because lanes from that membrane and the PTC membrane were sampled and extracted using excess proK and DTT buffers during preliminary studies. The RFSC extracts resulted in no profile and the PTC extracts in limited genotype information. While the technique was further refined prior to presentation in the thesis, the preliminary studies support the greater body of work incorporated into chapter 6 since the results ultimately obtained from the RFSC and PTC membranes were very similar to those obtained during preliminary extractions.

7.2.2 Contamination Studies and Considerations

Another important aspect of the work included extractions and amplification of samples by a graduate colleague. In some cases, the resulting types achieved indicated the presence of contaminant DNA. Some of the extraneous profiles could be attributed to the individual who conducted the analysis, while others were not as simply explained. This interfered greatly with the ability to present any of the data obtained throughout a period of the work in any meaningful manner, but it did give rise to a series of considerations surrounding the typing of archived samples initially processed decades prior when the concern for contamination was practically non-existent. It also stimulated considerations related to experimental design since several

studies were conducted using DNA samples obtained from the individual primarily conducting analysis.

PCR contamination was recognized as a concern almost simultaneously alongside PCR's emergence as a standard and relevant technique. During this time it was recognized that a different standard of precaution is required when PCR is used in the molecular laboratory than had been recognized previously. Strict protocols were developed, to include improved disposables and designated PCR areas within the lab [Kwok and Higuchi, 1989]. Interestingly, these ideas are now recognized as requirements within quality assurance standards set forth by the FBI [FBI, 2008]. In context of this thesis, contamination is considered to be the entry of exogenous DNA into the reaction that was not derived from the forensic specimen and can arise from reagents, neighboring samples, lab instrumentation and/or disposables, or post-amplification product re-entry. However, contamination of PCR can also be thought of in a much broader sense and can include things like inhibitors or non-specific DNA sources (such as bacterial DNA). In other areas of research, the entry of human DNA into a PCR reaction can interfere with the quantification and amplification of non-human targets. Therefore, laboratory background levels have been quantitatively studied to gain an understanding of expected environmental copy numbers and resulted in detection of high environmental levels [Urban *et. al*., 2000]. Therefore the idea of treating laboratory surfaces for removal of potential exogenous template is but one aspect of contamination. While physical barriers remain one of the most effective preventative measures for contamination, other techniques, such as UV irradiation became popular for sterilization of labware [Niederhauser, *et. al*., 1994]. Early studies indicated that the most dangerous source of exogenous DNA in forensic samples is the post-amplified product re-entry, however these studies were conducted using early quantification systems that were not adequate for identifying trace quantities of pre-amplification contaminants [Scherczinger, *et. al.*, 1999]. Because of this, cleaning laboratory surfaces with a dilution of bleach has become the standard practice for decontamination of surfaces and the technique has been widely applied in forensic studies involving DNA [Kemp and Smith, 2005]. Even clinical studies have focused on DNA damage induced by hypochlorite as a mechanism of cancer-related

genotoxicity [Ohnishi *et. al*., 2002]; therefore this mechanism is widely accepted and generally preferred over UV irradiation since not all laboratory surfaces can be irradiated, three dimensional objects can not be effectively decontaminated in this way, and short DNA fragments may not have sufficient pyrimidines for induction of sufficient damage [Cone and Fairfax, 1993].

In order to investigation environmental levels of DNA in the host laboratory, quarterly wipe tests were conducted throughout the course of the experimentation period and documented throughout September 2003-July 2008. Swabbings collected following standard surface cleaning with 10% bleach rarely resulted in detection of a DNA profile following standard organic extraction and consumption of extract via multiplex amplification. In the instances where alleles were detected above the threshold, they were sometimes attributable to the analyst collecting/extracting the swabs or analysts inhabiting the lab space sampled. For this reason, membrane tests were appropriately designed using DNA from a source other than the researcher. Nevertheless, a summary of the timeline and areas tested are detailed in Appendix 4.

In all cases where profiles were detected, cleaning of these areas and subsequent tests resulted in no profile; this indicated the decontamination procedure is working. However, the problem with this assay is that, in cases where the analyst collecting/processing the samples is the source of the contaminant, it is unknown if the environment was the true source of the DNA or if it was introduced via the assay itself. However the process was useful in gaining an understanding of labware levels of contaminant entry and also assists the trained analyst in differentiating and recognizing the types of profiles that must be interpreted with caution.

Archived membranes should be handled with labware and reagents of the utmost quality and purity, just as any other low copy number exhibit would be treated. One aspect of contamination to consider is deposition of DNA by the lab practitioner. However, the samples are not unlike any other cold case sample where a lesser degree of precaution was taken to reduce PCR contamination. The main advantage to the membrane as a cold case exhibit is that these specimens were prepared by laboratory

staff equipped with gloves and forceps who employed reagents that were filtered, autoclaved, or derived from other sterile solutions. Furthermore, upon identification of a membrane lane for possible extraction, the current analyst most likely has information regarding who prepared the membrane, and being former lab staff, usually has the STR profile of that individual which could be used for exclusion from any resulting membrane profile obtained. Laboratories process cold case exhibits routinely that were collected and stored with substandard technique and generally do not go through extensive effort to exclude investigators who previously had custody of the exhibit; in fact, that DNA is usually not even afforded the lab for exclusion. Nevertheless, the forensic scientist, with this information at his/her disposal, could exclude applicable analysts from profiles prior to further interpretation or comparison to membrane lane genotypes. While extensive studies could be conducted surrounding deposition of cellular material on membranes and extraction of exogenous touch DNA from nylon, any result obtained would not negate the need to exclude applicable parties from archived membrane lane profiles prior to interpretation. The other aspect of contamination to consider would be that co-extracted with the source of interest. However, it was reasoned that unless the contamination was severe, the relative amount of extraneous DNA present in the bound sample would likely not be recovered in a relative quantity conducive to end-point detection. For example, if the bound sample was 95% true source and the contaminant 5%, and 50 picograms total DNA was recovered, such a minute quantity of that DNA would be comprised of contaminant template that it would likely never be observed downstream upon conventional STR typing.

Finally, one observation that was made throughout the course of the colleague's analysis was that membrane lanes re-wetted and rinsed prior to extraction never produced exogenous alleles upon extraction and amplification. While studies were not specifically carried out to investigate whether pre-rinsing reduced the capture of post-archive deposited cellular DNA, a re-wetting/pre-rinse step was incorporated into the final archived membrane extractions conducted in Chapter 6 as a conservative measure.

7.3 Ongoing Studies

The systems investigated herein constitute forensic topics that have been highly prevalent and heavily researched during the past several years. Therefore, scientists across the world have contributed to research in these areas and those contributions have been reviewed and summarized to determine how the contributions of this thesis compare to other scholarly work.

7.3.1 Platform Comparisons

The decision of the host lab to initially purchase the gel based platform occurred long before the commencement of this work and was not selected by this research team. However, this platform was introduced to the forensic community just prior to capillary instrumentation and was purchased by many labs conducting RFLP testing simply because the platform produced familiar "banding patterns" rather than electropherograms with peaks, which constituted a far less familiar output. Many pioneers in the STR DNA typing community within the United States validated slab gel STR platforms. This included statewide lab networks in Pennsylvania, Maryland, Virginia, North Carolina, Atlanta, and Wyoming; this also included progressive private laboratories such as Cellmark, Bode, and Paternity Testing Corporation, and many of the early STR multiplex users working from regional labs in Sedgwick County Kansas, Detroit Michigan, and West Palm Beach Florida. The only substantial published work performed regarding platform comparison outside Sedgwick County occurred in Pennsylvania, which had long been equipped with multiple STR detection instruments throughout the statewide system [Tomsey, *et. al*. 2001]. The main contribution for platform conversion arose from work presented herein following the announcement that manufacturer support would no longer be available for the FMBIO® instrument and this work was widely presented across the United States in the form of posters and oral presentations in an effort to assist other FMBIO® labs in capillary validation efforts [Steadman, 2002b/2004; Steadman, 2005a/b; Steadman, 2006a/2008a]. Results obtained from each system were determined comparable by Pennsylvania and were highly consistent with the more abbreviated comparison presented here, as summarized in section 7.1.1.

7.3.2 Whole Genome Amplification

Whole genome amplification systems have been heavily marketed to the forensic DNA community in the last five years. Upon commencement of this project, only one manufacturer was known to be selling a kit for evaluation by the forensic community. Research and development of this kit was extensive, but Molecular Staging and Amersham Biosciences were the sources of most literature available regarding the product claims.

Because it has been shown to exhibit less bias than other WGA methods, the multiple displacement amplification (MDA) technology with ϕ29 polymerase offered the first non-PCR based isothermal method of amplification of large stretches of genomic DNA [Dean, *et. al*., 2002]. Since the release of the GenomiPhi™ kit by Amersham Biosciences/GE Healthcare, Qiagen has also introduced REPLI-g® technology to the forensic community. Having not tested the method directly, it is unknown how performance may compare to Amersham's kit; however the polymerase and mechanism of amplification are the same between kits.

Experiments reported by Barber and Foran compared I-PEP (improved primer extension preamplification) with random 15-mers to MDA with random hexamers (the GenomiPhi™ kit, specifically) for forensic applications. Agarose gel evaluation of amplified products and negative control gave results consistent with those generated in this thesis; even when no input DNA was included, random primers pairing and extending generated large quantities of high molecular weight products [Barber and Foran 2006]. Barber and Foran concluded that neither method performed well on degraded samples. Unfortunately, environmentally compromised samples are commonly encountered in forensics and this is one sample type of high interest for WGA applications. Ultimately, these authors agree with conclusions made in Chapter 3 of this thesis (and subsequent publications of this work), that allelic drop-out was prevalent and that the MDA kit needed to be retooled if it was to be of use to the forensic community to pre-amplify samples destined for STR multi-plex analysis [Steadman *et. al.*, 2005c/ 2006b]. This particular work is of interest since Barber and Foran clearly report the same difficulty with quantification as that reported/discussed

throughout Chapter 3, and as summarized in section 7.1.2. It is understood that real time quantification would be more suitable for estimating appropriate template quantities for STR reactions; however, real time does not alter the fact that the presence of large quantities of bacterial DNA is undesirable in the downstream multi-plex reaction. An abundance of non-human template has the potential to interfere with real-time based quantification assays. Moreover, these amplicons will be included with STR PCR template and will populate the amplification reaction with non-human DNA that inhibits balanced typing within the multi-plex system.

Both the GenomiPhi™ and REPLI-g® kits were evaluated by Schneider, *et. al*., and the imbalance within and between loci was reportedly problematic for both kits when tested by this group, as were concerns related to allelic drop-in. Therefore, conclusions were in agreement with those from this work. Balogh *et. al*. also summarized a collaborative investigation of the method for forensic applications and reported increasing numbers of allele dropout below 250 pg of DNA input to WGA-pre-amplification reactions followed by amplification with the SGM Plus multi-plex typing kit. The group seems to indicate a relationship between allele drop-out and WGA input template, which was not an immediately apparent trend observed from results herein; however, experimental design, typing kit, and data collected differed between groups and may account for this difference. Nevertheless, even though most groups agreed upon the limitations of the technology for forensic applications, these and others indicate promise for samples with greater amounts of starting template or for immortalization of clinical samples [Hosono *et. al*., 2003; Schneider, *et. al*., 2004].

It seems that the literature indicates reasonable success for WGA where downstream analysis is clinical or SNP-based [Lasken and Egholm, 2003; Barker *et. al*., 2004]; however, such success has yet to be achieved for applications like those explored here, even though this has been tested by several groups. After these studies were completed, some forensic investigators have met with moderate success using WGA technology, but marginally successful approaches have involved moderate to severe protocol modification to that suggested by the GenomiPhi™ kit manufacturer. Allelic balance was improved by combining amplification products that did and did not

undergo heat denaturation and using systems with different primer configurations has also shown improvement (Ballantyne *et. al*., 2007a/b]. Interestingly, programs funded by the National Institute of Justice in the United States after the commencement of this project originally considered MDA methods but have recently focused on DOP-PCR as the desired method when non-probative casework samples were tested [Brown, *et. al*., 2009].

In summary, review of literature reveals that the same difficulty for forensic samples was encountered by numerous groups with respect to MDA, regardless of kit tested. While this method seems highly applicable to the forensic lab theoretically, the technology is not yet refined to the point where it can directly be applied or validated for use in the casework lab.

7.3.3 Amplification Enhancement Additives

Repair cocktails have come to the forefront of forensic research also in the last five years, and proprietary enzyme mixtures are now commercially available that claim to repair various types of damage to DNA. The idea has met with some success in other areas of research such as improvements upon typing fixed tissue [Skage and Schander, 2007], but investigations beyond those conducted by developers was scarce upon commencement of work with Restorase®. An overview of findings from PCR enhancement additive experimentation performed for this project is offered in section 7.1.3. By way of comparison, the only presentation directly applicable to the enzyme was work performed at San Jose State University and presented at the 18th International Symposium on Human Identification in Hollywood CA, USA. The poster indicated UV repair was evident from samples pre-incubated with the Restorase® enzyme and then amplified with Taq polymerase [Odigie, *et. al*., 2007]. Interestingly, the end-point analysis used to draw these conclusions, which are noted as "preliminary", is not disclosed in the conference proceedings nor was it evident on the poster. The Restorase® patent itself does not illustrate examples of repair following UV irradiation. Therefore it could be that, although the samples were UV irradiated, the samples tested by the San Jose group suffered other forms of damage that were corrected by the repair enzyme. While the exact components of the enzyme

blend are proprietary, it is known that the main mechanism responsible for achieving better amplification is caused by combining polymerases, as information regarding the inclusion of other repair enzymes is less prevalent within the patent description. While it cannot be known whether or not there is a component of the enzyme blend specifically acting to repair damage caused by UV irradiation (previously described), there are considerations beyond the claims of the San Jose group that beg for further and more extensive testing.

Nevertheless, there are problems with using such a repair system in conjunction with a multi-plex kit in that the additional polymerases behave differently and have different buffer activity requirements. Even though preliminary amplifications following Restorase® incubation achieved a full profile, the reaction contained large quantities of template and the resulting STR profile was of diminished quality. Furthermore, ancillary studies resulted in the presence of PCR amplicons that did not size consistently within the PowerPlex® 16 bins, and the peak shifting, while predictable, complicated allele designations when samples recovered from nylon membrane were amplified.

The Restorase® system may offer significant improvement to template amplification in other disciplines, or in studies where PCR primers can be designed for optimal activity when used with the enzyme mix. However, at this juncture it is clear that the system may not be easily amenable to multiplex systems approved for use by NDIS and that the additional polymerases present in the mixture can cause amplification resulting in fragment sizing different than those generated by *Taq* polymerase validated with the multiplex system. Not unlike the WGA findings, it was finally concluded that this technology would require extensive retooling to have direct application to a broad variety of forensic specimens. Given each specimen may have suffered different types of damage, repairing these may need to be done stepwise by the addition of single repair enzymes, incubated each under their respective optimal conditions, prior to multiplex STR typing. Furthermore, it should be noted that most repair enzymes act on double stranded DNA template. Since that is not the predominant disposition of DNA recovered from nylon membranes, the applicability

of such repair mixes is probably very limited to the ultimate forensic question addressed in this thesis.

A second aspect of Chapter 4 addressed the use of an amplification additive for increasing yield from low quantity samples. PCRboost™ was tested according to manufacturer's suggested protocol and proved to offer no advantage for typing low quantity samples when compared to same quantities amplified using standard multiplex procedures. Again, the main body of literature existing prior to the tests conducted herein consisted of presentation material from Biomatrica, the manufacturer of the product. Samples of this mixture were provided to forensic scientists free of cost at multiple conferences and were undoubtedly tested in a manner similar to tests conducted for this thesis. However, little indication of success is found in the literature. The main contribution stems from the group at San Jose State University that conducted testing on Restorase®. The poster for this work was presented at the 61st Annual American Academy of Forensic Sciences Meeting in Denver CO, USA. According to Le, *et. al.*, amplification of 300 year old bone samples and samples in the presence of humic acid and indigo dye was enhanced when amplification reaction mixtures contained the PCRboost™ mixture [Le, *et. al.*, 2009]. However, conclusions state that the improvements were noted when a different formulation ("formulation C") were used in these reactions. This was verified as a formulation different than that used for the Chapter 4 experiments and personal communication with the vendor at the conference resulted in the supply of a new formulation for forensic testing, marketed as STRboost™, which is apparently more conducive to improving multiplex typing. STRboost™ was released for testing following completion of this work, therefore the potential for this has not been investigated.

With respect to additives in general, it is important to note that many of the additives discussed with introductory material for Chapter 4 are historic and actually exist in the 10X reaction buffer supplied with the PowerPlex® 16 amplification kit. For example, Triton and BSA are standard components of the buffer and manual addition of other potential enhancement agents is unlikely to improve a kit that has undergone

several generations of research, development, and improvement as these principles were well-documented prior to the development and evolution of the PowerPlex® series of typing kits.

7.3.4 Extraction Enhancement Studies

This area of work has probably experienced the greatest long-term attention within the forensic community, since it is the primary aspect to consider in the interest of obtaining the highest amount of template DNA possible. However, very little progress has been made in this area in the last several decades, and this is apparent because many forensic labs still use standard organic extraction to purify samples.

Much of the focus has been on the automation of the extraction process as the need for databasing samples has dramatically increased during the last decade. However, since these procedures are carried out on reference samples, yield is of little concern and is less than that obtained by standard extraction, as clearly reported in Chapter 5. The main reason paramagnetic extraction was tested in this thesis was because it offered a very different mechanism for DNA capture and purification than techniques used when testing other methods. As summarized in section 7.1.4, the method offered no yield advantage when blood stains were tested and compared against stains extracted manually, nor did it afford recovery from membrane bound samples. While performance verification of this instrument has been conducted for reference samples, it was not primarily intended for use with low copy number or highly degraded samples [Krnajski, *et. al*., 2007]. Although paramagnetic extraction can offer improved removal of inhibitors, the extraction method did not achieve dissociation between template and nylon fragments during these tests.

While experimental design may be modified to include the nylon fragment in the cartridge well following the lysis step, no data has been generated to indicate this method has ever outperformed standard organic extraction. Furthermore, the overall robotic action following the lysis incubation would not serve to dissociate molecular bonds. The practice would also have the potential to cause mechanical malfunction due to the presence of solids interacting with the plungers and could have a

detrimental effect on the performance of the robot from a mechanical perspective. Therefore, this avenue was not further pursued. Throughout this work, only Qiagen offered an alternative small scale extraction robot based on magnetic technology (QIAcube®, and EZ1®); but since then, iPrep™ has been released by Invitrogen and is partnering with Applied Biosystems and has claimed that extraction recoveries exceed that obtained by organic extraction. The technology has yet to be automated and is still undergoing development within the forensic community.

With respect to alternative processing methods prior to extraction that may serve to increase yield, the cryogenic pulverization was disregarded as an appropriate method for processing nylon membranes. While increasing surface area is important for extraction of stains from bulky substrate and was introduced to the forensic community as a desirable method for processing teeth over a decade ago [Sweet and Hildebrand, 1998], the pulverization procedure is undesirable here because of the large processing vial that is included with the freezer mill. The long narrow shape of the vial adheres to large quantities of the actual exhibit and even rinsing would be extremely difficult throughout the length of the cylinder if a small volume of liquid were to be employed. When the test samples were processed, a length of nylon consistent with that excised as a membrane lane was processed and there was no collectable mass in the vial. Instead, powder of the matrix was coating the vial and the impactor and could not be easily collected or transferred to an extraction tube. Although freezer mills are commonplace in forensic laboratories processing skeletal remains, this aspect of processing is generally considered sample preparation, and extensive validation of sample preparations are not conducted. However, work subsequently conducted by another group had identical findings and indicated that processing of samples in this manner did not increase DNA yield over that achieved by direct extraction [Morenos, 2008]. Therefore, further work using this instrument was not pursued relative to this thesis.

7.3.5 Recovery and Typing of DNA from Nylon Membrane

A unique forensic question, this particular aspect of the work has not been heavily pursued by other laboratories, which exemplifies the original value of the forensic question upon which the various aspects of this work lie.

While clinical literature has indicated rare instances of amplification of template directly from nylon membrane, these assays are generally conducted with DNA quantities magnitudes greater than that dealt with throughout the course of RFLP typing and is localized to specific small surface areas on the membrane [Sheikh and Lazarus, 1997]. Therefore, copies may be stacked upon transfer and if they are not contacting the surface area of the membrane for binding, may actually be more conducive to STR amplification in the stacked arrangement. More often, the PCR method is employed prior to nylon binding in an effort to increase gene copy number prior to sequence specific probing [Saiki, 1989].

With respect to other contributions in this area, the work described throughout Chapter 6 and summarized in section 7.1.5 marks a unique contribution to the scientific literature and has provided relevant data which supports the possibility that cold case material bound to nylon may hold important value for cold case investigation as technology advances over time [Steadman, *et. al*., 2008b].

7.4 Future Directions

As with any scientific study, a positive solution generally gives rise to additional studies. Throughout the course of this work, other possible long-term projects have come to mind. Although these are not within the timeline, resources, or scope of this thesis, they do warrant suggestion.

The most obvious, which was already suggested in Chapter 6, would be an experiment designed to test different membrane types that were stored in different conditions or for various lengths of time. The critical difficulty of such a study is two-fold. First, test membranes are rare and unavailable. While many casework membranes were archived, training membranes and quality control membranes were

not always kept in the interest of space. Therefore, experimental exhibits are extremely difficult to locate. Furthermore, none of the investigated variables were kept standard from lab to lab, so it would be very difficult to determine if trends observed were due to membrane type, storage conditions, or period of storage. If one lab had membranes of the same nylon that were stored the same way, it may be possible to investigate the effect of storage period. Or, if two different labs had membranes stored similarly and created at similar time points, it might be possible to look at matrix variability with respect to recovery. But again, these scenarios are limited due to the rarity of test membranes at the onset.

Expansion of the work involving different membrane types and storage conditions would only serve to further exercise the applicability of optimal methods discovered through this work. The problem is that the current technologies can give a partial result, but the real question surrounding future directions becomes whether more information could be taken. This prompts some discussion on what alternative approaches may be available that were not tested, or perhaps unavailable, throughout the course of this work.

One study that could easily be pursued from this point forward would be evaluation of post-amplification clean-up methods. Recent work presented by Milne and Mayntz-Press explored the usefulness of post-amp filtration and compared the effectiveness of two such clean up systems, Qiagen's MinElute® PCR Purification Kit and Promega's Wizard® SV Gel and PCR Clean-Up System [Milne and Mayntz-Press, 2008]. This process achieves cleaning and concentration of PCR product while salts, inhibitors, primers, dyes, and other electrokinetic competitors are removed. The study explored peak heights and allele recovery when sensitivity studies were conducted with and without post-amplification clean-up. The study also applied the method to bone cases and UV damaged samples. Much of the work was performed using half-volume reactions, to further increase the potential for amplification and detection of low quantity samples. Using both systems, the number of alleles and intensity of peaks was drastically increased following post-amplification purification. In one instance, a profile that would have had a random match probability of 1 in 73 was improved to a

probability of 1 in 24 quadrillion following clean-up with the Wizard® system. And somewhat surprisingly, a sample with no "called" alleles (using the laboratory standard analysis parameters) was cleaned up with the MinElute® system and achieved the detection of twelve peaks across seven loci. Prior to this work, the process had been routinely used in research conducted in Jack Ballantyne's lab and emerged as a prominent approach following publication by he and Smith in 2007 [Smith and Ballentyne, 2007]. Stemming from her graduate studies in Ballantyne's group, Mayntz-Press joined Arizona in further exploration of the devices for this purpose, which expanded on reports already generated in that lab by Merena, who reported in a comparison of three Qiagen products for the same application in 2006 [Merena, 2006]. Merena reported that QIAquick® PCR Purification Kit and the MinElute® Kit performed similarly, both successful in achieving detection at larger loci that was not possible prior to clean-up. Ballantyne and Smith presented a focused study on the MinElute® Kit and reported a four-fold increase in fluorescent signal when product was purified and achieved full profiles down to 20 pg of input template.

While the notion of post-amplification clean-up seems promising, the main deterrent is simply that this type of low-template processing is not allowed by NDIS for CODIS profile entry. The limitations of such a column would take considerable performance verification for use prior to casework application, but could be used thereafter for direct comparison between membrane samples and known reference samples in a case. The method is very appealing in that amplified product could first be analyzed using normal processes and clean-up could then be attempted on a sample derivative that is normally discarded; the method requires no additional sampling.

A fairly broad series of buffers and reagents were tested for the ability to dissociate DNA from nylon. While many promising methods have been explored or reasonably disregarded, one then turns to that which may be just theoretically possible. One such method that was not tested is that of nucleic acid isolation using supercritical fluids. This involves treating a sample with a supercritical fluid, that is, a substance above the critical temperature and pressure where the fluid has coexisting densities of gas and liquid phases and behaves much like a liquid solvent [Nivens and Applegate,

1999]. While the instrumentation for such a process is unavailable within the host lab and therefore the details of such work were not pursued, the theory has proven to capture DNA and RNA of high integrity from bacterial cells, fibers, and protozoa. Furthermore, the technology may not be limited to nylon extraction, but also soils and possibly other biological stains and substrates. The forensic implications surrounding the extraction of organic components on the surface of hair have been investigated, along with mitochondrial sequencing of such hairs [Benner, *et. al*., 2003]. However routine purification of RNA or DNA by this method would be a monumentally different approach to nucleic acid isolation and purification within the forensic community.

If the recovery method were to be improved to a point where dual amplification could be performed, future directives may include the design of a two-plex amplification containing novel TH01 and TPOX primers with recognition regions inside the *Hae*III cut sites. Sequences for these STR regions were analyzed for potential primer sets using Primer-BLAST primer designing tool, and two sets of primers with similar amplicon range were identified as feasible for co-amplification of TPOX and TH01. the primers are:

TH01-

Forward: TGTTCCTCCCTTATTTCCC (19-mer, 47.4% GC, Tm=56.5 ^{o}C)

Reverse: CACAGGGAACACAGACTCCA (20-mer, 55% GC, Tm=59.7 ^{o}C)

TPOX-

Forward: GCACAGAACAGGCACTTAGGGAACC (25-mer, 56% GC, Tm=59.7 ^{o}C)

Reverse: CTGTCCTTGTCAGCGTTTATTTGCC (25-mer, 48% GC, Tm=57.2 ^{o}C)

The TH01 set would result in an 81 bp fragment for an individual expressing nine repeats; therefore the amplicon range would be 61-100 bp. Likewise, for TPOX, a person expressing 11 repeats would result in a 105 bp product with a 95-113 bp amplicon size range. While both of these would be highly conducive to typing degraded samples, the product ranges do overlap and a system employing different fluorescent tags would be required to use such a primer scenario. Unfortunately, the limitations of primer sequences within the *Hae*III cut sites for these loci also greatly

restricts the ability to avoid overlap, as both loci become bound to this short range of possibilities.

As with all systems, the amplification parameters would need to be refined, but since these primers all have very similar GC content, a common annealing temperature may be achievable which would result in efficient yet specific binding of all primers [Coyne, *et. al*., 2001]. One challenge would be the design of reliable and reproducible ladders for sizing the amplified products compatible with GeneMapper™ ID software. While seemingly feasible, this aspect of work is outside the resources of this project. Furthermore, although the two CODIS eligible loci were selected in an effort to produce database-ready profiles, any system like this would require NDIS submission and approval prior to any amplified samples being entered into the database. Never having approved a novel system such as this for a small defined population of forensic samples, it is unlikely the FBI would approve the primer system. If the work were submitted, the review process alone would not meet the timeline of the thesis. Aside from compliance issues, problems with inadequate template recovery from the membrane to begin with are still looming and are only worsened by the prospect of additional amplification processes on the amount of existing recovered template consumed to attempt a 16-plex profile. For both compliance and scientific reasons, along with the absence of resources and test membrane lanes, the primers sets were not built or further pursued.

Finally, the newest technology that may be applicable to this project that is forthcoming include recently redesigned multi-plex kits that claim to improve low-template, degraded, or inhibited sample profiles. Applied Biosystems has embarked upon a next-generation of STR chemistries and will release the Identifiler® Plus PCR Amplification kit for use in the United States. The European equivalent, SEfiler® Plus PCR amplification kit is also on the horizon [Czar, 2009]. The main advantage to the next-generation kits as compared to old kits (the primer sequences of which are the same in both generations of kits), is that buffering systems discovered throughout the development of other technologies since the original release of the classic multi-plexes have been found to result in better profile quality for challenging samples.

This means increased sensitivity, lower baseline, better resolution of minor contributors in the presence of a major contributor and improved performance on inhibited samples by obtaining more peaks of greater intensity and improved heterozygote balance [Applied Biosystems, 2009]. Primer redesign did not take place upon development of this kit and was conserved from the SGM Plus® configuration.

In 2008, Promega responded to the need for a single multiplex that combined the 11 SGM Plus® loci (D8S1179, D18S51, D21S11, FGA, TH01, vWA, D2S1338, D3S1358, D16S539, D19S433 and Amelogenin) with the newly recommended loci from the European Network of Forensic Science Institutes (ENFSI); these are D2S44, D19S1248, D22S1045, D1S1656, and D12S391 [Sprecher, *et. al*., 2009]. In response to feedback from numerous labs, two basic systems were developed. One system incorporates the European Standard loci as mini STRs (D3S1358, D8S1179, D18S51, D21S11, FGA, TH01, and vWA), the other incorporates the new ENFSI loci as mini STRs [Sprecher, *et. al*., 2009]. Ultimately, both systems achieve genotypes across the same 16 loci and either kit can be purchased with or without SE33 primers present. Kits with the SE33 primers are denoted as PowerPlex® ESI 17 and PowerPlex® ESX 17 and produce amplicons that are the longest among those in the CXR-ET panel [Sprecher, *et. al*., 2009]. Amplicon arrangement within the five dye chemistry is depicted for these kits in Figures 7.1 and 7.2.

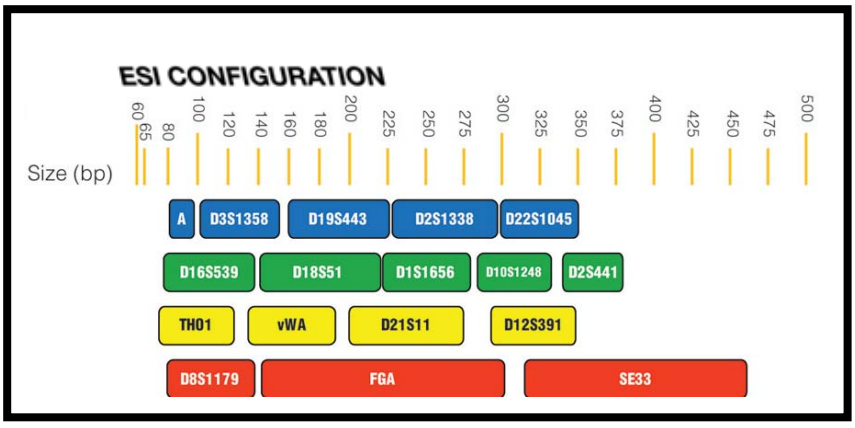

Figure 7.1: PowerPlex® ESI (European Standard Investigator) kit configuration. The ESI kit contains standard European loci as mini STRs. The figure represents the 17 plex kit with the SE33 included [Sprecher, *et. al*., 2009].

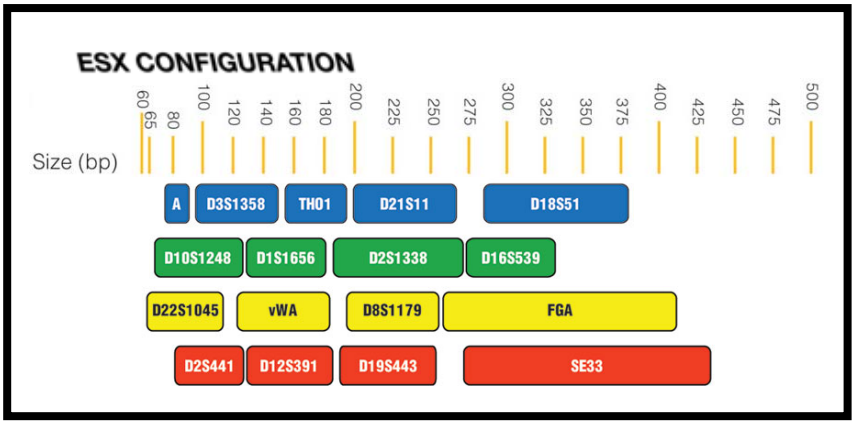

Figure 7.2: PowerPlex® ESX (European Standard Extended) kit configuration. The ESX kit contains ENFSI newly recommended loci as mini STRs. The figure represents the 17 plex kit with the SE33 included [Sprecher, *et. al*., 2009].

These kits designed for the European laboratories both contain hot start *Taq* polymerase in the master mix and boast a more robust buffering system that will result in greater sensitivity, decreased inhibition, and the ability to better type degraded or otherwise samples. The introduction of these kits marks a vast difference from previous multiplexes prepared for the European community. Improvements to kits used in the United States have been more subtle, consisting mainly of incorporation of *Taq* in the master mix and an improved buffer system. The PowerPlex® 16 kit went otherwise unchanged as primer sequences are concerned; the new kit reflects the inclusion of hot start (HS) *Taq* in the kit and is called PowerPlex® 16 HS [Green, 2008]. Data from this kit has been submitted and should be in the final stages of FBI approval as an NDIS approved typing kit [Green, 2008].

With these new and improved products forthcoming, it may become possible to test a new kit's ability to type DNA recovered from membranes. While the European kits are obviously not NDIS approved, it will be interesting to see if these platforms do improve the capability to produce profiles from challenging samples. One would begin to explore the possibility of using these kits by interrogating the primer sequences for *Hae*III restriction cut sites. Since the primer design is very different between the ESI and ESX kits, one system may prove to have a much greater advantage for amplifying restricted samples. The number of loci containing restriction sites would need to be weighed against which loci contain them, and the kit

to first test would be that with the least amount of affected loci in the mini STR regions.

With respect to the kit currently available in the United States, the PowerPlex® 16 HS may prove to out-perform the current 16 plex kit if it can improve specimen sensitivity. Since inhibition is not likely preventing typing of membrane-recovered samples and the primer sequences are conserved, this kit may not be sufficient for improving profiling unless the buffering system is truly capable of increased sensitivity for loci typed within the classic configuration of the 16-plex. Nevertheless, upon CODIS approval and widespread validation of this kit in the US community, this may be a reasonable future direction for this work. Finally, although future NDIS approval is unlikely, another candidate for testing would be Applied Biosystems' MiniFiler™ kit, a mini STR kit with primers designed to reduce amplicon products across 10 of the Identifiler® system loci as much as 201 base pairs per locus [Applied Biosystems, 2007b]. As with any Applied Biosystems kit, feasibility for recovered membrane template is more difficult to estimate due to the proprietary nature of the primer sequences. Without first interrogating the primer and intervening sequences for restriction sites, applicability remains a complete unknown and impedes the testing of this kit within the scope of this project.

Speculative efforts for any project may take a single direction or may employ a combination of new technology for improved profiling of challenging samples. Should supercritical fluid extraction have promise, products may be tested with current or future multi-plex kits following further research of kit configuration with respect to primer and amplicon sequence. Together, with post-amplification clean-up, one can foresee a separate volume of research in an effort to improve profile resolution from membrane-recovered template.

7.5 Overview of Scientific Contribution

The forensic laboratory can obtain an exceptional amount of information from forensic specimens of limited quantity and quality, including, potentially, the DNA preserved on archived membranes from RFLP analyses conducted on cases in the

past. Challenging samples presented to the casework lab drive the improvement of the capability of generating DNA profiles from difficult samples. This thesis explores and defines the sensitivity limits of current typing technologies, it reports the outcome of tests designed to investigate the utility of whole genome amplification (WGA), PCR enhancement agents, repair enzymes, and alternative processing/extraction methods for forensic applications where the quantity of DNA is limited. Ultimately, the purpose of this project is to develop methods that can be used to recover DNA from nylon supports for subsequent STR PCR testing. The applicability of this endeavor to the practical lab is broad since many cold cases exist where DNA evidence was consumed for RFLP testing and exists in a membrane-bound state in archived storage. Therefore, the thesis describes the systematic development of a procedure to recover DNA from archived RFLP membranes for subsequent STR PCR typing.

It was found that the WGA, PCR additives, repair polymerase systems, and alternative processing/extraction methods offered little or no advantage in enhancing forensic DNA profile information when used prior to multiplex typing. The results described herein concur with the reports of other groups who have subsequently conducted comparable studies and can eliminate these approaches in their current form.

With respect to recovery from archived membranes, an optimal procedure was identified and a common extraction buffer was successful in recovery of DNA from archived RFLP membranes. This demonstrates the ability to obtain partial STR profile information from samples previously considered to have been entirely consumed by the RFLP process.

The premise of this thesis was derived from a notorious serial murder case in the United States known as the BTK investigation; the investigation took place between 1973 and 2003, at which time a suspect was identified who later pled guilty for the crimes. Decades prior to the final conclusion of the case, DNA from seminal fluid left at one scene had undergone RFLP analysis and remained in archived storage at

the private lab that conducted the analysis. The work conducted herein coincided with the last couple years of the investigation and reflects a very true direct casework application, even though the case was resolved through other analytical techniques. While it is unknown how many cold cases this may affect, the demonstration of the generation of even partial STR profiles from archived RFLP membranes opens another possibility in the investigation and prosecution of cold cases.

8.0
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Appendices

Appendix 1: Definitions of Acronyms and Abbreviations

ACRONYM OR ABBREVIATION	DEFINITION
6-4PP	6-4 photoproducts
AB	Applied Biosystems
ACS	American Chemical Society
AP	apurinic or apyrimidinic
APS	ammonium persulfate
ANOVA	analysis of variance
ASCLD-LAB	American Society of Crime Lab Directors-Laboratory Accreditation Board
BER	base excision repair
BSA	bovine serum albumin
BTK	bind, torture, kill
CE	capillary electrophoresis
CDP	cyclobutane pyrimidine dimers
CODIS	Combined DNA Indexing System
CPS	cycles per second
Ct	cycle threshold
CXR	carboxy-X-rhodamine
DAB	DNA Advisory Board
DFX	Differex™
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOP-PCR	degenerate oligonucleotide-primed PCR
DSS	dual strip solution
DTT	dithiothreitol
EBSS	extraction buffer stock solution
EDTA	ethylenediamine tetraacetic acid
FBI	Federal Bureau of Investigation
FL	Fluorescein
FSS	Forensic Science Service
HEV	high elution volume
HMW	high molecular weight
HPLC	high pressure liquid chromatography
IPC	internal positive control
ILS	internal lane standard
JOE	6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein

Appendix 1: Definitions of Acronyms and Abbreviations

ACRONYM OR ABBREVIATION	DEFINITION
KCPD	Kansas City Police Department
L	liter
LCN	low copy number
LEV	low elution volume
LMP	ligation mediated PCR
HRP	horseradish peroxidase
HWE	Hardy Weinberg Equilibrium
MAX	Maxwell®
mito	mitochondrial
µL	microliter
mL	milliliter
µg	microgram
mg	milligram
MGMT	methyl guanine methyl transferase
NAC	negative amplification control
NDIS	national DNA indexing system
NER	nucleotide excision repair
NFW	nuclease free water
ng	nanogram
NIST	National Institute of Standards and Technology
NRC	National Research Council
OCME	City Office of the Chief Medical Examiner (New York City)
OD	optical density
PAC	positive amplification control
PASW	Predictive Analytics Software
PCI	phenol;chloroform;isoamyl alcohol
PCR	polymerase chain reaction
PEP	primer extension PCR
pg	picogram
POP4	performance optimized polymer 4%
PRO K	proteinase K
PRSG	PCR of randomly sheared genomic [DNA]

Appendix 1: Definitions of Acronyms and Abbreviations

ACRONYM OR ABBREVIATION	DEFINITION
PTC	Paternity Testing Corporation
RB	raised baseline
RD	restriction digest/digested
RE	restriction enzyme
RFLP	restriction fragment length polymorphism
RFSC	Regional Forensic Science Center
RFU	relative fluorescence unit
RNC	reagent negative control
RPM	revolutions per minute
RRX	rhodamine red™-X
RT-PCR	real time polymerase chain reaction
SCOMP	single cell comparative genomic hybridization
SDS	sodium dodecyl sulfate
SEB	stain extraction buffer
SNP	single nucleotide polymorphism
SP	sperm (when denoting extraction buffer type)
SPSS	Statistical Package for Social Sciences
SSC	sodium chloride sodium citrate buffer
SSPE	sodium chloride sodium phosphate EDTA buffer
STR	short tandem repeat
SWGDAM	Scientific Working Group for DNA Analysis Methods
TAE	tris acetic acid EDTA buffer
TBE	tris boric acid EDTA buffer
TE	tris EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TLAD	T7-based linear amplification of DNA
TMAC	tetra-methylammonium chloride
TMB	tetramethyl benzidine
TMR	carboxy-tetramethylrhodamine
TNE	tris sodium chloride EDTA buffer
TWGDAM	Technical Working Group for DNA Analysis Methods
UV	ultraviolet
V	Volt
VNTR	variable number tandem repeat
WGA	whole genome amplification

Appendix 2: Chemicals and Reagents

All reagents were prepared with ultra-purified, UV irradiated water and stored at room temperature unless otherwise indicated.

ACETONE was obtained commercially from Pharmco, Brookfield, CT, USA.

ACRYLAMIDE SOLUTION (6% Acrylamide) was prepared by dissolving 180 g urea in 240 mL water, followed by the addition of 50 mL 10X TBE and 75 mL PagePlus Concentrate. The mixture was filtered through a 0.45 μm filter and stored at 2-8^{o} C. Aliquots were filtered and degassed just prior to gel preparation.

AGAROSE, OmniPur® PCR Plus, was obtained commercially from EM Science, USA.

ALKALINE LYSIS SOLUTION was prepared by dissolving 2.24 g KOH (potassium hydroxide) and 1.54 g DTT (dithiothreitol) in 70 mL water. Following the addition of 2 mL 0.5M EDTA pH 8.00, the solution was brought to a final volume of 100 mL, autoclaved, and stored at 2-8^{o} C.

ALKALINE MEMBRANE STRIP SOLUTIONS were prepared for a dual step strip process. The STEP 1 WASH consisted of 0.4M NaOH (sodium hydroxide), which was prepared by mixing 800 μL 10% NaOH with 4.2 mL water. The STEP 2 WASH consisted of a 0.1X SSC/0.1% SDS solution which was prepared by combining 25 μL of 20X SSC and 25 μL 20% SDS with 4.95 mL water.

AMMONIUM PERSULFATE (APS), molecular biology grade, was obtained commercially from Promega Corporation (Madison, WI, USA). AMMONIUM PERSULFATE SOLUTION -10% was prepared by dissolving 0.5 g ammonium persulfate in a final volume of 5 mL water. The mixture was filtered through a 0.45 μm filter, aliquotted, and stored frozen.

AmpliTaq GOLD® DNA POLYMERASE (5 U/μL) was obtained commercially from Applied Biosystems, Foster City, CA, USA. The enzyme was stored frozen.

BORIC ACID, proteomics grade, was obtained commercially from Amresco, Solon, OH, USA.

BROMOPHENOL BLUE LOADING SOLUTION used as loading dye for agarose gels was supplied by Promega Corporation, Madison, WI, USA, and stored frozen. The solution consists of 95% formamide, 0.05% bromophenol blue, and 10mM NaOH (sodium hydroxide).

BUFFER WITH EDTA – 10X used for capillary electrophoresis instrumentation was obtained commercially from Applied Biosystems, Foster City, CA, USA. The stock was stored at 2-8° C. Working solutions (1X) were prepared by mixing 3 mL of 10X buffer with 27 mL water.

CHLOROFORM was obtained commercially from Sigma, St. Louis, MO, USA.

CHROMOGEN:TMB was received from Applied Biosystems, Foster City, CA, USA, stored at 2-8° C, and brought to room temperature prior to preparing working solutions. CHROMOGEN:TMB SOLUTION was prepared by the addition of 30 mL of room temperature 100% ethanol to the powder. The mixture was thoroughly mixed and stored at 2-8° C.

CITRIC ACID ($C_6H_8O_7$), anhydrous, was obtained commercially from Sigma, St. Louis, MO, USA.

CITRATE BUFFER - 10X (0.01M sodium citrate, pH 5.0) was prepared by dissolving 368 g $Na_3C_6H_5O_7$-$2H_2O$ (sodium citrate tribasic dihydrate) in 1600 mL water. The pH was adjusted to 5.00 by addition of citric acid (free acid, anhydrous). This mixture was adjusted to a final volume of 2 L with water. Working solutions (1X) were prepared by mixing 100 mL of 10X citrate buffer with 900 mL water.

DITHIOTHREITOL (DTT), molecular biology grade, was obtained commercially from Sigma, St. Louis, MO, USA.

DMSO was obtained commercially from Sigma, St. Louis, MO, USA.

DNA QUANTIFICATION STANDARDS for agarose gel analyses were prepared from a stock solution of Human Genomic DNA obtained commercially from Promega Corporation, Madison, WI, USA. Using the Human Genomic DNA, 400 µL of a 100 ng/µL stock concentration was prepared. From this stock, 50, 25, 10, and 5 ng/µL concentrations were prepared as described in Table 2.9. The standard series was stored frozen.

Target Concentration	**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

Table 9.1: Preparation of agarose gel standard series. A 100 ng/µL stock solution was mixed with TE in for the preparation of 50, 25, 10, and 5 ng/µL concentrations of human genomic DNA.

DNA IQ™ CASEWORK SAMPLE KIT FOR MAXWELL® 16 was obtained commercially from Promega Corporation, Madison, WI, USA. The kit contains sample cartridges, LEV plungers, elution tubes, elution buffer, and lysis buffer.

ETHANOL, 100% (200 proof) molecular biology grade, was obtained commercially from Aldrich, Milwaukee, WI, USA.

ETHYLENEDIAMINETETRACETIC ACID - 0.5M pH 8.0 (EDTA) was obtained commercially from Sigma, St. Louis, MO, USA.

ETHIDIUM BROMIDE, biotechnology grade powder, was received from Amresco, Solon, OH, USA. ETHIDIUM BROMIDE SOLUTION was prepared by the addition of 1 mL water to 5 mg of ethidium bromide; this solution was stored in the dark.

ETHIDIUM BROMIDE STAIN SOLUTION was prepared by the addition of 50 µL ethidium bromide solution to 200 mL 1X TAE. This solution was stored in the dark.

EXTRACTION BUFFER STOCK SOLUTION (EBSS) was prepared by dissolving 1.21 g $C_4H_{11}NO_3$ (Tris base) and 5.84 g NaCl (sodium chloride) in a total volume of 500 mL of water. The pH was adjusted to 8.0 with HCl (hydrochloric acid) and then 100 mL 20% SDS and 20 mL 0.5M EDTA pH 8.0 were added. The solution was brought to a final volume of 1 L and autoclaved prior to storage.

GenomiPhi™ DNA AMPLIFICATION KITS were obtained commercially from Amersham Biosciences, Buckinghamshire, UK. Enzyme was ultra-cooled (-80^{o} C); other components were stored frozen. The kit includes sample buffer, reaction buffer, enzyme mix, and Lambda control DNA (10 ng/µL).

*Hae*III RESTRICTION ENZYME was obtained commercially from Promega Corporation, Madison, WI, USA at a concentration of 10 u/µL. The enzyme was provided with 10X MULTI-CORE™ buffer and acetylated BSA at a concentration of 10 µg/µL.

HYBRIDIZATION SOLUTION (5X SSPE, 0.5% w/v SDS) was prepared by the addition of 250 mL 20X SSPE and 25 mL 20% SDS to 725 mL water.

HYDROCHLORIC ACID (HCl), concentrated, was obtained commercially from Sigma, St. Louis, MO, USA. HYDROCHLORIC ACID SOLUTION – 1M was prepared by mixing 4.17 mL concentrated HCl (hydrochloric acid) with 45.83 mL water.

HYDROGEN PEROXIDE - 30% (H_2O_2) was obtained commercially from Sigma, St. Louis, MO, USA and stored at 2-8^{o} C.

METHYLENE BLUE was obtained commercially from Sigma, St. Louis, MO, USA.

METHYLENE BLUE STAINING SOLUTION was prepared by dissolving 0.03 g of methylene blue in 100 mL a 0.3M sodium acetate solution (pH 5.2).

ISOPROPANOL was obtained commercially from Fisher Scientific, Fair Lawn, NJ, USA.

METHANOL (HPLC grade) was obtained commercially from Pharmco, Brookfield, CT, USA.

MICROCON® 100 centrifugal filter devices used for DNA concentration and purification were obtained from Millipore Corporation, Bedford, MA, USA.

NEUTRALIZING SOLUTION for membrane binding applications was prepared by mixing 500 mL 3.0M NaCl (sodium chloride) with 500 mL 2.0M Tris-HCl pH 8.0.

NUCLEASE-FREE WATER (NFW) was prepared by autoclaving ultrafiltered water.

NYLON MEMBRANES for human DNA quantification applications (Biodyne® B 0.45 μm) were obtained commercially from Pall Corporation, Pensacola, FL, USA. Nylon membranes for cross-linking experiments (MagnaGraph® transfer membranes), were obtained commercially from MSI, Westborough, MA, USA.

PAGE PLUS CONCENTRATE, biotechnology grade, was obtained from Amresco, Solon, OH, USA.

PCRboost™ was obtained commercially from Biomatrica and stored at room temperature.

PERFORMANCE OPTIMIZED POLYMER 4 (POP4) was obtained commercially from Applied Biosystems, Foster City, CA, USA. The polymer was stored at 2-8° C.

PHENOL (saturated biotech grade) was obtained commercially from Fisher Scientific, Fair Lawn, NJ, USA.

PHENOL/CHLOROFORM/ISOAMYL ALCOHOL (PCI - 25:24:1) was obtained at pH 7.0-8.0 from Amresco, Solon, OH, USA and stored at 2-8° C.

PHOSPHATE BUFFERED SALINE (PBS) was prepared by dissolving 0.2 g KCl (potassium chloride), 8.0 g NaCl (sodium chloride), 0.24 g KH_2PO_4 (potassium phosphate monobasic anhydrous), and 1.44 g Na_2HPO_4 (sodium phosphate dibasic anhydrous) in 800 mL water. The pH was adjusted to 7.4 with HCl (hydrochloric acid) and the solution then brought to a final volume of 1.0 L with water. This solution was autoclaved and stored at 2-8° C.

POTASSIUM HYDROXIDE (KOH), ACS reagent, was obtained commercially from Sigma, St. Louis MO, USA.

POTASSIUM CHLORIDE (KCl), ReagentPlus grade, was obtained commercially from Sigma, St. Louis MO, USA.

PowerPlex® 2.1, PowerPlex® 16 BIO, and PowerPlex® 16 TYPING KITS were obtained from Promega Corporation, Madison, WI, USA. Kits include Gold ST*R 10X buffer, primer mix, allelic ladder mix, internal lane standard, and control DNA

(K562 or 9947A); gel tracking dye and blue dextran loading solution are also included in the 2.1 and BIO kits. All components were stored frozen.

PRE-WETTING SOLUTION (0.4N NaOH, 25 mM EDTA) was prepared by adding 40 mL of 10N NaOH (sodium hydroxide) and 50 mL of 0.5M EDTA to 910 mL of water.

PROTEINASE K (20 mg/mL) was obtained commercially from Amresco and stored at 2-8^{o} C.

QuantiBlot® HUMAN DNA QUANTIFICATION KITS were obtained commercially from Applied Biosystems, Foster City, CA, USA and stored at 2-8^{o} C. The kit includes D17Z1 probe, HRP-SA enzyme conjugate, bromothymol blue solution, DNA standard A, and calibrators 1 and 2.

Quantifiler™ HUMAN DNA QUANTIFICATION KIT was obtained commercially from Applied Biosystems, Foster City, CA, USA and stored at 2-8^{o} C. The kit includes PCR reaction mix, human DNA standard, and human primer mix.

RESTORASE® DNA POLYMERASE was obtained commercially from Sigma, St. Louis, MO, USA. The enzyme was stored frozen.

SODIUM ACETATE (anhydrous) was obtained commercially from Sigma, St. Louis, MO, USA. SODIUM ACETATE SOLUTION (0.3M) was prepared by dissolving 2.641 g of sodium acetate in 70 mL of water; this mixture was then treated with glacial acetic acid to a pH of 5.2. The solution was brought to a final volume of 100 mL.

SODIUM CITRATE TRIBASIC DIHYDRATE ($Na_3C_6H_5O_7$-$2H_2O$), ACS reagent, was obtained commercially from Sigma, St. Louis, MO, USA.

SODIUM DODECYL SULFATE - 20% (SDS), electrophoresis grade, was obtained commercially from Fisher Scientific, Fair Lawn, NJ, USA.

SODIUM CHLORIDE (NaCl), molecular biology grade, was obtained commercially from Sigma, St. Louis, MO, USA. SODIUM CHLORIDE SOLUTION - 3M was prepared by dissolving 175.32 g NaCl (sodium chloride) in a final volume of 1.0 L with water. The solution was autoclaved prior to storage.

SODIUM HYDROXIDE (NaOH), SigmaUltra grade anhydrous pellet form, was obtained commercially from Sigma, St. Louis, MO, USA.

SODIUM HYDROXIDE SOLUTION–10% was prepared by dissolving 10 g NaOH (sodium hydroxide) in 80 mL of water. The solution was brought to a final volume of 100 mL with water. SODIUM HYDROXIDE SOLUTION-0.4M was prepared by mixing 800 µL of 10% NaOH (sodium hydroxide) with 4.2 mL NFW. Solutions were stored in plastic bottles.

SODIUM HYDROXIDE (NaOH - 0.5M) / SODIUM CHLORIDE (NaCl - 0.5M) was prepared by dissolving 2.0 g NaOH (sodium hydroxide) and 2.9 g NaCl (sodium chloride) in a final volume of 100 mL of water. Following autoclaving, the solution was stored in a plastic bottle.

SODIUM PHOSPHATE DIBASIC ANHYDROUS (Na_2HPO_4), SigmaUltra grade, was obtained commercially from Sigma, St. Louis, MO, USA.

SODIUM PHOSPHATE MONOBASIC ANHYDROUS (NaH_2PO_4), SigmaUltra grade, was obtained commercially from Sigma, St. Louis, MO, USA.

SPOTTING SOLUTION (0.4N NaOH, 25mM EDTA, 0.00008% bromothymol blue) was prepared by adding 3 mL of 10N NaOH (sodium hydroxide), 3.75 mL of 0.5M EDTA, and 150 µL 0.04% bromothymol blue to 65 mL water.

SSC-20X was prepared by dissolving 175.3 g NaCl (sodium chloride) and 88.2 g $C_6H_5Na_3O_7$-$2H_2O$ (sodium citrate tribasic dehydrate) in 800 mL water. The pH was adjusted to 7.0 with HCl (hydrochloric acid) and bring to a final volume of 1L. A 2X solution was prepared by adding 100 mL 20X SSC to 900 mL water.

SSPE BUFFER – 20X (3.6M NaCl, 200 mM NaH_2PO_4-H_2O , 20 mM EDTA, pH 7.4) was prepared by dissolving 210 g NaCl (sodium chloride) in approximately 800 mL water. Following the addition of 40 mL 0.5M EDTA and 24 g NaH_2PO_4 (sodium phosphate, monobasic, anhydrous), the pH was adjusted to 7.4 using 10N NaOH (sodium hydroxide). The solution was then brought to a final volume of 1 L with water.

STAIN EXTRACTION BUFFER WORKING SOLUTION (SEB) was prepared by dissolving 0.3 g DTT in 50 mL extraction buffer stock solution (EBSS).

STRIP SOLUTION (0.1X SSC, 0.1% SDS) was prepared by mixing 25 µL 20% SDS, 25 µL of 20X SSC and 4.95 µL NFW.

STRONG STRIP SOLUTION (0.5M NaOH, 1% SDS) was prepared by dissolving 2.0 g NaOH (sodium hydroxide) in 80 mL of water. Following the addition of 5 mL 20% SDS, the solution was brought to a final volume of 100 mL.

TAE - 20X was prepared by dissolving 96.6 g $C_4H_{11}NO_3$ (Tris base), 22.8 mL glacial acetic acid, and 40.0 mL 0.5M EDTA pH 8.0 in a final volume of 1.0 L with water. A 1X working solution was prepared by mixing 50 mL 20X TAE with 950 mL water.

TBE - 10X was prepared by dissolving 107.81 g $C_4H_{11}NO_3$ (Tris base) and 55.0 g H_3BO_3 (boric acid) in 800 mL water. To this, 40.0 mL 0.5M EDTA pH 8.0 was added and the solution was then brought to a final volume of 1.0 L with water. A 1X working solution was prepared by mixing 100 mL 10X TBE with 900 mL water.

TE BUFFER was prepared by dissolving 1.21 g $C_4H_{11}NO_3$ (Tris base) in 800 mL water. The pH was adjusted to 7.5 with HCl (hydrochloric acid). The solution was brought to a final volume of 1.0 L with water and autoclaved prior to storage.

N, N, N', N'-TETRAMETHYLETHYLENEDIAMINE (TEMED), electrophoresis grade, was obtained commercially from Promega Corporation, Madison, WI, USA.

TNE BUFFER was prepared by mixing 1.21 g $C_4H_{11}NO_3$ (Tris base), 5.84 g NaCl (sodium chloride), and 2.0 mL 0.5M EDTA in 800 mL water. The pH was adjusted to 8.0 with HCl (hydrochloric acid). The solution was brought to a final volume of 1.0 L with water and autoclaved prior to storage.

TRIS BASE ($C_4H_{11}NO_3$), crystallized molecular biology grade, was obtained commercially from Fisher Scientific, Fair Lawn, NJ, USA.

TRIS-HCl – 1.0M pH 7.5 was prepared by dissolving 12.11 g Tris base in 80 mL of water. The pH was adjusted to 7.5 with HCl (hydrochloric acid). The solution was brought to a final volume of 100 mL with water and autoclaved prior to storage.

TRIS-HCl - 2.0M pH 8.0 was prepared by dissolving 242.2 g $C_4H_{11}NO_3$ (Tris base) in ~800 mL water. The pH was adjusted to 8.0 with HCl (hydrochloric acid). The solution was brought to a final volume of 1.0 L with water and autoclaved prior to storage.

TRIS-0.2M: SSC-2X was prepared by combining 100 mL 2.0M Tris-HCl pH 8.00 and 100 mL 20X SSC with 800 mL water.

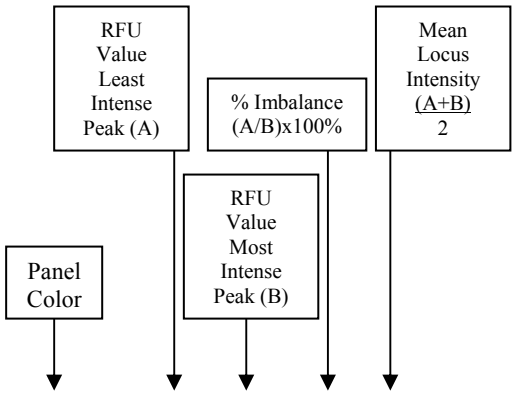
UREA, ultra pure grade, was obtained commercially from Amresco, Solon, OH, USA.

WASH SOLUTION (1.5X SSPE, 0.5% w/v SDS) was prepared by adding 150 mL of 20X SSPE and 50 mL of 20% SDS to 1,800 mL of water. The solution was stored at room temperature and warmed prior to use to ensure that any precipitates were in solution.

WATER was ultrapurified and UV irradiated for reagent preparation applications.

Appendix 3: Performance Verification Sensitivity Study-Intensity Data

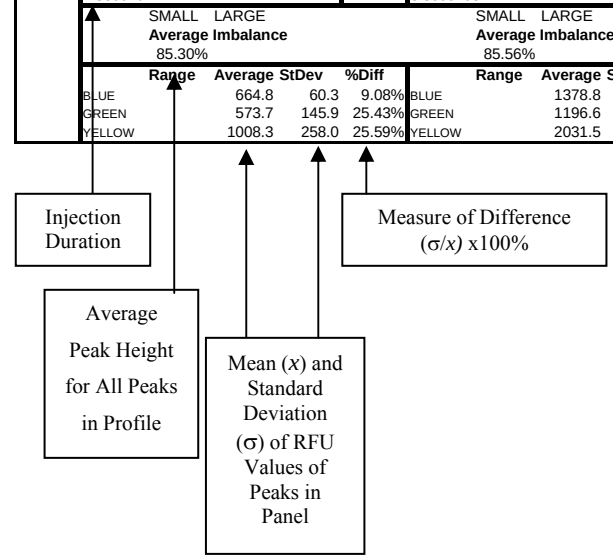
Appendix legend for 3100-*Avant* capillary data:



	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean
BLUE	D3	543	624	87.02%	583.50	D3	1145	1299	88.14%	1222.00	D3	1633	1902	85.86%	1767.50
BLUE	Th01	743	754	98.54%	748.50	Th01	1543	1545	99.87%	1544.00	Th01	2204	2240	98.39%	2222.00
BLUE	D21	571	777	73.49%	674.00	D21	1180	1589	74.26%	1384.50	D21	1650	2270	72.69%	1960.00
BLUE	D18	644	714	90.20%	679.00	D18	1339	1459	91.78%	1399.00	D18	1853	2066	89.69%	1959.50
BLUE	Penta E	467	811	57.58%	639.00	Penta E	996	1693	58.83%	1344.50	Penta E	1185	2188	54.16%	1686.50
GREEN	D5	748	821	91.11%	784.50	D5	1567	1679	93.33%	1623.00	D5	2228	2426	91.84%	2327.00
GREEN	D13	639	752	84.97%	695.50	D13	1358	1592	85.30%	1475.00	D13	1890	2234	84.60%	2062.00
GREEN	D7	435	442	98.42%	438.50	D7	906	907	99.89%	906.50	D7	1256	1279	98.20%	1267.50
GREEN	D16	376	528	71.21%	452.00	D16	778	1124	69.22%	951.00	D16	1099	1557	70.58%	1328.00
GREEN	CSF1PO	552	669	82.51%	610.50	CSF1PO	1146	1361	84.20%	1253.50	CSF1PO	1583	1895	83.54%	1739.00
GREEN	Penta D	392	530	73.96%	461.00	Penta D	809	1132	71.47%	970.50	Penta D	1050	1480	70.95%	1265.00
YELLOW	AMEL	1282				AMEL	2616				AMEL	3773			
YELLOW	vWA	1986				vWA	3867				vWA	5324			
YELLOW	D8	1265	1280	98.83%	1272.50	D8	2531	2605	97.16%	2568.00	D8	3580	3684	97.18%	3632.00
YELLOW	TPOX	712	802	88.78%	757.00	TPOX	1418	1621	87.48%	1519.50	TPOX	2004	2264	88.52%	2134.00
YELLOW	FGA	983	1008	97.52%	995.50	FGA	1976	2038	96.96%	2007.00	FGA	2728	2782	98.06%	2755.00
	1 second					3 seconds					5 seconds				

	SMALL	LARGE		SMALL	LARGE		SMALL	LARGE	
	Average Imbalance			**Average Imbalance**			**Average Imbalance**		
	85.30%			85.56%			84.59%		

	Range	Average	StDev	%Diff		Range	Average	StDev	%Diff		Range	Average	StDev	%Diff
BLUE		664.8	60.3	9.08%	BLUE		1378.8	115.6	8.39%	BLUE		1919.1	207.4	10.81%
GREEN		573.7	145.9	25.43%	GREEN		1196.6	302.7	25.30%	GREEN		1664.8	454.5	27.30%
YELLOW		1008.3	258.0	25.59%	YELLOW		2031.5	524.7	25.83%	YELLOW		2840.3	752.6	26.50%



Appendix 3: Performance Verification Sensitivity Study-Intensity Data

Table A3.1

2.0 ng template 3100-*Avant* capillary data

	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean
BLUE	D3	543	624	87.02%	583.50	D3	1145	1299	88.14%	1222.00	D3	1633	1902	85.86%	1767.50
BLUE	Th01	743	754	98.54%	748.50	Th01	1543	1545	99.87%	1544.00	Th01	2204	2240	98.39%	2222.00
BLUE	D21	571	777	73.49%	674.00	D21	1180	1589	74.26%	1384.50	D21	1650	2270	72.69%	1960.00
BLUE	D18	644	714	90.20%	679.00	D18	1339	1459	91.78%	1399.00	D18	1853	2066	89.69%	1959.50
BLUE	Penta E	467	811	57.58%	639.00	Penta E	996	1693	58.83%	1344.50	Penta E	1185	2188	54.16%	1686.50
GREEN	D5	748	821	91.11%	784.50	D5	1567	1679	93.33%	1623.00	D5	2228	2426	91.84%	2327.00
GREEN	D13	639	752	84.97%	695.50	D13	1358	1592	85.30%	1475.00	D13	1890	2234	84.60%	2062.00
GREEN	D7	435	442	98.42%	438.50	D7	906	907	99.89%	906.50	D7	1256	1279	98.20%	1267.50
GREEN	D16	376	528	71.21%	452.00	D16	778	1124	69.22%	951.00	D16	1099	1557	70.58%	1328.00
GREEN	CSF1PO	552	669	82.51%	610.50	CSF1PO	1146	1361	84.20%	1253.50	CSF1PO	1583	1895	83.54%	1739.00
GREEN	Penta D	392	530	73.96%	461.00	Penta D	809	1132	71.47%	970.50	Penta D	1050	1480	70.95%	1265.00
YELLOW	AMEL	1282				AMEL	2616				AMEL	3773			
YELLOW	vWA	1986				vWA	3867				vWA	5324			
YELLOW	D8	1265	1280	98.83%	1272.50	D8	2531	2605	97.16%	2568.00	D8	3580	3684	97.18%	3632.00
YELLOW	TPOX	712	802	88.78%	757.00	TPOX	1418	1621	87.48%	1519.50	TPOX	2004	2264	88.52%	2134.00
YELLOW	FGA	983	1008	97.52%	995.50	FGA	1976	2038	96.96%	2007.00	FGA	2728	2782	98.06%	2755.00
	1 second					3 seconds					5 seconds				

	SMALL	LARGE		SMALL	LARGE		SMALL	LARGE	
	Average Imbalance			**Average Imbalance**			**Average Imbalance**		
	85.30%			85.56%			84.59%		

	Range	Average	StDev	%Diff		Range	Average	StDev	%Diff		Range	Average	StDev	%Diff
BLUE		664.8	60.3	9.08%	BLUE		1378.8	115.6	8.39%	BLUE		1919.1	207.4	10.81%
GREEN		573.7	145.9	25.43%	GREEN		1196.6	302.7	25.30%	GREEN		1664.8	454.5	27.30%
YELLOW		1008.3	258.0	25.59%	YELLOW		2031.5	524.7	25.83%	YELLOW		2840.3	752.6	26.50%

Table A3.1a: 2.0 ng template 3100-*Avant* capillary data (1, 3, and 5 second).

	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean
BLUE	D3	2238	2564	87.29%	2401.00	D3	2843	3277	86.76%	3060.00	D3	3388	3905	86.76%	3646.50
BLUE	Th01	3015	3046	98.98%	3030.50	Th01	3834	3875	98.94%	3854.50	Th01	4525	4601	98.35%	4563.00
BLUE	D21	2282	3111	73.35%	2696.50	D21	2909	3988	72.94%	3448.50	D21	3513	4628	75.91%	4070.50
BLUE	D18	2633	2902	90.73%	2767.50	D18	3367	3689	91.27%	3528.00	D18	3981	4411	90.25%	4196.00
BLUE	Penta E	1916	3305	57.97%	2610.50	Penta E	2437	4210	57.89%	3323.50	Penta E	2893	5032	57.49%	3962.50
GREEN	D5	3076	3331	92.34%	3203.50	D5	3904	4242	92.03%	4073.00	D5	4561	5038	90.53%	4799.50
GREEN	D13	2609	3067	85.07%	2838.00	D13	3309	3903	84.78%	3606.00	D13	3905	4637	84.21%	4271.00
GREEN	D7	1761	1781	98.88%	1771.00	D7	2261	2267	99.74%	2264.00	D7	2649	2654	99.81%	2651.50
GREEN	D16	1544	2162	71.42%	1853.00	D16	1951	2782	70.13%	2366.50	D16	2325	3281	70.86%	2803.00
GREEN	CSF1PO	2255	2727	82.69%	2491.00	CSF1PO	2932	3476	84.35%	3204.00	CSF1PO	3437	4087	84.10%	3762.00
GREEN	Penta D	1586	2233	71.03%	1909.50	Penta D	2082	2818	73.88%	2450.00	Penta D	2453	3385	72.47%	2919.00
YELLOW	AMEL	5038			5038.00	AMEL	6363				AMEL	7162			
YELLOW	vWA	6924			6924.00	vWA	7543				vWA	7406			
YELLOW	D8	4884	5077	96.20%	4980.50	D8	6235	6431	96.95%	6333.00	D8	7155	7205	99.31%	7180.00
YELLOW	TPOX	2737	3105	88.15%	2921.00	TPOX	3496	3899	89.66%	3697.50	TPOX	4104	4665	87.97%	4384.50
YELLOW	FGA	3827	3936	97.23%	3881.50	FGA	4877	4999	97.56%	4938.00	FGA	5752	5876	97.89%	5814.00
	7 seconds					9 seconds					11 seconds				

	SMALL	LARGE		SMALL	LARGE		SMALL	LARGE	
	Average Imbalance			**Average Imbalance**			**Average Imbalance**		
	85.09%			85.49%			85.42%		

	Range	Average	StDev	%Diff		Range	Average	StDev	%Diff		Range	Average	StDev	%Diff
BLUE		2701.2	229.8	8.51%	BLUE		3442.9	290.5	8.44%	BLUE		4087.7	334.8	8.19%
GREEN		2344.3	593.7	25.33%	GREEN		2993.9	749.1	25.02%	GREEN		3534.3	881.8	24.95%
YELLOW		3927.7	1030.5	26.24%	YELLOW		4989.5	1318.5	26.43%	YELLOW		5792.8	1397.9	24.13%

Table A3.1b: 2.0 ng template 3100-*Avant* capillary data (7, 9, and 11 second).

Appendix 3: Performance Verification Sensitivity Study-Intensity Data

Table A3.2

1.0 ng template 3100-*Avant* capillary data

	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean
BLUE	D3	543	624	87.02%	583.50	D3	1145	1299	88.14%	1222.00	D3	1633	1902	85.86%	1767.50
BLUE	Th01	743	754	98.54%	748.50	Th01	1543	1545	99.87%	1544.00	Th01	2204	2240	98.39%	2222.00
BLUE	D21	571	777	73.49%	674.00	D21	1180	1589	74.26%	1384.50	D21	1650	2270	72.69%	1960.00
BLUE	D18	644	714	90.20%	679.00	D18	1339	1459	91.78%	1399.00	D18	1853	2066	89.69%	1959.50
BLUE	Penta E	467	811	57.58%	639.00	Penta E	996	1693	58.83%	1344.50	Penta E	1185	2188	54.16%	1686.50
GREEN	D5	748	821	91.11%	784.50	D5	1567	1679	93.33%	1623.00	D5	2228	2426	91.84%	2327.00
GREEN	D13	639	752	84.97%	695.50	D13	1358	1592	85.30%	1475.00	D13	1890	2234	84.60%	2062.00
GREEN	D7	435	442	98.42%	438.50	D7	906	907	99.89%	906.50	D7	1256	1279	98.20%	1267.50
GREEN	D16	376	528	71.21%	452.00	D16	778	1124	69.22%	951.00	D16	1099	1557	70.58%	1328.00
GREEN	CSF1PO	552	669	82.51%	610.50	CSF1PO	1146	1361	84.20%	1253.50	CSF1PO	1583	1895	83.54%	1739.00
GREEN	Penta D	392	530	73.96%	461.00	Penta D	809	1132	71.47%	970.50	Penta D	1050	1480	70.95%	1265.00
YELLOW	AMEL	1282				AMEL	2616				AMEL	3773			
YELLOW	vWA	1986				vWA	3867				vWA	5324			
YELLOW	D8	1265	1280	98.83%	1272.50	D8	2531	2605	97.16%	2568.00	D8	3580	3684	97.18%	3632.00
YELLOW	TPOX	712	802	88.78%	757.00	TPOX	1418	1621	87.48%	1519.50	TPOX	2004	2264	88.52%	2134.00
YELLOW	FGA	983	1008	97.52%	995.50	FGA	1976	2038	96.96%	2007.00	FGA	2728	2782	98.06%	2755.00
	1 second					3 seconds					5 seconds				

SMALL LARGE Average Imbalance				SMALL LARGE Average Imbalance				SMALL LARGE Average Imbalance			
85.30%				85.56%				84.59%			
	Range	**Average**	**StDev**	**%Diff**		**Range**	**Average**	**StDev**	**%Diff**		**Range**	**Average**	**StDev**	**%Diff**
BLUE		664.8	60.3	9.08%	BLUE		1378.8	115.6	8.39%	BLUE		1919.1	207.4	10.81%
GREEN		573.7	145.9	25.43%	GREEN		1196.6	302.7	25.30%	GREEN		1664.8	454.5	27.30%
YELLOW		1008.3	258.0	25.59%	YELLOW		2031.5	524.7	25.83%	YELLOW		2840.3	752.6	26.50%

Table A3.2a: 1.0 ng template 3100-*Avant* capillary data (1, 3, and 5 second).

	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean
BLUE	D3	2238	2564	87.29%	2401.00	D3	2843	3277	86.76%	3060.00	D3	3388	3905	86.76%	3646.50
BLUE	Th01	3015	3046	98.98%	3030.50	Th01	3834	3875	98.94%	3854.50	Th01	4525	4601	98.35%	4563.00
BLUE	D21	2282	3111	73.35%	2696.50	D21	2909	3988	72.94%	3448.50	D21	3513	4628	75.91%	4070.50
BLUE	D18	2633	2902	90.73%	2767.50	D18	3367	3689	91.27%	3528.00	D18	3981	4411	90.25%	4196.00
BLUE	Penta E	1916	3305	57.97%	2610.50	Penta E	2437	4210	57.89%	3323.50	Penta E	2893	5032	57.49%	3962.50
GREEN	D5	3076	3331	92.34%	3203.50	D5	3904	4242	92.03%	4073.00	D5	4561	5038	90.53%	4799.50
GREEN	D13	2609	3067	85.07%	2838.00	D13	3309	3903	84.78%	3606.00	D13	3905	4637	84.21%	4271.00
GREEN	D7	1761	1781	98.88%	1771.00	D7	2261	2267	99.74%	2264.00	D7	2649	2654	99.81%	2651.50
GREEN	D16	1544	2162	71.42%	1853.00	D16	1951	2782	70.13%	2366.50	D16	2325	3281	70.86%	2803.00
GREEN	CSF1PO	2255	2727	82.69%	2491.00	CSF1PO	2932	3476	84.35%	3204.00	CSF1PO	3437	4087	84.10%	3762.00
GREEN	Penta D	1586	2233	71.03%	1909.50	Penta D	2082	2818	73.88%	2450.00	Penta D	2453	3385	72.47%	2919.00
YELLOW	AMEL	5038			5038.00	AMEL	6363				AMEL	7162			
YELLOW	vWA	6924			6924.00	vWA	7543				vWA	7406			
YELLOW	D8	4884	5077	96.20%	4980.50	D8	6235	6431	96.95%	6333.00	D8	7155	7205	99.31%	7180.00
YELLOW	TPOX	2737	3105	88.15%	2921.00	TPOX	3496	3899	89.66%	3697.50	TPOX	4104	4665	87.97%	4384.50
YELLOW	FGA	3827	3936	97.23%	3881.50	FGA	4877	4999	97.56%	4938.00	FGA	5752	5876	97.89%	5814.00
	7 seconds					9 seconds					11 seconds				

SMALL LARGE Average Imbalance				SMALL LARGE Average Imbalance				SMALL LARGE Average Imbalance			
85.09%				85.49%				85.42%			
	Range	**Average**	**StDev**	**%Diff**		**Range**	**Average**	**StDev**	**%Diff**		**Range**	**Average**	**StDev**	**%Diff**
BLUE		2701.2	229.8	8.51%	BLUE		3442.9	290.5	8.44%	BLUE		4087.7	334.8	8.19%
GREEN		2344.3	593.7	25.33%	GREEN		2993.9	749.1	25.02%	GREEN		3534.3	881.8	24.95%
YELLOW		3927.7	1030.5	26.24%	YELLOW		4989.5	1318.5	26.43%	YELLOW		5792.8	1397.9	24.13%

Table A3.2b: 1.0 ng template 3100-*Avant* capillary data (7, 9, and 11 second).

Appendix 3: Performance Verification Sensitivity Study-Intensity Data

Table A3.3

0.75 ng template 3100-*Avant* capillary data.

	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean
BLUE	D3	538	598	89.97%	568.00	D3	1087	1211	89.76%	1149.00	D3	1686	1910	88.27%	1798.00
BLUE	Th01	625	689	90.71%	657.00	Th01	1281	1413	90.66%	1347.00	Th01	1985	2157	92.03%	2071.00
BLUE	D21	513	695	73.81%	604.00	D21	1061	1442	73.58%	1251.50	D21	1618	2206	73.35%	1912.00
BLUE	D18	581	784	74.11%	682.50	D18	1155	1598	72.28%	1376.50	D18	1782	2426	73.45%	2104.00
BLUE	Penta E	599	830	72.17%	714.50	Penta E	1230	1679	73.26%	1454.50	Penta E	1901	2619	72.58%	2260.00
GREEN	D5	725	794	91.31%	759.50	D5	1482	1604	92.39%	1543.00	D5	2282	2520	90.56%	2401.00
GREEN	D13	640	642	99.69%	641.00	D13	1292	1312	98.48%	1302.00	D13	2014	2039	98.77%	2026.50
GREEN	D7	435	455	95.60%	445.00	D7	897	931	96.35%	914.00	D7	1378	1424	96.77%	1401.00
GREEN	D16	458	502	91.24%	480.00	D16	927	997	92.98%	962.00	D16	1427	1593	89.58%	1510.00
GREEN	CSF1PO	527	583	90.39%	555.00	CSF1PO	1087	1179	92.20%	1133.00	CSF1PO	1680	1839	91.35%	1759.50
GREEN	Penta D	421	572	73.60%	496.50	Penta D	873	1170	74.62%	1021.50	Penta D	1352	1809	74.74%	1580.50
YELLOW	AMEL	978				AMEL	1933				AMEL	2961			
YELLOW	vWA	1647				vWA	3174				vWA	4624			
YELLOW	D8	862	1002	86.03%	932.00	D8	1699	2032	83.61%	1865.50	D8	2611	3094	84.39%	2852.50
YELLOW	TPOX	582	591	98.48%	586.50	TPOX	1151	1173	98.12%	1162.00	TPOX	1741	1780	97.81%	1760.50
YELLOW	FGA	800	879	91.01%	839.50	FGA	1571	1712	91.76%	1641.50	FGA	2408	2641	91.18%	2524.50
	1 second					3 seconds					5 seconds				

	SMALL	LARGE		SMALL	LARGE		SMALL	LARGE	
	Average Imbalance			Average Imbalance			Average Imbalance		
	87.01%			87.15%			86.77%		

	Range	Average	StDev	%Diff		Range	Average	StDev	%Diff		Range	Average	StDev	%Diff
BLUE		645.2	59.1	9.17%	BLUE		1315.7	118.2	8.98%	BLUE		2029.0	178.7	8.81%
GREEN		562.8	118.3	21.02%	GREEN		1145.9	238.9	20.85%	GREEN		1779.8	375.0	21.07%
YELLOW		786.0	178.9	22.76%	YELLOW		1556.3	359.4	23.09%	YELLOW		2379.2	560.3	23.55%

Table A3.3a: 0.75 ng template 3100-*Avant* capillary data (1, 3, and 5 second).

	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean
BLUE	D3	2255	2588	87.13%	2421.50	D3	2172	2283	95.14%	2227.50	D3	2868	3272	87.65%	3070.00
BLUE	Th01	2616	2892	90.46%	2754.00	Th01	2639	2682	98.40%	2660.50	Th01	3289	3642	90.31%	3465.50
BLUE	D21	2177	2930	74.30%	2553.50	D21	2906	3018	96.29%	2962.00	D21	2760	3709	74.41%	3234.50
BLUE	D18	2427	3265	74.33%	2846.00	D18	2633	3387	77.74%	3010.00	D18	3056	4122	74.14%	3589.00
BLUE	Penta E	2549	3510	72.62%	3029.50	Penta E	2912	4230	68.84%	3571.00	Penta E	3257	4498	72.41%	3877.50
GREEN	D5	3062	3322	92.17%	3192.00	D5	2639	2797	94.35%	2718.00	D5	3898	4242	91.89%	4070.00
GREEN	D13	2642	2722	97.06%	2682.00	D13	2002	2554	78.39%	2278.00	D13	3366	3402	98.94%	3384.00
GREEN	D7	1834	1895	96.78%	1864.50	D7	1648	1863	88.46%	1755.50	D7	2337	2415	96.77%	2376.00
GREEN	D16	1921	2114	90.87%	2017.50	D16	1691	2297	73.62%	1994.00	D16	2437	2635	92.49%	2536.00
GREEN	CSF1PO	2251	2471	91.10%	2361.00	CSF1PO	2797	2828	98.90%	2812.50	CSF1PO	2853	3123	91.35%	2988.00
GREEN	Penta D	1804	2442	73.87%	2123.00	Penta D	2253	2470	91.21%	2361.50	Penta D	2296	3055	75.16%	2675.50
YELLOW	AMEL	3917				AMEL	4715				AMEL	4982			
YELLOW	vWA	5989				vWA	4761				vWA	7180			
YELLOW	D8	3449	4131	83.49%	3790.00	D8	3388	4060	83.45%	3724.00	D8	4390	5196	84.49%	4793.00
YELLOW	TPOX	2328	2373	98.10%	2350.50	TPOX	2290	2781	82.34%	2535.50	TPOX	2937	2988	98.29%	2962.50
YELLOW	FGA	3202	3522	90.91%	3362.00	FGA	3284	3337	98.41%	3310.50	FGA	4031	4423	91.14%	4227.00
	7 seconds					9 seconds					11 seconds				

	SMALL	LARGE		SMALL	LARGE		SMALL	LARGE	
	Average Imbalance			Average Imbalance			Average Imbalance		
	86.66%			87.54%			87.10%		

	Range	Average	StDev	%Diff		Range	Average	StDev	%Diff		Range	Average	StDev	%Diff
BLUE		2720.9	239.6	8.81%	BLUE		2886.2	493.7	17.10%	BLUE		3447.3	313.5	9.09%
GREEN		2373.3	492.7	20.76%	GREEN		2319.9	407.3	17.56%	GREEN		3004.9	632.8	21.06%
YELLOW		3167.5	739.2	23.34%	YELLOW		3190.0	603.3	18.91%	YELLOW		3994.2	937.2	23.46%

Table A3.3b: 0.75 ng template 3100-*Avant* capillary data (7, 9, and 11 second).

Appendix 3: Performance Verification Sensitivity Study-Intensity Data

Table A3.4

0.50 ng template 3100-Avant capillary data

	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean
BLUE	D3	351	376	93.35%	363.50	D3	808	851	94.95%	829.50	D3	1277	1340	95.30%	1308.50
BLUE	Th01	428	437	97.94%	432.50	Th01	981	1009	97.22%	995.00	Th01	1534	1589	96.54%	1561.50
BLUE	D21	471	483	97.52%	477.00	D21	1076	1130	95.22%	1103.00	D21	1726	1808	95.46%	1767.00
BLUE	D18	422	549	76.87%	485.50	D18	981	1250	78.48%	1115.50	D18	1546	1964	78.72%	1755.00
BLUE	Penta E	464	649	71.49%	556.50	Penta E	1069	1559	68.57%	1314.00	Penta E	1718	2464	69.72%	2091.00
GREEN	D5	415	449	92.43%	432.00	D5	975	1026	95.03%	1000.50	D5	1554	1650	94.18%	1602.00
GREEN	D13	328	412	79.61%	370.00	D13	759	954	79.56%	856.50	D13	1161	1489	77.97%	1325.00
GREEN	D7	265	294	90.14%	279.50	D7	618	680	90.88%	649.00	D7	956	1082	88.35%	1019.00
GREEN	D16	275	370	74.32%	322.50	D16	624	855	72.98%	739.50	D16	1012	1342	75.41%	1177.00
GREEN	CSF1PO	444	447	99.33%	445.50	CSF1PO	1034	1043	99.14%	1038.50	CSF1PO	1651	1661	99.40%	1656.00
GREEN	Penta D	363	392	92.60%	377.50	Penta D	836	897	93.20%	866.50	Penta D	1325	1438	92.14%	1381.50
YELLOW	AMEL	770				AMEL	1799				AMEL	2792			
YELLOW	vWA	907				vWA	2042				vWA	3039			
YELLOW	D8	531	645	82.33%	588.00	D8	1244	1515	82.11%	1379.50	D8	1954	2371	82.41%	2162.50
YELLOW	TPOX	373	453	82.34%	413.00	TPOX	857	1044	82.09%	950.50	TPOX	1358	1653	82.15%	1505.50
YELLOW	FGA	524	530	98.87%	527.00	FGA	1219	1246	97.83%	1232.50	FGA	1927	1949	98.87%	1938.00
	1 second					3 seconds					5 seconds				

	SMALL	LARGE		SMALL	LARGE		SMALL	LARGE	
	Average Imbalance			Average Imbalance			Average Imbalance		
	87.80%			87.66%			87.62%		

	Range	Average	StDev	%Diff		Range	Average	StDev	%Diff		Range	Average	StDev	%Diff
BLUE		463.0	71.2	15.38%	BLUE		1071.4	177.6	16.58%	BLUE		1696.6	288.4	17.00%
GREEN		371.2	63.3	17.06%	GREEN		858.4	148.8	17.33%	GREEN		1360.1	244.0	17.94%
YELLOW		509.3	88.8	17.44%	YELLOW		1187.5	218.0	18.36%	YELLOW		1868.7	333.9	17.87%

Table A3.4a: 0.50 ng template 3100-*Avant* capillary data (1, 3, and 5 second).

	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean
BLUE	D3	1737	1836	94.61%	1786.50	D3	2172	2283	95.14%	2227.50	D3	2631	2757	95.43%	2694.00
BLUE	Th01	2096	2152	97.40%	2124.00	Th01	2639	2682	98.40%	2660.50	Th01	3138	3226	97.27%	3182.00
BLUE	D21	2304	2419	95.25%	2361.50	D21	2906	3018	96.29%	2962.00	D21	3439	3646	94.32%	3542.50
BLUE	D18	2098	2668	78.64%	2383.00	D18	2633	3387	77.74%	3010.00	D18	3162	4068	77.73%	3615.00
BLUE	Penta E	2315	3379	68.51%	2847.00	Penta E	2912	4230	68.84%	3571.00	Penta E	3520	5073	69.39%	4296.50
GREEN	D5	2082	2220	93.78%	2151.00	D5	2639	2797	94.35%	2718.00	D5	3146	3343	94.11%	3244.50
GREEN	D13	1593	1985	80.25%	1789.00	D13	2002	2554	78.39%	2278.00	D13	2386	2996	79.64%	2691.00
GREEN	D7	1280	1465	87.37%	1372.50	D7	1648	1863	88.46%	1755.50	D7	1966	2224	88.40%	2095.00
GREEN	D16	1358	1830	74.21%	1594.00	D16	1691	2297	73.62%	1994.00	D16	2042	2731	74.77%	2386.50
GREEN	CSF1PO	2212	2223	99.51%	2217.50	CSF1PO	2797	2828	98.90%	2812.50	CSF1PO	3378	3402	99.29%	3390.00
GREEN	Penta D	1770	1931	91.66%	1850.50	Penta D	2253	2470	91.21%	2361.50	Penta D	2720	2951	92.17%	2835.50
YELLOW	AMEL	3796				AMEL	4715				AMEL	5628			
YELLOW	vWA	3963				vWA	4761				vWA	5515			
YELLOW	D8	2673	3227	82.83%	2950.00	D8	3388	4060	83.45%	3724.00	D8	4027	4824	83.48%	4425.50
YELLOW	TPOX	1840	2219	82.92%	2029.50	TPOX	2290	2781	82.34%	2535.50	TPOX	2764	3325	83.13%	3044.50
YELLOW	FGA	2609	2619	99.62%	2614.00	FGA	3284	3337	98.41%	3310.50	FGA	3892	3959	98.31%	3925.50
	7 seconds					9 seconds					11 seconds				

	SMALL	LARGE		SMALL	LARGE		SMALL	LARGE	
	Average Imbalance			Average Imbalance			Average Imbalance		
	87.61%			87.54%			87.67%		

	Range	Average	StDev	%Diff		Range	Average	StDev	%Diff		Range	Average	StDev	%Diff
BLUE		2300.4	388.8	16.90%	BLUE		2886.2	493.7	17.10%	BLUE		3466.0	590.4	17.03%
GREEN		1829.1	322.6	17.64%	GREEN		2319.9	407.3	17.56%	GREEN		2773.8	494.5	17.83%
YELLOW		2531.2	465.8	18.40%	YELLOW		3190.0	603.3	18.91%	YELLOW		3798.5	699.2	18.41%

Table A3.4b: 0.50 ng template 3100-*Avant* capillary data (7, 9, and 11 second).

Appendix 3: Performance Verification Sensitivity Study-Intensity Data

Table A3.5

0.25 ng template 3100-*Avant* capillary data

	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean
BLUE	D3	120	223	53.81%	171.50	D3	256	488	52.46%	372.00	D3	410	773	53.04%	591.50
BLUE	Th01	158	253	62.45%	205.50	Th01	354	546	64.84%	450.00	Th01	547	865	63.24%	706.00
BLUE	D21	267	308	86.69%	287.50	D21	589	658	89.51%	623.50	D21	945	1046	90.34%	995.50
BLUE	D18	328	334	98.20%	331.00	D18	682	725	94.07%	703.50	D18	1115	1150	96.96%	1132.50
BLUE	Penta E	420	574	73.17%	497.00	Penta E	786	1168	67.29%	977.00	Penta E	1421	2039	69.69%	1730.00
GREEN	D5	169	289	58.48%	229.00	D5	374	678	55.16%	526.00	D5	599	1058	56.62%	828.50
GREEN	D13	160	191	83.77%	175.50	D13	331	433	76.44%	382.00	D13	544	676	80.47%	610.00
GREEN	D7	175	243	72.02%	209.00	D7	384	527	72.87%	455.50	D7	622	830	74.94%	726.00
GREEN	D16	154	239	64.44%	196.50	D16	322	499	64.53%	410.50	D16	515	787	65.44%	651.00
GREEN	CSF1PO	238	290	82.07%	264.00	CSF1PO	507	615	82.44%	561.00	CSF1PO	833	1011	82.39%	922.00
GREEN	Penta D	238	281	84.70%	259.50	Penta D	459	572	80.24%	515.50	Penta D	787	993	79.25%	890.00
YELLOW	AMEL	433				AMEL	961				AMEL	1476			
YELLOW	vWA	524				vWA	1130				vWA	1673			
YELLOW	D8	368	474	77.64%	421.00	D8	822	1066	77.11%	944.00	D8	1308	1686	77.58%	1497.00
YELLOW	TPOX	261	354	73.73%	307.50	TPOX	581	787	73.82%	684.00	TPOX	917	1241	73.89%	1079.00
YELLOW	FGA	412	417	98.80%	414.50	FGA	871	911	95.61%	891.00	FGA	1422	1471	96.67%	1446.50
	1 second					3 seconds					5 seconds				

	SMALL	LARGE		SMALL	LARGE		SMALL	LARGE	
	Average Imbalance			Average Imbalance			Average Imbalance		
	76.43%			74.74%			75.75%		

	Range	Average	StDev	%Diff	Range	Average	StDev	%Diff	Range	Average	StDev	%Diff
BLUE		298.5	127.8	42.82%		625.2	237.0	37.91%		1031.1	446.9	43.34%
GREEN		222.3	35.2	15.84%		475.1	70.5	14.83%		771.3	128.5	16.67%
YELLOW		381.0	63.7	16.73%		839.7	137.4	16.36%		1340.8	228.2	17.02%

Table A3.5a: 0.25 ng template 3100-*Avant* capillary data (1, 3, and 5 second).

	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean
BLUE	D3	557	1060	52.55%	808.50	D3	687	1323	51.93%	1005.00	D3	783	1531	51.14%	1157.00
BLUE	Th01	758	1179	64.29%	968.50	Th01	946	1450	65.24%	1198.00	Th01	1059	1690	62.66%	1374.50
BLUE	D21	1279	1425	89.75%	1352.00	D21	1561	1754	89.00%	1657.50	D21	1702	1923	88.51%	1812.50
BLUE	D18	1505	1590	94.65%	1547.50	D18	1843	1925	95.74%	1884.00	D18	1595	1732	92.09%	1663.50
BLUE	Penta E	1958	2750	71.20%	2354.00	Penta E	2354	3352	70.23%	2853.00	Penta E	1359	2297	59.16%	1828.00
GREEN	D5	815	1444	56.44%	1129.50	D5	999	1788	55.87%	1393.50	D5	1151	2037	56.50%	1594.00
GREEN	D13	719	918	78.32%	818.50	D13	890	1127	78.97%	1008.50	D13	1014	1268	79.97%	1141.00
GREEN	D7	838	1148	73.00%	993.00	D7	1048	1403	74.70%	1225.50	D7	1119	1502	74.50%	1310.50
GREEN	D16	696	1072	64.93%	884.00	D16	878	1310	67.02%	1094.00	D16	796	1261	63.12%	1028.50
GREEN	CSF1PO	1148	1390	82.59%	1269.00	CSF1PO	1389	1688	82.29%	1538.50	CSF1PO	1132	1415	80.00%	1273.50
GREEN	Penta D	1097	1344	81.62%	1220.50	Penta D	1316	1625	80.98%	1470.50	Penta D	900	1133	79.44%	1016.50
YELLOW	AMEL	1988				AMEL	2477				AMEL	2876			
YELLOW	vWA	2191				vWA	2639				vWA	3000			
YELLOW	D8	1766	2308	76.52%	2037.00	D8	2189	2852	76.75%	2520.50	D8	2405	3180	75.63%	2792.50
YELLOW	TPOX	1225	1683	72.79%	1454.00	TPOX	1511	2077	72.75%	1794.00	TPOX	1539	2038	75.52%	1788.50
YELLOW	FGA	1926	1996	96.49%	1961.00	FGA	2342	2426	96.54%	2384.00	FGA	1943	2096	92.70%	2019.50
	7 seconds					9 seconds					11 seconds				

	SMALL	LARGE		SMALL	LARGE		SMALL	LARGE	
	Average Imbalance			Average Imbalance			Average Imbalance		
	75.37%			75.57%			73.64%		

	Range	Average	StDev	%Diff	Range	Average	StDev	%Diff	Range	Average	StDev	%Diff
BLUE		1406.1	606.2	43.11%		1719.5	724.3	42.12%		1567.1	292.8	18.68%
GREEN		1052.4	183.1	17.40%		1288.4	213.0	16.53%		1227.3	216.8	17.66%
YELLOW		1817.3	316.9	17.44%		2232.8	386.1	17.29%		2200.2	525.8	23.90%

Table A3.5b: 0.25 ng template 3100-*Avant* capillary data (7, 9, and 11 second).

Appendix 3: Performance Verification Sensitivity Study-Intensity Data

Table A3.6

0.10 ng template 3100-*Avant* capillary data

	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean
BLUE	D3					D3					D3	123	235	52.34%	179.00
BLUE	Th01	107	107	100.00%	107.00	Th01	232	234	99.15%	233.00	Th01	353	360	98.06%	356.50
BLUE	D21	120	121	99.17%	120.50	D21	256	271	94.46%	263.50	D21	386	408	94.61%	397.00
BLUE	D18	190	236	80.51%	213.00	D18	412	510	80.78%	461.00	D18	640	782	81.84%	711.00
BLUE	Penta E	214	222	96.40%	218.00	Penta E	464	483	96.07%	473.50	Penta E	726	731	99.32%	728.50
GREEN	D5	100	113	88.50%	106.50	D5	212	239	88.70%	225.50	D5	328	380		354.00
GREEN	D13					D13	131	136	96.32%	133.50	D13	191	209	91.39%	200.00
GREEN	D7					D7	97	140	69.29%	118.50	D7	151	215	70.23%	183.00
GREEN	D16					D16	123	238	51.68%	180.50	D16	193	258	74.81%	225.50
GREEN	CSF1PO					CSF1PO	128	313	40.89%	220.50	CSF1PO	191	471	40.55%	331.00
GREEN	Penta D	158	205	77.07%	181.50	Penta D	362	437	82.84%	399.50	Penta D	558	675	82.67%	616.50
YELLOW	AMEL	343				AMEL	739				AMEL	1144			
YELLOW	vWA	245				vWA	517				vWA	763			
YELLOW	D8	206	217	94.93%	211.50	D8	441	473	93.23%	457.00	D8	674	732	92.08%	703.00
YELLOW	TPOX	148	225	65.78%	186.50	TPOX	328	473	69.34%	400.50	TPOX	505	734	68.80%	619.50
YELLOW	FGA	151	178	84.83%	164.50	FGA	339	378	89.68%	358.50	FGA	521	588	88.61%	554.50
	1 second					3 seconds					5 seconds				

SMALL	LARGE	SMALL	LARGE	SMALL	LARGE
Average Imbalance		Average Imbalance		Average Imbalance	
87.47%		80.96%		79.64%	

	Range	Average	StDev	%Diff		Range	Average	StDev	%Diff		Range	Average	StDev	%Diff
BLUE		164.6	59.0	35.86%	BLUE		357.8	127.2	35.54%	BLUE		474.4	238.6	50.29%
GREEN		144.0	53.0	36.83%	GREEN		213.0	101.3	47.56%	GREEN		318.3	162.0	50.90%
YELLOW		187.5	23.5	12.54%	YELLOW		405.3	49.4	12.19%	YELLOW		625.7	74.4	11.90%

Table A3.6a: 0.10 ng template 3100-*Avant* capillary data (1, 3, and 5 second).

	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean
BLUE	D3	167	321	52.02%	244.00	D3	219	394	55.58%	306.50	D3	257	475	54.11%	366.00
BLUE	Th01	495	501	98.80%	498.00	Th01	596	605	98.51%	600.50	Th01	715	720	99.31%	717.50
BLUE	D21	535	570	93.86%	552.50	D21	658	688	95.64%	673.00	D21	802	829	96.74%	815.50
BLUE	D18	869	1066	81.52%	967.50	D18	1054	1310	80.46%	1182.00	D18	1273	1575	80.83%	1424.00
BLUE	Penta E	986	1011	97.53%	998.50	Penta E	1225	1231	99.51%	1228.00	Penta E	1453	1512	96.10%	1482.50
GREEN	D5	456	506	90.12%	481.00	D5	541	634	85.33%	587.50	D5	655	761	86.07%	708.00
GREEN	D13	267	284	94.01%	275.50	D13	327	355	92.11%	341.00	D13	396	418	94.74%	407.00
GREEN	D7	207	295	70.17%	251.00	D7	259	359	72.14%	309.00	D7	309	423	73.05%	366.00
GREEN	D16	249	492	50.61%	370.50	D16	312	620	50.32%	466.00	D16	387	740	52.30%	563.50
GREEN	CSF1PO	266	644	41.30%	455.00	CSF1PO	334	797	41.91%	565.50	CSF1PO	410	957	42.84%	683.50
GREEN	Penta D	749	935	80.11%	842.00	Penta D	924	1145	80.70%	1034.50	Penta D	1117	1382	80.82%	1249.50
YELLOW	AMEL	1530				AMEL	1886				AMEL	2243			
YELLOW	vWA	1021				vWA	1201				vWA	1411			
YELLOW	D8	932	997	93.48%	964.50	D8	1154	1245	92.69%	1199.50	D8	1372	1464	93.72%	1418.00
YELLOW	TPOX	684	999	68.47%	841.50	TPOX	850	1241	68.49%	1045.50	TPOX	1017	1480	68.72%	1248.50
YELLOW	FGA	716	815	87.85%	765.50	FGA	875	987	88.65%	931.00	FGA	1048	1194	87.77%	1121.00
	7 seconds					9 seconds					11 seconds				

SMALL	LARGE	SMALL	LARGE	SMALL	LARGE
Average Imbalance		Average Imbalance		Average Imbalance	
78.56%		78.72%		79.08%	

	Range	Average	StDev	%Diff		Range	Average	StDev	%Diff		Range	Average	StDev	%Diff
BLUE		652.1	323.9	49.67%	BLUE		798.0	396.4	49.68%	BLUE		961.1	479.8	49.92%
GREEN		445.8	214.9	48.20%	GREEN		550.6	262.8	47.72%	GREEN		662.9	319.4	48.18%
YELLOW		857.2	100.4	11.72%	YELLOW		1058.7	134.7	12.73%	YELLOW		1262.5	149.0	11.80%

Table A3.6b: 0.10 ng template 3100-*Avant* capillary data (7, 9, and 11 second).

Appendix 3: Performance Verification Sensitivity Study-Intensity Data

Table A3.7

0.05 ng template 3100-*Avant* capillary data

	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean
BLUE	D3					D3					D3				
BLUE	Th01					Th01	109	114	95.61%	111.50	Th01	168	171	98.25%	169.50
BLUE	D21					D21					D21	131	344	38.08%	237.50
BLUE	D18	110	144	76.39%	127.00	D18	251	318	78.93%	284.50	D18	375	482	77.80%	428.50
BLUE	Penta E	137	149	91.95%	143.00	Penta E	258	305	84.59%	281.50	Penta E	399	455	87.69%	427.00
GREEN	D5					D5	110	141	78.01%	125.50	D5	160	215	74.42%	187.50
GREEN	D13					D13	83	126	65.87%	104.50	D13	121	180	67.22%	150.50
GREEN	D7					D7					D7	127	138	92.03%	132.50
GREEN	D16					D16					D16	107	161	66.46%	134.00
GREEN	CSF1PO					CSF1PO					CSF1PO	101	198	51.01%	149.50
GREEN	Penta D					Penta D	185	206	89.81%	195.50	Penta D	281	306	91.83%	293.50
YELLOW	AMEL	156				AMEL	325				AMEL	488			
YELLOW	vWA	166				vWA	348				vWA	509			
YELLOW	D8					D8	179	196	91.33%	187.50	D8	266	304	87.50%	285.00
YELLOW	TPOX					TPOX	196	224	87.50%	210.00	TPOX	291	339	85.84%	315.00
YELLOW	FGA					FGA	133	210	63.33%	171.50	FGA	192	316	60.76%	254.00
	1 second					3 seconds					5 seconds				

	SMALL	LARGE			SMALL	LARGE			SMALL	LARGE		
	Average Imbalance				Average Imbalance				Average Imbalance			
	84.17%				81.67%				75.30%			
	Range	Average	StDev	%Diff	Range	Average	StDev	%Diff	Range	Average	StDev	%Diff
BLUE		135.0	11.3	8.38%	225.8	99.0	43.85%		315.6	132.4	41.95%	
GREEN					141.8	47.6	33.59%		174.6	61.5	35.25%	
YELLOW					189.7	19.3	10.20%		284.7	30.5	10.71%	

Table A3.7a: 0.05 ng template 3100-*Avant* capillary data (1, 3, and 5 second).

	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean
BLUE	D3					D3	115	284	40.49%	199.50	D3	130	342	38.01%	236.00
BLUE	Th01	230	235	97.87%	232.50	Th01	301	311	96.78%	306.00	Th01	347	357	97.20%	352.00
BLUE	D21	181	472	38.35%	326.50	D21	226	608	37.17%	417.00	D21	287	720	39.86%	503.50
BLUE	D18	514	637	80.69%	575.50	D18	669	832	80.41%	750.50	D18	773	989	78.16%	881.00
BLUE	Penta E	546	608	89.80%	577.00	Penta E	691	788	87.69%	739.50	Penta E	832	947	87.86%	889.50
GREEN	D5	226	297	76.09%	261.50	D5	285	375	76.00%	330.00	D5	343	455	75.38%	399.00
GREEN	D13	166	250	66.40%	208.00	D13	205	318	64.47%	261.50	D13	244	380	64.21%	312.00
GREEN	D7	179	186	96.24%	182.50	D7	218	238	91.60%	228.00	D7	263	282	93.26%	272.50
GREEN	D16	151	220	68.64%	185.50	D16	197	275	71.64%	236.00	D16	226	339	66.67%	282.50
GREEN	CSF1PO	137	284	48.24%	210.50	CSF1PO	170	369	46.07%	269.50	CSF1PO	204	424	48.11%	314.00
GREEN	Penta D	379	426	88.97%	402.50	Penta D	488	526	92.78%	507.00	Penta D	576	636	90.57%	606.00
YELLOW	AMEL	661				AMEL	846				AMEL	997			
YELLOW	vWA	670				vWA	827				vWA	973			
YELLOW	D8	375	415	90.36%	395.00	D8	471	523	90.06%	497.00	D8	563	631	89.22%	597.00
YELLOW	TPOX	399	458	87.12%	428.50	TPOX	497	582	85.40%	539.50	TPOX	605	690	87.68%	647.50
YELLOW	FGA	261	431	60.56%	346.00	FGA	338	546	61.90%	442.00	FGA	402	656	61.28%	529.00
	7 seconds					9 seconds					11 seconds				

	SMALL	LARGE			SMALL	LARGE			SMALL	LARGE		
	Average Imbalance				Average Imbalance				Average Imbalance			
	76.10%				73.03%				72.68%			
	Range	Average	StDev	%Diff	Range	Average	StDev	%Diff	Range	Average	StDev	%Diff
BLUE	351	427.9	175.6	41.03%	482.5	251.7	52.17%		572.4	300.9	52.58%	
GREEN	99	241.8	83.7	34.62%	305.3	105.1	34.43%		364.3	126.5	34.72%	
YELLOW	120	389.8	41.5	10.64%	492.8	48.9	9.92%		591.2	59.5	10.06%	

Table A3.7b: 0.05 ng template 3100-*Avant* capillary data (7, 9, and 11 second).

Appendix 3: Performance Verification Sensitivity Study-Intensity Data

Table A3.8

0.025 ng template 3100-*Avant* capillary data

	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean
BLUE	D3					D3					D3				
BLUE	Th01					Th01					Th01				
BLUE	D21					D21					D21	146	210	69.52%	178.00
BLUE	D18					D18	191	226	84.51%	208.50	D18	283	339	83.48%	311.00
BLUE	Penta E					Penta E					Penta E	157	444	35.36%	300.50
GREEN	D5					D5					D5				
GREEN	D13					D13					D13	115	223	51.57%	169.00
GREEN	D7					D7					D7				
GREEN	D16					D16					D16				
GREEN	CSF1PO					CSF1PO					CSF1PO				
GREEN	Penta D					Penta D	124	195	63.59%	159.50	Penta D	178	291	61.17%	234.50
YELLOW	AMEL					AMEL	196				AMEL	293			
YELLOW	vWA					vWA	128				vWA	180			
YELLOW	D8					D8					D8				
YELLOW	TPOX					TPOX	101	106	95.28%	103.50	TPOX	155	162	95.68%	158.50
YELLOW	FGA					FGA					FGA				
	1 second					3 seconds					5 seconds				

	SMALL	LARGE				SMALL	LARGE				SMALL	LARGE			
	Average Imbalance					Average Imbalance					Average Imbalance				
	#DIV/0!					81.13%					66.13%				
	Range	Average	StDev	%Diff		Range	Average	StDev	%Diff		Range	Average	StDev	%Diff	
BLUE		#DIV/0!			BLUE		208.5	#DIV/0!	#DIV/0!	BLUE		263.2	73.9	28.10%	
GREEN					GREEN					GREEN		201.8	46.3		
YELLOW					YELLOW		103.5		0.00%	YELLOW		158.5		0.00%	

Table A3.8a: 0.025 ng template 3100-*Avant* capillary data (1, 3, and 5 second).

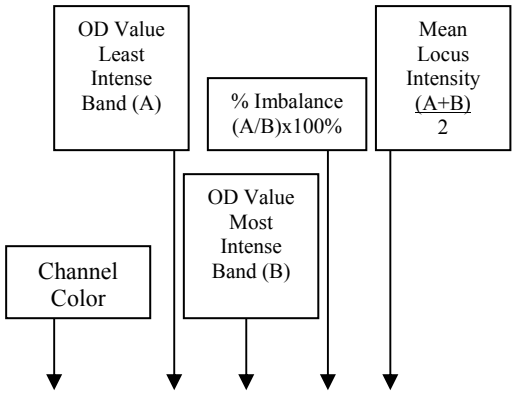
	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean
BLUE	D3					D3					D3				
BLUE	Th01					Th01					Th01	111	112	99.11%	111.50
BLUE	D21	196	284	69.01%	240.00	D21	249	365	68.22%	307.00	D21	302	423	71.39%	362.50
BLUE	D18	385	460	83.70%	422.50	D18	487	576	84.55%	531.50	D18	569	681	83.55%	625.00
BLUE	Penta E	210	610	34.43%	410.00	Penta E	274	772	35.49%	523.00	Penta E	319	918	34.75%	618.50
GREEN	D5					D5	117	221	52.94%	169.00	D5	132	268	49.25%	200.00
GREEN	D13	148	303	48.84%	225.50	D13	191	382	50.00%	286.50	D13	220	448	49.11%	334.00
GREEN	D7					D7					D7				
GREEN	D16					D16					D16				
GREEN	CSF1PO	130	324	40.12%	227.00	CSF1PO	161	402	40.05%	281.50	CSF1PO	198	474	41.77%	336.00
GREEN	Penta D	245	392	62.50%	318.50	Penta D	323	492	65.65%	407.50	Penta D	373	588	63.44%	480.50
YELLOW	AMEL	389				AMEL	497				AMEL	581			
YELLOW	vWA	231				vWA	288				vWA	328			
YELLOW	D8					D8	111	178	62.36%	144.50	D8	127	211	60.19%	169.00
YELLOW	TPOX	213	225	94.67%	219.00	TPOX	267	283	94.35%	275.00	TPOX	318	336	94.64%	327.00
YELLOW	FGA					FGA					FGA				
	7 seconds					9 seconds			39.54%		11 seconds				

	SMALL	LARGE				SMALL	LARGE				SMALL	LARGE			
	Average Imbalance					Average Imbalance					Average Imbalance				
	61.90%					61.51%					64.72%				
	Range	Average	StDev	%Diff		Range	Average	StDev	%Diff		Range	Average	StDev	%Diff	
BLUE	351	357.5	101.9	28.52%	BLUE		453.8	127.2	28.04%	BLUE		429.4	244.6	56.98%	
GREEN	99	257.0	53.3	20.73%	GREEN		286.1	97.4	34.05%	GREEN		337.6	114.6	33.93%	
YELLOW	120	219.0	#DIV/0!	#DIV/0!	YELLOW		209.8	92.3	43.99%	YELLOW		248.0	111.7	45.05%	

Table A3.8b: 0.025 ng template 3100-*Avant* capillary data (7, 9, and 11 second).

Appendix 3: Performance Verification Sensitivity Study-Intensity Data

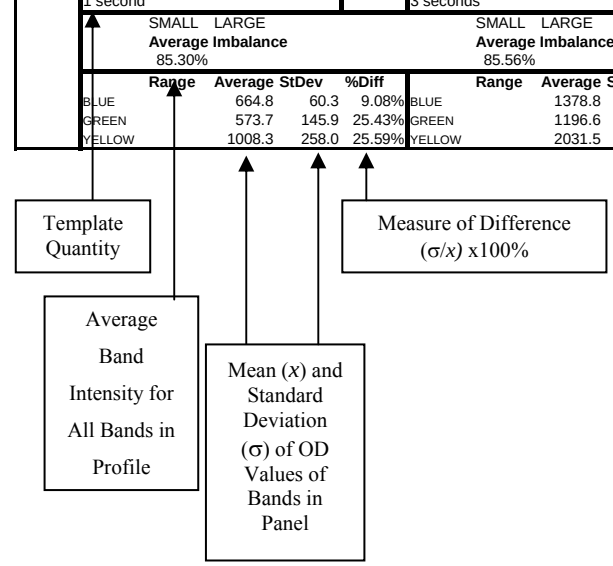
Appendix legend for gel platform (FMBIO®) data:



	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean
BLUE	D3	543	624	87.02%	583.50	D3	1145	1299	88.14%	1222.00	D3	1633	1902	85.86%	1767.50
BLUE	Th01	743	754	98.54%	748.50	Th01	1543	1545	99.87%	1544.00	Th01	2204	2240	98.39%	2222.00
BLUE	D21	571	777	73.49%	674.00	D21	1180	1589	74.26%	1384.50	D21	1650	2270	72.69%	1960.00
BLUE	D18	644	714	90.20%	679.00	D18	1339	1459	91.78%	1399.00	D18	1853	2066	89.69%	1959.50
BLUE	Penta E	467	811	57.58%	639.00	Penta E	996	1693	58.83%	1344.50	Penta E	1185	2188	54.16%	1686.50
GREEN	D5	748	821	91.11%	784.50	D5	1567	1679	93.33%	1623.00	D5	2228	2426	91.84%	2327.00
GREEN	D13	639	752	84.97%	695.50	D13	1358	1592	85.30%	1475.00	D13	1890	2234	84.60%	2062.00
GREEN	D7	435	442	98.42%	438.50	D7	906	907	99.89%	906.50	D7	1256	1279	98.20%	1267.50
GREEN	D16	376	528	71.21%	452.00	D16	778	1124	69.22%	951.00	D16	1099	1557	70.58%	1328.00
GREEN	CSF1PO	552	669	82.51%	610.50	CSF1PO	1146	1361	84.20%	1253.50	CSF1PO	1583	1895	83.54%	1739.00
GREEN	Penta D	392	530	73.96%	461.00	Penta D	809	1132	71.47%	970.50	Penta D	1050	1480	70.95%	1265.00
YELLOW	AMEL	1282				AMEL	2616				AMEL	3773			
YELLOW	vWA	1986				vWA	3867				vWA	5324			
YELLOW	D8	1265	1280	98.83%	1272.50	D8	2531	2605	97.16%	2568.00	D8	3580	3684	97.18%	3632.00
YELLOW	TPOX	712	802	88.78%	757.00	TPOX	1418	1621	87.48%	1519.50	TPOX	2004	2264	88.52%	2134.00
YELLOW	FGA	983	1008	97.52%	995.50	FGA	1976	2038	96.96%	2007.00	FGA	2728	2782	98.06%	2755.00
	1 second					3 seconds					5 seconds				

	SMALL	LARGE		SMALL	LARGE		SMALL	LARGE	
	Average Imbalance			**Average Imbalance**			**Average Imbalance**		
	85.30%			85.56%			84.59%		

	Range	Average	StDev	%Diff		Range	Average	StDev	%Diff		Range	Average	StDev	%Diff
BLUE		664.8	60.3	9.08%	BLUE		1378.8	115.6	8.39%	BLUE		1919.1	207.4	10.81%
GREEN		573.7	145.9	25.43%	GREEN		1196.6	302.7	25.30%	GREEN		1664.8	454.5	27.30%
YELLOW		1008.3	258.0	25.59%	YELLOW		2031.5	524.7	25.83%	YELLOW		2840.3	752.6	26.50%



Appendix 3: Performance Verification Sensitivity Study-Intensity Data

Table A3.9

Gel Platform FMBIO® II data (standard loading volume for each template quantity):

	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean
BLUE	D3	545	567	96.12%	556.00	D3	500	567	88.18%	533.50	D3	365	427	85.48%	396.00
BLUE	Th01	703	704	99.86%	703.50	Th01	570	571	99.82%	570.50	Th01	312	367	85.01%	339.50
BLUE	D21	649	673	96.43%	661.00	D21	543	557	97.49%	550.00	D21	432	536	80.60%	484.00
BLUE	D18	643	765	84.05%	704.00	D18	680	692	98.27%	686.00	D18	628	678	92.63%	653.00
BLUE	Penta E	658	793	82.98%	725.50	Penta E	664	876	75.80%	770.00	Penta E	755	979	77.12%	867.00
GREEN	D5	756	996	75.90%	876.00	D5	781	871	89.67%	826.00	D5	718	731	98.22%	724.50
GREEN	D13	1404	1476	95.12%	1440.00	D13	1234	1293	95.44%	1263.50	D13	931	935	99.57%	933.00
GREEN	D7	621	693	89.61%	657.00	D7	570	607	93.90%	588.50	D7	444	508	87.40%	476.00
GREEN	D16	407	430	94.65%	418.50	D16	335	415	80.72%	375.00	D16	254	402	63.18%	328.00
GREEN	CSF1PO	634	797	79.55%	715.50	CSF1PO	576	659	87.41%	617.50	CSF1PO	428	583	73.41%	505.50
GREEN	Penta D	381	472	80.72%	426.50	Penta D	371	405	91.60%	388.00	Penta D	343	364	94.23%	353.50
YELLOW	AMEL	1103				AMEL	1070				AMEL	1210			
YELLOW	vWA	3531				vWA	3193				vWA	2234			
YELLOW	D8	2634	2819	93.44%	2726.50	D8	2679	2720	98.49%	2699.50	D8	1761	2152	81.83%	1956.50
YELLOW	TPOX	1367	1432	95.46%	1399.50	TPOX	1194	1283	93.06%	1238.50	TPOX	864	1198	72.12%	1031.00
YELLOW	FGA	1805	2149	83.99%	1977.00	FGA	1698	2361	71.92%	2029.50	FGA	1564	2065	75.74%	1814.50
	3 ng					2 ng					1 ng				

	SMALL	LARGE		SMALL	LARGE		SMALL	LARGE	
	Average Imbalance			Average Imbalance			Average Imbalance		
	89.13%			90.13%			83.32%		

	Range	Average	StDev	%Diff		Range	Average	StDev	%Diff		Range	Average	StDev	%Diff
BLUE		670.0	67.9	10.13%	BLUE		622.0	102.1	16.41%	BLUE		547.9	214.2	39.09%
GREEN		755.6	378.5	50.09%	GREEN		676.4	332.3	49.13%	GREEN		553.4	233.4	42.18%
YELLOW		2034.3	665.4	32.71%	YELLOW		1989.2	731.3	36.77%	YELLOW		1600.7	498.4	31.14%

Table A3.9a: Gel Platform FMBIO® II data (3, 2, and 1 ng template).

	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean	LOCUS	Small	Large	%	Mean
BLUE	D3	246	289	85.12%	267.50	D3	161	172	93.60%	166.50	D3	63	83	75.90%	73.00
BLUE	Th01	196	235	83.40%	215.50	Th01	86	130	66.15%	108.00	Th01	7			7.00
BLUE	D21	224	366	61.20%	295.00	D21	141	219	64.38%	180.00	D21	160	166	96.39%	163.00
BLUE	D18	521	788	66.12%	654.50	D18	381	497	76.66%	439.00	D18	162	190	85.26%	176.00
BLUE	Penta E	633	786	80.53%	709.50	Penta E	563	621	90.66%	592.00	Penta E	198	250	79.20%	224.00
GREEN	D5	525	635	82.68%	580.00	D5	353	393	89.82%	373.00	D5	170	199	85.43%	184.50
GREEN	D13	655	691	94.79%	673.00	D13	308	381	80.84%	344.50	D13	153	200	76.50%	176.50
GREEN	D7	247	371	66.58%	309.00	D7	230	234	98.29%	232.00	D7	41	115	35.65%	78.00
GREEN	D16	192	220	87.27%	206.00	D16	84	101	83.17%	92.50	D16	32			32.00
GREEN	CSF1PO	343	486	70.58%	414.50	CSF1PO	262	350	74.86%	306.00	CSF1PO	146	188	77.66%	167.00
GREEN	Penta D	444	531	83.62%	487.50	Penta D	210	530	39.62%	370.00	Penta D	142	197	72.08%	169.50
YELLOW	AMEL	1099				AMEL	641				AMEL	307			
YELLOW	vWA	1393				vWA	1005				vWA	524			
YELLOW	D8	1105	1341	82.40%	1223.00	D8	901	1054	85.48%	977.50	D8	202	295	68.47%	248.50
YELLOW	TPOX	756	902	83.81%	829.00	TPOX	511	753	67.86%	632.00	TPOX	254	410	61.95%	332.00
YELLOW	FGA	1392	1629	85.45%	1510.50	FGA	872	943	92.47%	907.50	FGA	217	257	84.44%	237.00
	0.5 ng					0.25 ng					0.1 ng				

	SMALL	LARGE		SMALL	LARGE		SMALL	LARGE	
	Average Imbalance			Average Imbalance			Average Imbalance		
	79.54%			78.85%			74.91%		

	Range	Average	StDev	%Diff		Range	Average	StDev	%Diff		Range	Average	StDev	%Diff
BLUE		428.4	234.1	54.64%	BLUE		297.1	208.3	70.13%	BLUE		128.6	87.2	67.80%
GREEN		445.0	172.4	38.74%	GREEN		286.3	108.4	37.87%	GREEN		134.6	63.6	47.28%
YELLOW		1187.5	342.1	28.81%	YELLOW		839.0	182.7	21.77%	YELLOW		272.5	51.8	19.03%

Table A3.9b: Gel Platform FMBIO® II data (0.5, 0.25, and 0.1 ng template).

Appendix 4: Contamination Study Data and Overview

Wipe tests were conducted quarterly during the period the studies herein were conducted to monitor laboratory surface contamination. Key areas such as the extraction hood, PCR amplification set-up area, PCR room barrier (door and knob), and equipment (centrifuges) were swabbed following routine decontamination to determine levels of potential environmental contamination present on these surfaces. Table A4.1 summarizes results and each profile event is subsequently summarized.

Date mo/day/yr	Extraction Hood	PCR Set-up	PCR Room Doorknob – Lab Side	Centrifuge	Analyst Bench
9/5/2003	np	np	np	np	np
12/8/2003	np	np	np	np	np
3/5/2004	np	np	np	np	np
5/7/2004	np	np	np	np	np
8/6/2004	np	np	1		
11/5/2004	np	np	2		
2/9/2005	np	np	np		np
5/6/2005	np	3	np		
8/4/2005	np	np	np		np
11/4/2005	np	np	np		
2/9/2006	np	np	np		
5/5/2006	np	np	np		
8/4/2006	np	np	np		
11/3/2006	np	np	np		
2/2/2007	np	np	np		4
5/9/2007	np	np	np		np
8/6/2007	np	np	np		
11/5/2007	np	np	np		
2/19/2008	np	np	np		
5/6/2008	np	np	5		
7/13/2008	np	6	np		
11/7/2008	np	np	np		

Table A4.1 Overview of the environmental contamination assessment. The dates the wipe test swabs were collected are indicated. Samples were extracted and consumed for amplification using PowerPlex® 16. Gel profiles were analyzed for bands and electropherograms were analyzed for peaks above 100 RFU; capillary data was also verified below the threshold visually to verify absence of low-level profiles. Events where alleles were detected are numbered 1-6. The absence of data indicates that that surface was not collected, np=no profile.

Appendix 4: Contamination Study Data and Overview

Event 1 involved the detection of three bands from the PCR room door knob on the pre-amplification side of the door. This was X at Amelogenin (OD=107), and 11/13 at D5S818 (OD=60/64). The alleles are frequently observed in the population and the absence of a Y at this level does not mean the source is female. However, it was noted the peaks were not attributable to the female analysts collecting or running the wipe test. Due to the common nature of the alleles, attribution was not further pursued.

The second event involved just two bands at a single locus; this was 5/9 at Penta E (OD=60/107). The alleles were not attributable to the two female analysts that collected and processed the samples; however they were attributable to a third male analyst who accesses the room.

Event 3 arose from the PCR set-up area and consisted of one peak above 100 RFU in the form of an X at Amelogenin (103 RFU). Analysis below the threshold noted six other peaks ranging from31-74 RFU that were attributable to the analyst who extracted and amplified the wipe test samples.

Regarding event 4, an analyst's work bench resulted in the detection of an 11 at TPOX (106 RFU). Other possible peaks below the threshold were noted at four loci with RFU ranging from 38-76. All five of the noted peaks were attributable to two of the analysts, one of whom was assigned that bench space for screening at that time.

Event 5 was the most severe. The swabbing originated from the PCR door and resulted in six peaks across six loci exceeding 100 RFU and an additional twelve peaks at ten loci ranging from 35-98 RFU. All peaks were consistent with the analyst that collected and processed the wipe test samples.

Regarding event 6, at PCR set-up, no peaks were detected above 100 RFU, but a low grade profile consistent with the laboratory analyst other than the person who conducted the wipe test was determined the source of the low-level genotype detected (average peak heights were 60 RFU).