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EVALUATION OF THE SLICPREP™ 96 DEVICE FOR USE IN EXTRACTION OF FORENSIC SAMPLES ON THE BIOMEK® 2000 LABORATORY AUTOMATION WORKSTATION

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CHAPTER 1

INTRODUCTION

Extraction is a critical step in DNA analysis, with all subsequent steps dependant on the quality and quantity of DNA extracted. The most common extraction technique used in forensic laboratories is organic extraction. This technique is time-consuming, labor intensive and uses hazardous reagents (1). Promega's DNA IQ™ System (Madison, WI) for DNA extraction is non-hazardous and makes use of a silica-coated magnetic bead resin to which deoxyribonucleic acid (DNA) has an affinity, thus doing away with lengthy centrifugation steps (1). However, manual DNA IQ™ extraction is still time consuming. With the number of backlogged cases increasing in forensic laboratories across the country, efficiency is essential. The less time an analyst spends performing DNA extractions, the more time the analyst can spend analyzing and interpreting DNA results.

The Palm Beach County Sheriff's Office (PBSO) Crime Laboratory is a small casework laboratory with seven DNA Analysts, a Laboratory Analyst and an Evidence Coordinator. Yet, Palm Beach County is one of the largest counties in Florida, with a population of over 1.2 million citizens. The Serology/DNA Section of the PBSO Crime Laboratory services over 34 law enforcement agencies, including the Florida State Highway Patrol and the county Medical Examiners Office among others (2). During the past ten years there has been an increased demand for DNA testing, but little change in

the number of analysts and laboratory space. In 2004, the PBSO Crime Laboratory DNA/Serology section experienced a 50% increase in casework requests and a 40% increase in the number of samples tested per case. Therefore, to increase efficiency of DNA analysis, PBSO validated and implemented the BioMek® 2000 Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA) and have been using it for forensic casework since June 2003. This has enabled PBSO to convert the extraction process from manual to semi-automated. The ultimate goal is to shift further towards complete automation. Currently, sample cuttings and swabs must be digested in 1.5 ml microcentrifuge tubes and then manually transferred into a spin basket. Following centrifugation, the samples must be transferred once again to a 96 deep-well plate before starting automated DNA purification.

Scientists at the Promega Corporation have developed a Slicprep™ 96 Device (Figure 1) which has three components: a 96-well spin basket plate, a collar and a 96 deep-well plate. The 96-well spin basket plate fits into the 96 deep-well plate and the collar is used as a separation device between the two plates. The use of the Slicprep™ 96 device for DNA extraction eliminates the need for most manual preprocessing, with both digestion and centrifugation carried out in the same unit, improving the efficiency of the DNA extraction process. Following centrifugation, the 96 deep-well plate can be placed directly onto the BioMek® 2000 deck for automated DNA purification.

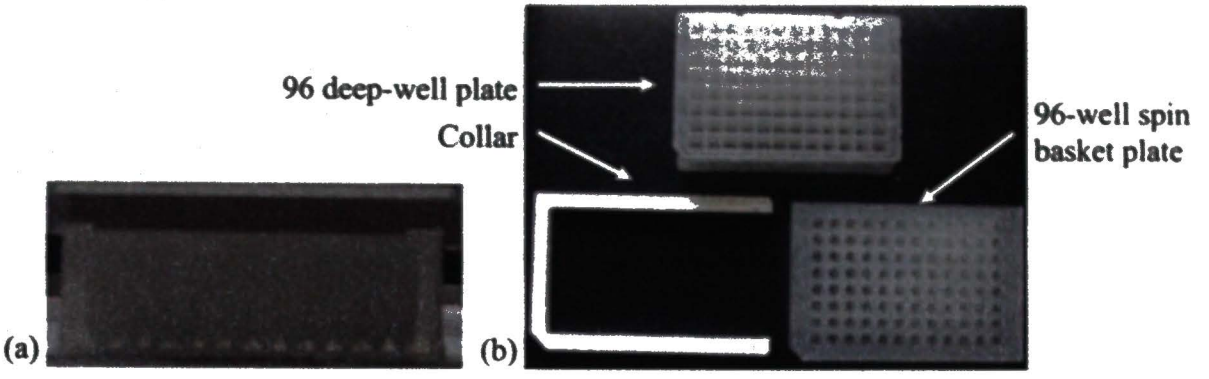

Figure 1. The Slicprep™ 96 Device. Figure 1a shows the device put together. In Figure 1b the three components are shown separately.

However, before the Slicprep™ 96 Device can be implemented for forensic casework, it must be validated. Standard 8.1 of the FBI Quality Assurance Standards set forth by the DNA Advisory Board (DAB) states that "the laboratory shall use validated methods and procedures for forensic casework analyses" (3). The purpose of this study was to evaluate the Slicprep™ 96 Device for extraction of forensic samples on the BioMek® 2000 robot to determine if it can further automate and increase the efficiency of the extraction process as well as generate optimal DNA yields with no contamination. This validation study was not as comprehensive as PBSO's validation of the BioMek® 2000, because the platform and reagents used were the same. The differences include the use of the Slicprep™ 96 Device during extraction and the slight modifications to protocol that were made to accommodate this device.

The first studies were designed to determine if there are risks for contamination with the Slicprep™ plate. Both a Checkerboard and a Zebra Stripe test were conducted to determine if the introduction of the Slicprep™ 96 Device into the extraction process would result in any carryover contamination between wells. A variety of samples,

including buccal swabs, blood standards, mixtures and touch evidence were tested. This was done to determine the reliability and capability of the Slicprep™ 96 Device to successfully extract various concentrations of DNA typically present in forensic evidence samples. Mock sexual assault samples were not processed because the current Slicprep™ 96 Device is not designed for use with differential extractions. All samples were run in triplicate to assess the reproducibility of the procedure.

After the samples were extracted on the BioMek® 2000, samples were quantified using real-time PCR (polymerase chain reaction). Real-time PCR (qPCR) was performed on the ABI PRISM® 7000 Sequence Detection System using the Quantifiler™ Human DNA Quantification Kit (Applied Biosystems, Foster City, CA). Amplification of all samples was performed using the PowerPlex 16® BIO system (Promega Corp.), followed by post-amplification electrophoresis on 3% Embitec (EmbiTec, San Diego, CA) agarose gels in some instances and then 6% PAGE Plus™ (Amresco) polyacrylamide gel electrophoresis. The Hitachi FMBIO® II Fluorescent Imaging System (MiraiBio, Alameda, CA) and FMBIO® Analysis 8.0 Software in conjunction with STaRCALL™ Software were utilized for allele detection and typing.

CHAPTER II

BACKGROUND

DNA Extraction

Numerous extraction techniques have been developed over the years. Commonly used methods include organic extraction with phenol/chloroform and Chelex® extraction. Organic extraction is laborious, involves the use of hazardous reagents and does not allow for automation (1). It may also result in significant DNA loss. Even with concentration devices, organic extraction does not optimize the extraction of low template DNA samples and there is a potential for PCR inhibitors to be extracted with the DNA. Chelex® extraction is much quicker, but does not remove inhibitors that may interfere with DNA amplification (4). Promega's DNA IQ™ System makes use of a silica-coated magnetic bead resin that binds to DNA in the presence of chaotropic agents (4), separating the DNA from the rest of the cellular components. As a result, no hazardous chemicals or time-consuming centrifugation steps are necessary. There is also no observed inhibition in later stages of DNA analysis (6). Another major benefit of the DNA IQ™ System is that it is amenable to automation.

The DNA IQ™ System contains four different reagents: DNA IQ™ Lysis Buffer, DNA IQ™ Resin, DNA IQ™ Wash Buffer and DNA IQ™ Elution Buffer. Samples are incubated in the presence of DNA IQ™ Lysis Buffer and dithiothreitol (DTT) to digest the cells. After a 30 minute incubation period, the samples are placed in a spin basket

and centrifuged to allow for optimal DNA recovery (6). The addition of the silica-coated magnetic resin, results in DNA binding to the resin with high affinity (1). The samples are then transferred to a shallow Greiner plate which fits on top of a MagnaBot (Promega Corp.), consisting of 24 separate magnets, one for every four sample wells (Figure 2). As a result, the magnetic resin is drawn towards a magnet, pulling the DNA along with it. The remaining solution can then be drawn out and the resin is washed once with DNA IQ™ Lysis Buffer and then three times with DNA IQ™ Wash Buffer to purify the DNA. The addition of DNA IQ™ Elution Buffer releases DNA that is bound to the resin, allowing the supernatant containing the DNA to be transferred to a 1.5 ml microcentrifuge tube.

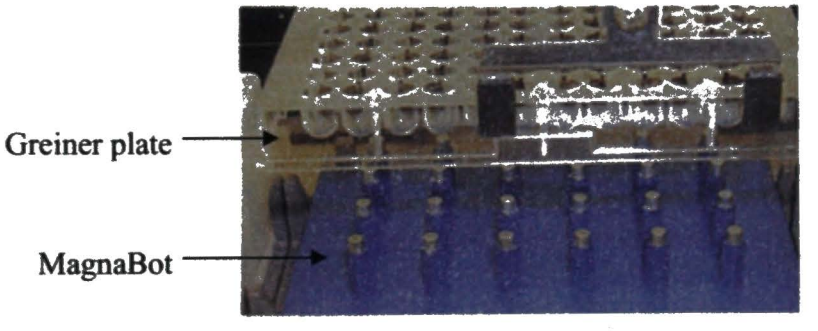

Figure 2. Greiner plate and MagnaBot® (7).

The first automated DNA extraction systems utilized in the forensic field were installed in convicted offender laboratories. This was to aid those laboratories in processing large volumes of single source samples for DNA profiling and submission into CODIS (Combined DNA Index System). Due to various substrates and varying DNA template concentrations of forensic evidentiary samples, it has taken longer to

implement robotics for processing forensic casework samples (8). A few of the robotic platforms that have been made available for forensic DNA extraction include the BioRobot™ EZ1 (QIAGEN, Hilden, Germany), the BioRobot™ M48 (QIAGEN, Hilden, Germany) and the BioMek® 2000 Laboratory Automation Workstation. All three of these workstations utilize silica-coated magnetic bead technology. The BioRobot™ EZ1 is a small throughput robot that uses protocol cards which are preprogrammed and single use reagent cartridges (9). With this workstation, DNA extraction occurs within a barrier pipette tip. The pipette tip also serves as the reaction chamber for the BioRobot™ M48 (10). However, the BioRobot™ M48 can process up to 48 samples per run compared to only six for the BioRobot™ EZ1. It also contains a UV light source which decontaminates the worksurface between extraction runs (11).

Beckman Coulter's BioMek® 2000 Laboratory Automation Workstation (Figure 3) can extract DNA from as many as 88 samples per run and was the workstation of choice for PBSO. Different tools such as single or multi-channel pipettes and a gripper can be attached to the tool rack so that the robot can pipette various volumes and move plates around. One disadvantage of the BioMek® 2000 is its lack of liquid and motion sensors, therefore, the robot arm can not sense any possible obstructions in the pipette tips or on the deck during extraction.

Promega has developed new components for Beckman Coulter's BioMek® 2000 and has modified computer applications to accommodate the DNA IQ™ System for DNA extraction. These components include the MagnaBot® as well as a shaking platform and a thermal exchange unit which is connected to a water bath by plastic tubing

(6). The Magnabot® separates the magnetic resin from the lysate solution and the thermal exhange unit is necessary for elution of the DNA from the magnetic resin (8). Therefore, the BioMek® 2000 robot can perform all the steps necessary for extraction via the DNA IQ™ System, including pipetting, magnetic separation, incubation and shaking (8).

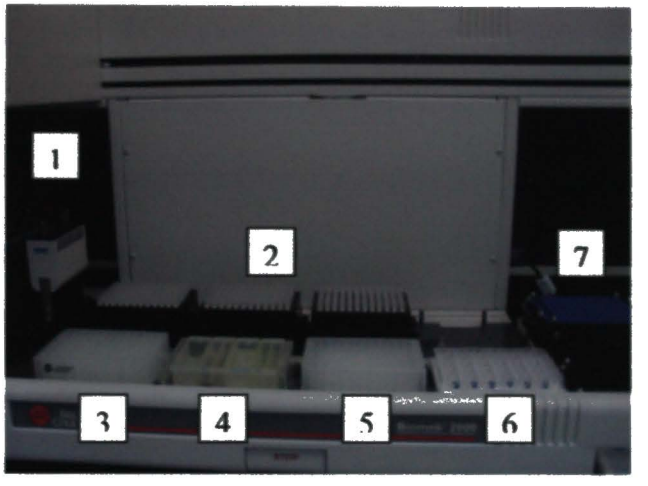

Figure 3. BioMek® 2000 Laboratory Automation Workstation. Picture shows (1) tool rack – position A1, (2) tip box holders – positions A2, A3 and A4, (3) 96 deep-well plate containing samples – position B2, (4) reservoirs containing required reagents – position B3, (5) 96 deep-well plate for disposal of waste – position B4, (6) Greiner plate on MagnaBot – position B5 and (7) heat transfer block on thermal exchange unit.

Use of the BioMek® 2000 can reduce pipetting errors and it can be modified to automate other stages of DNA analysis, such as quantification setup, pre-amplification setup and amplification. Also, no carryover contamination has been observed during extraction with the robot (8). With the current method employed at PBSO, a series of manual preprocessing steps are required prior to automated DNA purification. Use of the Slicprep™ 96 Device reduces the number of manual preprocessing steps so that the

majority of the DNA extraction is performed on the BioMek® 2000. As was mentioned previously, the Slicprep™ 96 Device is comprised of three parts: a 96-well spin basket plate, a 96 deep-well plate and a collar which separates these two plates. The 96 deep-well plate that is currently being sold with the device is manufactured by ABgene (Rochester, NY). Allan Tereba, a scientist at Promega, mentioned that the 96-well spin basket plate was designed to fit into a number of deep-well plates (personal communication, June 2, 2005). However, the ABgene plate was chosen because it requires the smallest amount of DNA IQ™ Lysis Buffer. The dimensions of the Beckman Coulter Square Well plate currently in use for extraction at PBSO are slightly different than the ABgene plate. Therefore, the BioMek® 2000 methods will require definition changes in order to optimally accommodate the ABgene plate. If the Beckman Coulter Square Well plate is used, no changes to the method labware definitions are necessary, however, more DNA IQ™ Lysis Buffer must be added to fully cover the sample during incubation.

Quantification Using Real-Time PCR (qPCR)

According to the national standards and guidelines set forth by the DNA Advisory Board (DAB), all forensic casework unknown DNA samples must be quantified (3). More specifically, the amount of human DNA in the sample must be determined. This is necessary to ensure that the optimal amount of DNA (~1 ng) is added to the multiplex PCR amplification during the next stage of the process (12) as well as to determine if a low template DNA sample should be concentrated prior to amplification. Real-time polymerase chain reaction (qPCR) enables the PCR process to be monitored as it occurs

rather than after the process is complete (13). This enables quantification measurements to be taken during the exponential phase of PCR, during which ample reagents are available and the amplicons are doubling with each cycle (14).

Applied Biosystems has developed a Quantifiler™ Human DNA Quantification Kit for use on the ABI PRISM® 7000 Sequence Detection System, which is based on the 5' nuclease assay and TaqMan® probe-based technology (15). The TaqMan® probe contains a reporter fluorescent dye on the 5′ end and a quencher dye on the 3′ end. As long as the probe is intact, the fluorescence emitted by the reporter dye is quenched due to fluorescence resonance energy transfer (FRET) (13). In the presence of target sequence, the probe is cleaved by the 5' nuclease activity of *Taq* DNA polymerase during primer extension. This results in a separation of the reporter dye from the quencher dye increasing reporter dye signal (Figure 4). As more reporter dye molecules are cleaved with each cycle, the fluorescence intensity increases proportionally to the number of amplicons produced (13).

Following analysis of quantification results, a cycle threshold (C_T) value is assigned for each sample. This value represents the exact cycle number where the amplification curve crosses a set fluorescence detection threshold and is inversely proportional to the DNA concentration (13). This can be observed in Figure 5. The quantity of unknown sample can be extrapolated by comparison to a standard curve of known concentrations that was generated during the qPCR reaction.

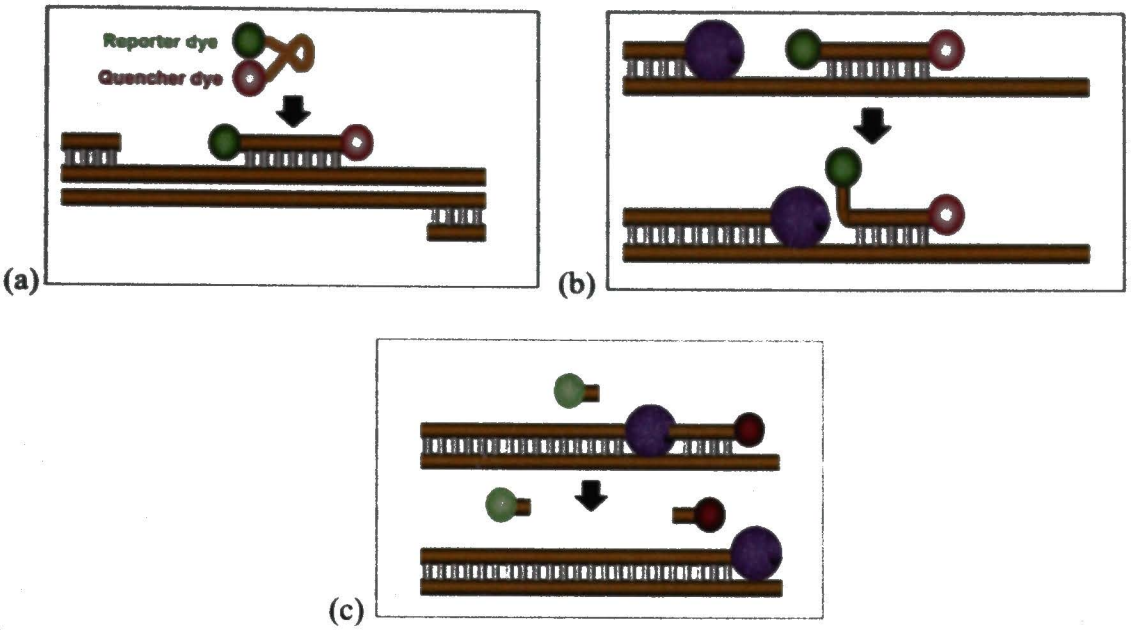

Figure 4. The 5' Nuclease Activity. In figure (a), the probe anneals to the target-specific area on the template. In figure (b), the polymerase collides with the Taqman® probe. Figure (c) shows the Taqman® probe being cleaved (14).

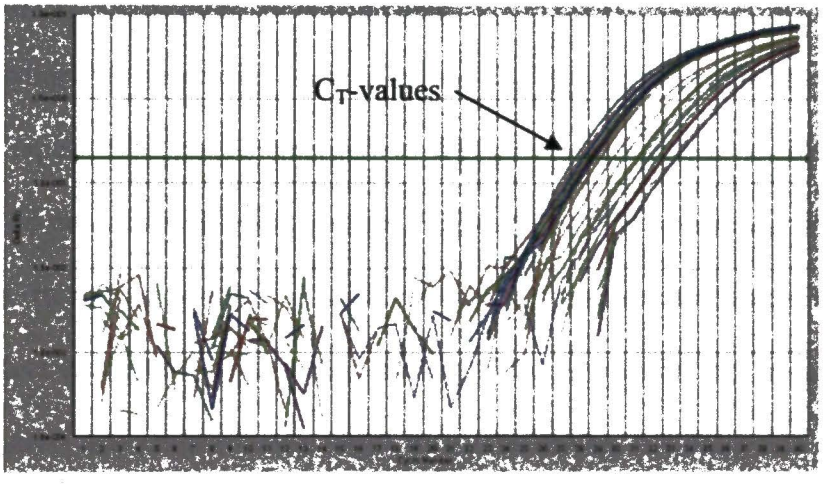

Figure 5. Amplification Plot of Delta Rn vs. Cycle Number.

Real-time PCR is often preferred over slot-blot hybridization techniques due to its increased sensitivity and ease of use. The technique is extremely sensitive, detecting DNA quantities ranging from 0.023 – 50 ng/2μL (14). Since the Quantifiler™ Human DNA Quantification Kit is based on PCR technology, it can provide a more comparative indication of what will occur downstream in STR analysis than other quantification methods (15). Very little manual work is required, especially when the reaction setup is performed on the BioMek® 2000. This significantly reduces the likelihood of human error and hands on time allowing the analyst to focus on other laboratory work. In addition, the kit contains an internal PCR control (IPC) which makes it possible to identify samples which do not contain human DNA or those that contain PCR inhibitors (15).

PCR, STRs and the PowerPlex® 16 BIO System

Following DNA extraction and quantification, specific regions of the DNA called short tandem repeat (STR) loci are amplified. STRs or microsatellites are the most commonly used markers in forensic science. They consist of a core repeat of 2-6 base pairs (bp) that is repeated in tandem (16). The number of repeats and the size of the PCR product are highly polymorphic, making STRs a powerful tool in forensic DNA testing. The small size range of these target loci, 100-500 bp, is what makes STRs amenable to multiplex PCR (12).

PCR is an enzymatic process that replicates a specific region of DNA repeatedly, yielding millions of copies of a target sequence. Multiplex PCR is a type of PCR in which more than one pair of primers is included in the same reaction enabling two or

more target sequences to be amplified simultaneously (17). Therefore, many STR loci can be typed in a single PCR reaction. Many different STR multiplex kits have been developed over the years, amplifying as few as three to as many as 16 loci simultaneously. The PowerPlex® 16 BIO System (Promega Corp.), which was introduced in May 2000, allows for amplifcation of the 13 CODIS STR loci as well as two pentanucleotide repeat loci and the sex-determining locus, Amelogenin. Specifically, this kit contains the loci FGA, TPOX, D8S1179, vWA, Amelogenin, Penta E, D18S51, D21S11, TH01, D3S1358, Penta D, CSF1PO, D16S539, D7S820, D13S317 and D5S818(12). The PowerPlex® 16 BIO System is optimized for use with Hitachi's FMBIO II® Fluorescent Imaging System and a primer for each locus is fluorescently labeled with either Rhodamine Red™-X (RRX), fluorescein (FL) or 6-carboxy-4',5'-dichloro-2',7'-dimethoxy-fluorescein (JOE) so that all 16 loci can later be analyzed in a single gel lane (12).

Polyacrylamide Gel Electrophoresis and the FMBIO II®

After the samples have been amplified, the amplicons must be separated in order to obtain a DNA profile. The two major methods used by forensic laboratories for separation are capillary electrophoresis (CE) and polyacrylamide gel electrophoresis (PAGE). In the past, the separation method chosen was dependent on which multiplex PCR kit was used. Kits manufactured by Promega were designed for PAGE and the Hitachi FMBIO, whereas kits manufactured by Applied Biosystems were designed for the ABI 310 or 3100 CE instruments (2). However, Promega now manufacturers two PowerPlex® 16 kits, one designed for ABI platforms (PowerPlex® 16) and the other designed for FMBIO® platforms (PowerPlex® 16 BIO).

During capillary electrophoresis (CE), DNA samples are injected into a polymer-filled capillary by electrokinetic injection and the negatively-charged DNA molecules move away from the electrode at one end of the capillary towards the anode at the other end (18). When the fluorescently-tagged DNA fragments reach the detection window, they are excited by a laser and the emitted fluorescence is captured by a charged-coupled device (CCD) camera (19). Prior to the run, a denaturant (formamide) is added to the samples and the samples are then heat denatured and snapped cool on ice in order to separate the two DNA strands of each amplicon. Two different polymers are available: POP-4 and POP-6. POP-4 polymer, the less stringent of the two, is typically used for STR typing (20). Color separation is performed by a mathematical matrix which separates out the contributions of other dyes in areas of spectral overlap. The four dyes utilized for CE are 5-FAM, ROX, JOE and NED. Both an internal size standard and an allelic ladder are run as well so that the correct size of fragments and allele calls can be made.

Polyacrylamide gels consist of long strands of acrylamide with occasional crosslinks. The higher the acrylamide concentration is, the longer the chain length and the higher the resolution (21). DNA fragments which are smaller in size will migrate further through the gel than larger sized fragments and therefore will have higher resolution (20). As with CE, the samples are heat denatured and snapped cooled on ice prior to electrophoresis. A denaturant such as urea is also added to the gel to keep the DNA strands separated (20). This is to ensure that the rate by which the DNA fragments migrate through the gel is independent of the DNA sequence.

Unlike with CE, fluorescence detection is performed after electrophoresis by scanning the gel on the Hitachi FMBIO® II Fluorescent Imaging System, a flat bed laser-scanning instrument. As a result, more than one gel can be running simultaneously while another is being scanned. The PowerPlex® 16 BIO STR loci are fluorescently labeled with RRX, FL or JOE, which are detected at wavelengths of 598 nm, 505 nm and 577 nm, respectively (12). A fourth dye, Texas Red®-X, is utilized for the internal lane sizing standard (ILS 600 BIO). It is detected at 665 nm. Once the gel has been scanned, the colored bands can be separated using the FMBIO® Analysis 8.0 Software program. Every laboratory must define its own color separation strategy. A color matrix is run on each gel to aid in color separation and a mathematical matrix is used to separate the overlapping dyes from one another, similarly to CE. Only the dyes used are different. The FMBIO® Analysis 8.0 Software program sizes the DNA fragments using the internal lane standard (ILS 600 BIO) and calculates optical density values. After color separation and sizing is performed, allele calls can be made using the STaRCall™ Genotyping Software program by comparison to allelic ladders (20).

CHAPTER 3

MATERIALS AND METHODS

Samples

Buccal swabs, bloodstains, mixtures and touch evidence (low template DNA) samples were utilized to determine the feasibility, applicability, and efficiency of incorporating the Slicprep™ 96 Device into the PBSO robotic extraction procedure. Buccal swabs, including inheritance samples, were collected from ten male and female volunteers using sterile cotton-tipped swabs. Blood standards were collected previously from two PBSO staff members and stored in purple-top tubes. The following ratios of male and female whole blood samples were mixed: 1:0, 3:1, 1:3 and 0:1. In addition, a 3-individual mixture consisting of whole blood and buccal cells was prepared. This was done by soaking a buccal swab overnight in a 1:1 mixture of whole blood from two different individuals. Touch evidence, including a keyboard, television remote, microwave oven, soda can, car steering wheel and a light switch were swabbed using sterile cotton-tipped swabs to obtain any epithelial cells that may be present. Cuttings (~2 mm x 3.2 mm) from blood stain cards (Whatman), which contained blood from a single individual, were used for two contamination studies. All samples were run in triplicate to assess the reproducibility of the technique. To do so, three cuttings were taken from each swab. With the exception of the contamination studies, all extraction

were done using appropriate controls including a reagent control positive (RCP), also known as P2, and a reagent control negative (RCN).

Contamination Studies

Prior to applying this protocol to the samples collected, both the Checkerboard test and the Zebra Stripe test were utilized to determine how well the protocol was working and also to monitor for any contamination that may have been introduced during sample loading, centrifugation or the automated extraction steps. For the Checkerboard test, blood samples were placed in every other well of the Slicprep™ 96 Device. The remaining wells served as reagent blanks so that every sample was surrounded by a blank (Figure 6). In total, 44 samples and 44 reagent blanks were tested. The last column of the plate was not utilized. For the Zebra Stripe test, blood samples were placed in alternating columns in which every sample column was surrounded by a column of reagent blanks (Figure 7). In total, 40 samples and 48 reagent blanks were tested. Again, the last column of the plate was left empty.

DNA Extraction

The DNA IQ™ System in conjunction with the BioMek® 2000 Laboratory Automation Workstation was utilized for all extractions. In addition, Promega's Slicprep™ 96 device was evaluated with hopes to further increase the automation and efficiency of the overall process. Cuttings from swabs or blood stain cards were placed directly into the 96-well spin basket plate, ensuring that the samples were pushed to the bottom of the wells. A master mix of DNA IQ™ Lysis Buffer and DTT was made which

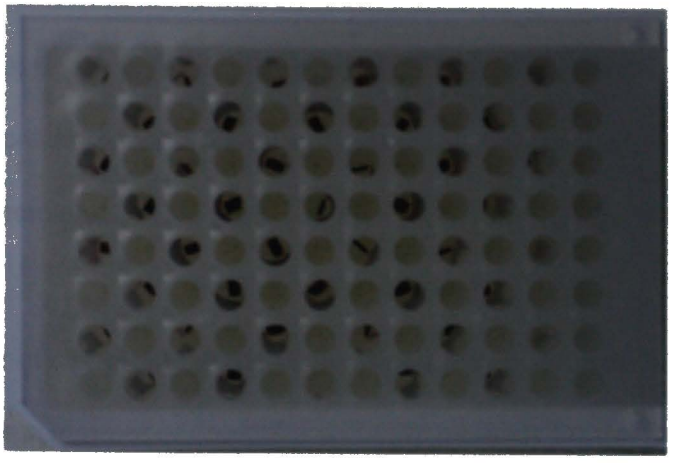

Figure 6. Set up of the Checkerboard test using the Slicprep™ 96 Device. Cuttings from a blood stain card have been placed in every other well. The last column (row12) is empty.

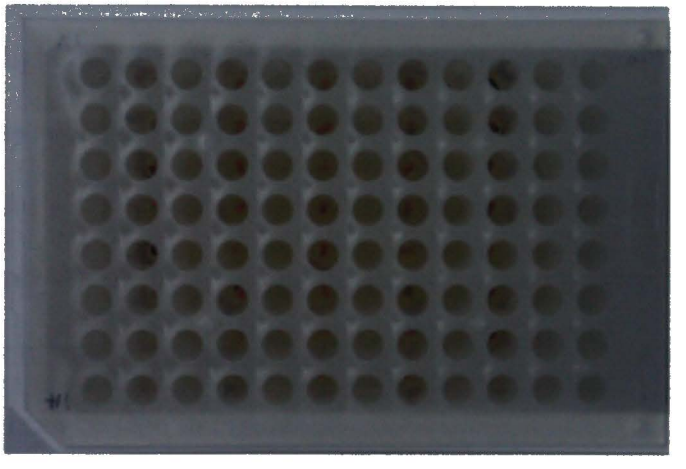

Figure 7. Set up of the Zebra Stripe test using the Slicprep™ 96 Device. Cuttings from a blood stain card have been placed in every other column. The last column (row12) is empty.

contained 500 µl of DNA IQ™ Lysis Buffer and 50 µl of 1M DTT per sample. Then 500 µl of this master mix was added to each sample. The Slicprep™ 96 Device was sealed with a foil film and then allowed to incubate for 1 hour in a 70° C water bath in

order to facilitate cell lysis. Following this incubation period, the collar was inserted and the Slicprep™ 96 Device was then centrifuged at 1500 g for 5 minutes. After centrifugation, the collar and the 96-well spin basket plate were removed so that only the 96 deep-well plate remained. This plate was then placed directly on the deck of the BioMek® 2000.

The number of samples to extract determined which software method was chosen. Promega has written a number of computer software methods for the BioMek® 2000 that are specialized for use with their DNA IQ™ System. PBSO has five major BioMek® 2000 methods in use: 16, 24, 40, 56 and 88 sample methods. The only differences between these methods are the amount of tips and reagents required. However, these methods required slight modifications to accommodate the 500 µl of DNA IQ™ Lysis Buffer/DTT solution added during the Slicprep™ method compared to the 150 µl added using PBSO's current validated method. A 72 sample method was also written for this study by PBSO. Once the method was chosen, the edit screen was opened to display on the computer where to place tips, reagents and plates (Figure 8). An example of a deck set up for extraction can be seen in Figure 9.

The reagents required and their volume is also reported for the particular method chosen. Reservoir 1 contained a DNA IQ™ Lysis Buffer/DTT solution. For every100 µl of Lysis Buffer, 1 µl of DTT must be added. Reservoir 2 consisted of a Lysis Buffer/DTT solution and DNA IQ™ Resin of which 120 µl was added to each sample. More specifically, 113 µl of DNA IQ™ Lysis Buffer and 7 µl of DNA IQ™ Resin was added to each sample. Reservoir 4 contained DNA IQ™ 1X Wash Buffer and the fifth reservoir

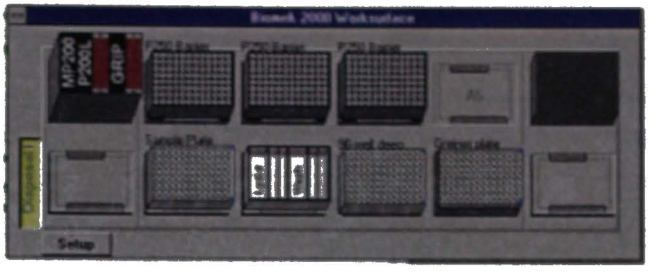

Figure 8. View from Edit Screen on how to set up the BioMek® 2000 Laboratory Automation Workstation for extraction using the 88 Samples for 96-well method.

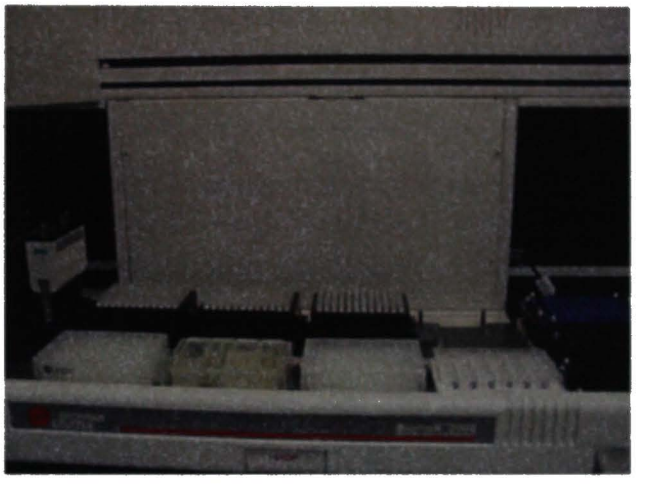

Figure 9. Deck setup of the BioMek® 2000 Laboratory Automation Workstation for extraction using the 88 Samples for 96-well method.

held DNA IQ™ Elution Buffer. For each sample, three washings of 100 µl with DNA IQ™ Wash Buffer are required. Reservoirs 3 and 6 were left empty. Once the appropriate reagents were added to the reservoirs, the run was executed. A summary of the reagents is provided in Table 1.

Reservoir	Reagents	Volume Added to Each Sample (µl)
1	9.7 ml DNA IQ™ Lysis Buffer 97 µl DTT	100
2	10.7 ml DNA IQ™ Lysis Buffer 107 µl DTT 665 µl DNA IQ™ Resin	120
3	Empty	N/A
4	30 ml DNA IQ™ 1X Wash Buffer	100 x 3
5	5 ml DNA IQ™ Elution Buffer	50
6	Empty	N/A

Table 1. Reagents required for 88 Samples for 96-well method. For every 100 µl of DNA IQ™ Lysis Buffer, 1 µl of 1M DTT was added. N/A = not applicable.

For the elution step, 24-Place Tube Racks which contain labeled microcentrifuge tubes were placed on the robot deck (Figure 10). The samples were resuspended in 50 µl of DNA IQ™ Elution Buffer and then transferred into individually-labeled microcentrifuge tubes. Following elution, the microcentrifuge tubes containing DNA extract were capped and the robot deck was wiped down with ethanol.

Quantification

Quantification was performed on the ABI PRISM® 7000 Sequence Detection System using the Quantifiler™ Human DNA Quantification Kit. The BioMek® 2000 was used to set up the PCR reactions as well as the DNA standard curve, helping to eliminate any pipetting errors that may occur when done manually. Only preparation of the qPCR master mix was done manually.

Figure 10. Deck setup of the BioMek® 2000 Laboratory Automation Workstation for the 96-well 88 Sample Elution method.

A divided reservoir containing a mixture of Quantifiler™ PCR Reaction Mix and Quantifiler™ Human Primer Mix (qPCR master mix) on the left side and TE^{-4} Buffer (10 mM Tris-HCl, 0.1 mM EDTA) on the right side was prepared. One row of strip tubes was then placed into the last column (A12-H12) of a 96-well support base. Quantifiler™ Human DNA Standard was vortexed for 15 seconds and 20 µl of it was added to the strip tube in position A12. The BioMek® 2000 uses the strip tubes and TE^{-4} Buffer for dilution of the DNA standard and generation of the standard curve. For every plate quantified, an Optical Reaction Plate Control (OPC) was present. The OPC contains TE^{-4} Buffer in place of DNA extract. It monitors for any contaminants that may be present in the reagents or that are introduced during the BioMek® method. For each qPCR reaction, the BioMek® 2000 aliquotted 23 µl of master mix and 2 µl of DNA extract into a MicroAmp® 96-well Optical Reaction Plate. The standard curve was aliquotted in duplicate and was located in rows 11 and 12 of the plate. Meanwhile, the sample

position on the Optical Reaction Plate was recorded using the Quantifiler Total Human 96-well template worksheet.

After the qPCR reactions were set up, the optical reaction plate was sealed with an Optical Adhesive Cover (Applied Biosystems) and then centrifuged at 3700 RPM for 2 minutes to remove any bubbles that may be present. The plate was then placed into the ABI PRISM® 7000 Sequence Detection System and topped with the Compression Pad (Applied Biosystems). The reaction was carried out under the following parameters: 95° C for 10 minutes and 40 cycles of 95° C for 15 seconds/60° C for 60 seconds. Total reaction time was 1 hour and 46 minutes. Following quantification, the results were analyzed and exported into a Microsoft® Excel spreadsheet.

Microcon® Concentration

All samples that contained <0.1 ng/µl according to the quantification results were concentrated with the exception of those samples for which the DNA quantity was "unknown undetermined". Microcon® concentration was performed according to manufacturer's instructions with slight variations in centrifugation speed and time (22). The first centrifugation step was carried out at 5200 g for 10 minutes. In the second spin, where the sample reservoir is inverted, the Microcon® assembly was centrifuged at 5200 g for 2 minutes. Then 5 µl of TE^{-4} was added to each concentrated sample.

Amplification

The DNA quantification results obtained for Quantifiler™ determined whether or not the samples needed to be diluted prior to amplification. To each PCR reaction, 20 µl

of master mix and 5 µl of DNA/sterile water were added for a total reaction volume of 25 µl. The master mix contained the following (for one reaction): 14.55 µl sterile water, 2.5 µl 10X GoldSTaR Buffer, 2.5 µl 10X PowerPlex® 16 BIO primers and 0.45 µl AmpliTaq Gold Polymerase. Both a positive (ACP) and negative (ACN) amplification control were included for every amplification. The ACP was prepared with DNA standard 9947A and the ACN contained only sterile water. All amplifications were carried out with the PowerPlex16® BIO system on a Perkin-Elmer GeneAmp® PCR System 9600 Thermal Cycler. The PCR parameters are included in Table 2.

Step in Protocol	PowerPlex® 16 BIO System
Initial Incubation	95°C for 11 minutes; 96°C for 1 minute
Thermal Cycling Denature Anneal Extend	32 Cycles 94°C for 30 seconds (cycles 1-10) ramp 68 seconds to 60° C (hold for 30 seconds) ramp 50 seconds to 70° C (hold for 45 seconds) 90°C for 30 seconds (cycles 11-32) ramp 68 seconds to 60° C (hold for 30 seconds) ramp 50 seconds to 70° C (hold for 45 seconds) 60°C for 30 seconds 70°C for 45 seconds
Final Extension	60°C for 30 minutes
Final Soak	4°C (until samples removed)

Table 2. PCR cycling parameters for the PowerPlex® 16 BIO System (12).

Post-amplification Agarose Gel Electrophoresis

Agarose gel electrophoresis is used to rapidly access the outcome of the amplification reaction prior to performing polyacrylamide gel electrophoresis. Amplified products from all reagent blanks and samples which had been concentrated were run on a

post-amplification agarose gel to determine if any DNA was present. The gel utilized was an Embitec precast 3% agarose gel with ethidium bromide. Tubes (0.5 ml) containing a mixture of 6 µl of amplified sample and 1 µl of 3X loading solution were prepared. To each gel well, 6 µl of this mixture was loaded. For every row, a KB ladder was included. Electrophoresis was conducted at 100 volts for ~40 minutes in an Embitec electrophoretic unit. Following electrophoresis, the gels were scanned on the FMBIO® II for visual detection of amplified product.

Polyacrylamide Gel Electrophoresis and Detection

Prior to preparing the polyacrylamide gel, the glass plates were cleaned with Liqui-Nox® detergent and rinsed with deionized water. Both the large and small glass plate were then wiped down with 95% ethanol and allowed to dry for 5 minutes. The small glass plate was then treated with Bind Silane and allowed to dry for 5 minutes. Excess Bind Silane was removed by wiping the small glass plate with 95% ethanol. Spacers (0.4 mm) were then placed between the two treated sides of the glass plates and clamps were used to hold the plates and spacers in place. The polyacrylamide gel matrix was prepared and filtered. A cross linker (TEMED) and a polymerizer (Ammonium persulfate) were added to the filtered 6% PAG matrix and poured between the two gel plates. The components for the 6% Page Plus™ gel are included in Table 3. Once the gel was poured, a 30 flat well comb was inserted.

After polymerization had occurred, the clamps and comb were removed so that the plates could be placed in a SA43 vertical gel apparatus. The gels were then pre-electrophoresed for ~40 minutes at 60 watts prior to sample loading.

Component	Amount Added
Urea	18 g
Deionized water	24 ml
10X TBE	5 ml
Page Plus™ Gel solution	7.5 ml (40% stock)
TEMED	50 µl
10 % Ammonium Persulfate	500 µl

Table 3. Components of a 6% Page Plus™ polyacrylamide gel.

To each gel lane, 2 µl of PCR product and 4 µl of master mix were added with the exception of DNA extracts which under went Microcon® concentration. For those samples, 4 µl of PCR product and 4 µl of master mix were added to the gel lane. The master mix consisted of Bromophenol Blue dye and ILS 600 BIO in a 3:1 ratio. Prior to loading the samples, the tubes were denatured on a 95° C heat block for 2 minutes and snapped cool on ice for 5 minutes. Other lanes were occupied by a tracking dye, a color matrix, and allelic ladders. Total run time was ~2 hours at 60 watts.

The plates were cleaned once again with Liqui-Nox® detergent and then scanned on the FMBIO II® Fluorescent Imaging System. FMBIO® Analysis 8.0 Software was then utilized for allele detection. Color separation was performed according to Promega's PowerPlex® 16 BIO Technical User's Manual (13). Finally, the Hitachi STaRCALL™ Software program was used for allele sizing and designation.

CHAPTER IV

RESULTS AND DISCUSSION

Contamination Study 1

The first Checkerboard test utilized the Slicprep™ 96 Device as it was received from Promega, i.e. with the ABgene plate instead of the Beckman Coulter Square Well plate that PBSO currently uses during extractions. The blood used to prepare the blood stain card had been drawn from a PBSO employee in 2001 and stored at 4° C in a purple-top tube. It was also diluted 1:2 prior to blotting it on the blood stain card. Blood stain cuttings of ~2 mm x 3.2 mm were placed in alternating wells in the Slicprep™ 96 Device. Every other well was left blank. With the ABgene plate, less DNA IQ™ Lysis Buffer was needed during sample preparation than with the Beckman Coulter plate, 350 µl versus 500 µl, respectively. Although both the ABgene and Beckman Coulter 96 Square Well plates are 2.2 ml deep, the dimensions of the two plates are slightly different. The BioMek® 2000 at PBSO is not properly defined for the ABgene plate. Therefore, a few challenges arose during extraction. Filters in several of the barrier pipette tips became wet and when those same tips were re-used later in the process, they failed to draw up any reagents. Following this observation, the tip box was replaced after the first wash step. After the extraction process was complete, sample containing resin remained in the ABgene plate.

The quantification results obtained were expected considering the challenges encountered during the extraction procedure. The DNA concentrations obtained for the blood stain cuttings are summarized in Figure 11 below.

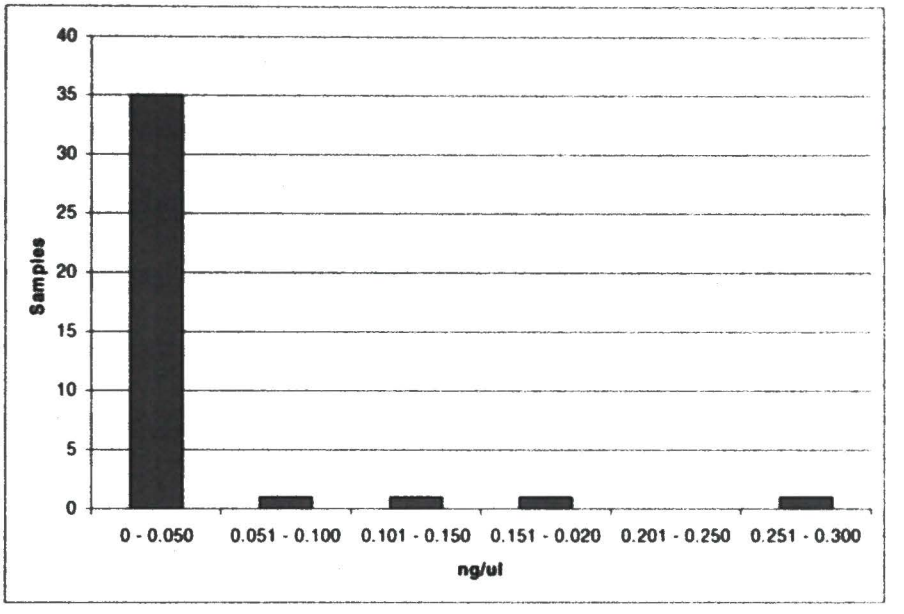

Figure 11. DNA concentrations for 39 blood stain cuttings from Checkerboard Test 1.

The C_T values for those samples ranged from 30.84 – 38.03. According to PBSO protocol, the target C_T value range for DNA amplification is 27 – 30 (4). Samples with a C_T value between 30 and 36 should be concentrated prior to amplification. Samples with a C_T value 36 and higher are considered DNA negative samples. All reagent blank samples quantified had C_T values >36, with the exception of three (C_T values = 35.89, 35.99 and 34.57). No further analysis was done for this Checkerboard test based on the low DNA yield obtained from the blood samples.

The same blood stain card was used to make cuttings for the Zebra Stripe test. This time the Beckman Coulter 96 Square Well plate was used with the Slicprep™ 96 Device instead of the ABgene plate. The cuttings were placed in alternating columns so that every other column was blank. Since the BioMek® 2000 is defined for the Beckman Coulter Square Well plate, no wet filter tips were observed and little to no sample/resin was left in the plate following extraction.

Even though the previously described challenges did not occur during extraction for the Zebra Stripe test, the quantification results did not improve. Once again the DNA concentrations obtained for the blood stain cuttings were low. The results are summarized in Figure 12.

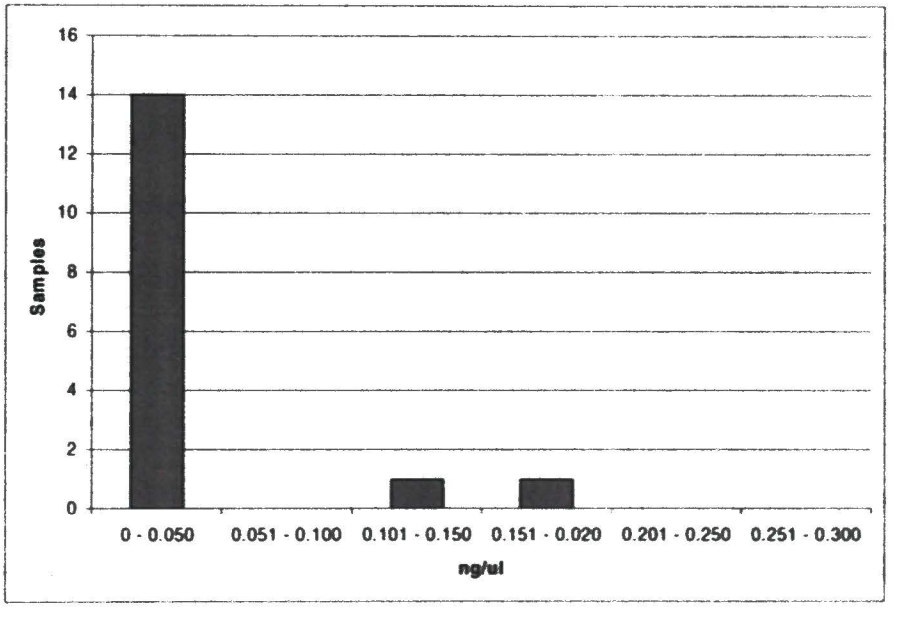

Figure 12. DNA concentrations for 16 blood stain cuttings from Zebra Stripe Test 1.

The C_T values for these samples ranged from 31.35 – unknown undetermined. All reagent blank samples quantified, with the exception of one (C_T value = 35.69), had C_T values >36 and were considered negative. No further analysis was performed on these samples based on the quantification results.

Since poor quantification results were still obtained for Zebra Stripe Test 1 when no challenges were encountered during extraction, it was hypothesized that the low DNA yields resulted from sample degradation of the original sample source as opposed to a problem with the extraction method. In order to test this hypothesis, a mini Checkerboard test was set up so as not to waste sample and reagents. This time blood stain cuttings from a different blood source, Reagent Control Positive (RCP/P2), were utilized, since they were known to produce good quantification results. Once again, the Beckman Coulter 96 Square Well plate was inserted in place of the ABgene plate. Cuttings were placed in only the first two columns of the Slicprep™ 96 Device for a total of 8 samples and 8 reagent blanks.

While the robot was performing the extraction, it was observed that some wells of the Greiner plate did not contain any DNA IQ™ Elution Buffer. In order to investigate this further, the analyst opened the glass doors housing the BioMek® 2000 and inadvertently obstructed the movement of the robotic arm. A tip containing P2 sample was broken upon contact and that sample was lost. It was determined that the DNA IQ™ Elution Buffer was not added to a few samples as a result of uneven distribution of the buffer in the reservoir. Following this incident, the run was aborted and

the remaining wells containing DNA IQ™ Elution Buffer were manually transferred to their corresponding 1.5 ml microcentrifuge tubes.

The samples were then quantified and indicated higher DNA yields. The average C_T value and DNA concentration (ng/µl) obtained for the P2 samples were 30.52 and 0.622, respectively. The lost sample, although quantified, was not factored into the calculations. Two of the reagent blanks had C_T values of 35.49 and 33.73. Since those C_T values were <36, they were not considered negative. Those samples (A2, E2) as well as the other six reagent blanks (B1, D1, F1, C2, G2) were concentrated, amplified and run out on a post-amplification agarose gel. The gel can be seen in Figure 13. All results were negative except for the two reagent blank samples which had positive quantification results. Of those two, sample A2, which had the C_T value of 35.49 was very weak. The other positive sample as observed by the agarose gel was sample E2 (C_T value of 33.73).

Figure 13. 3% Embitec gel of mini Checkerboard test reagent blank samples. Key: KB = KB ladder, + = positive control (9947A), - = amplification negative control (ACN), B1, D1, F1, A2, C2, E2 and G2 are reagent blank samples.

It is possible that the broken pipette tip resulted in the contamination of surrounding reagent blank wells. All reagent blank samples were run on a polyacrylamide gel and

were negative, with the exception of E2. Sample E2 yielded a weak, but complete DNA profile that was consistent with P2.

Contamination Study 2

Once it was established that the second blood source (P2) was not degraded, the 88-sample Checkerboard and Zebra Stripe tests were repeated. The setup for each test was the same as the first contamination study, except a different blood source was used. Blood that had been used to make the P2 cuttings was blotted onto a clean blood stain card. Cuttings from that card were designated as P3.

As a result of the obstruction to the robotic arm during the mini Checkerboard Test, the BioMek® 2000 fell out of alignment. The BioMek® was not realigned prior to performing Checkerboard Test 2 causing a pipette tip to be displaced on top of another pipette tip during the transfer of samples from the Greiner plate to the 1.5 ml tubes. The run was paused and then aborted. DNA extracts were manually transferred to the labeled microcentrifuge tubes for the remaining three columns of samples. The alignment problem most likely resulted from contact with the robot arm during the mini Checkerboard test. Following this extraction, the BioMek® 2000 was manually realigned. No issues were encountered during extraction for Zebra Stripe Test 2.

The Quantifiler™ Human DNA Quantification Kit was used for quantification of all 88 samples from both Checkerboard Test 2 and Zebra Stripe Test 2. DNA concentrations obtained for the P3 samples for those tests can be seen in Figures 14 and 15. For Checkerboard Test 2, the C_T values for P3 samples ranged from 27.27 – 33.11. The sample with a C_T value of 33.11 (0.063 ng/µl) was later concentrated. All reagent

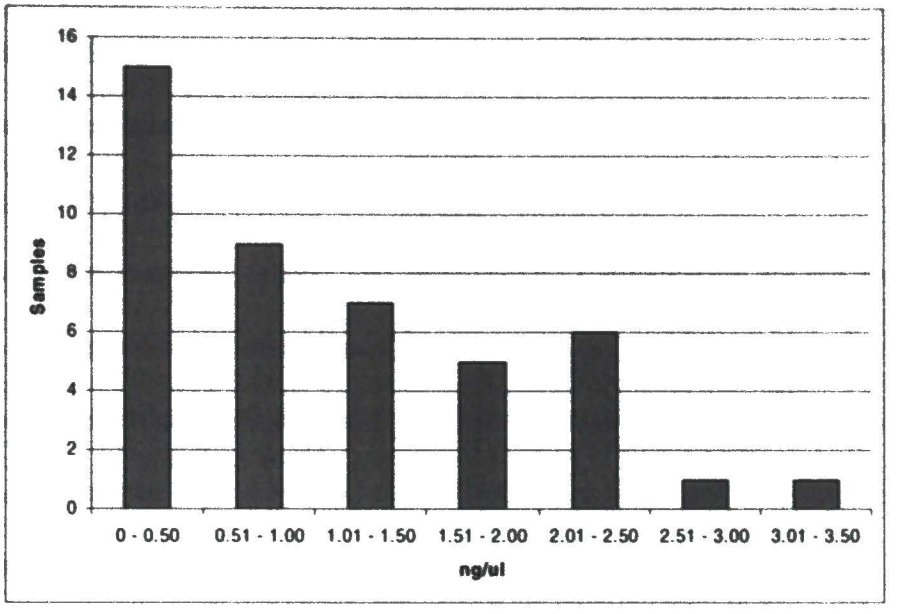

Figure 14. DNA concentrations obtained for the P3 samples from Checkerboard Test 2.

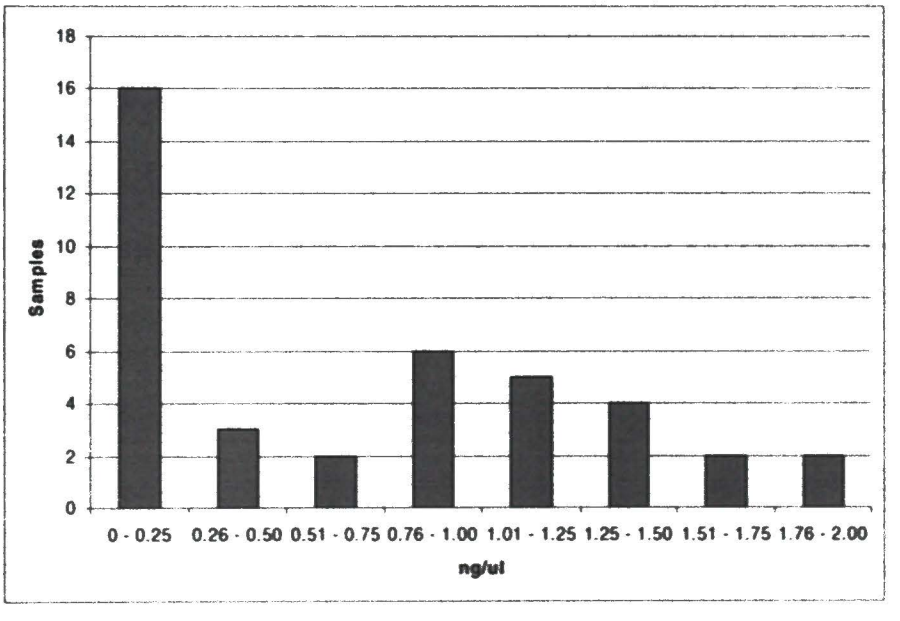

Figure 15. DNA concentrations obtained for the P3 samples from Zebra Stripe Test 2.

blank samples had C_T values >36 except for two – C_T values = 35.38 and 35.41. This corresponded to 0.014 ng/μl and 0.013 ng/μl, respectively. Those two samples were also concentrated. For Zebra Stripe Test 2, the C_T values for P3 samples ranged from 27.75 to 33.23. Eight P3 samples contained <0.1 ng/μl, four of which were Microcon® concentrated prior to amplification. The other four P3 samples were not processed further. All reagent blank samples had C_T values >36.

The reagent blank samples and five P3 samples, one from every other column of the plates, were amplified and run on both a 3% Embitec and a polyacrylamide gel for both contamination studies. All reagent blank samples were negative on post amplification gels. Fluorescence detection of the DNA fragments separated by PAGE revealed no contamination in any of the reagent blank samples. Complete DNA profiles were obtained and typed correctly for all ten P3 samples. Therefore, there was no indication of any robotic DNA carryover during either the Checkerboard or Zebra Strip contamination tests. Checkerboard Test 2 PAGE results for 21 reagent blank samples scanned at 577 nm are shown in Figure 16. Zebra Stripe Test 2 PAG results can be seen in Figure 17.

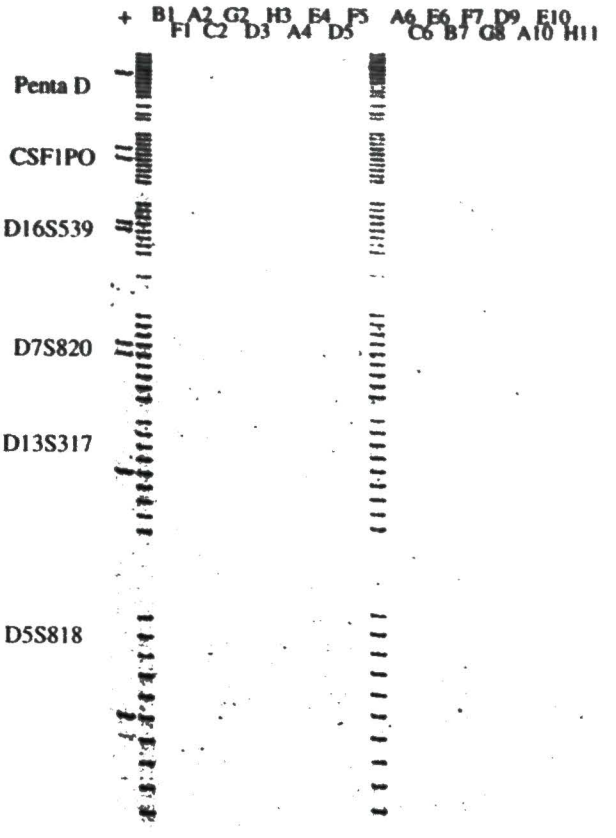

Figure 16. PowerPlex®16 BIO System 577 nm scan of polyacrylamide gel image for reagent blank samples from Checkerboard Test 2. Loci are indicated on the left. Key: + = positive control (9947A), all other lanes are reagent blanks.

Forensic Samples

Three separate cuttings (~ 2 mm x 3.2 mm) from ten oral swabs, two blood swabs, three prepared mixtures and six touch evidence swabs were taken for a total of 63 samples. All samples were extracted using the Slicprep™ method. Although, this time the ABgene plate supplied with the Slicprep™ 96 device was utilized instead of the Beckman Coulter plate for which the BioMek® 2000 at PBSO is currently defined. As a result, approximately 25 – 50 µl of sample/resin remained in the majority of the ABgene

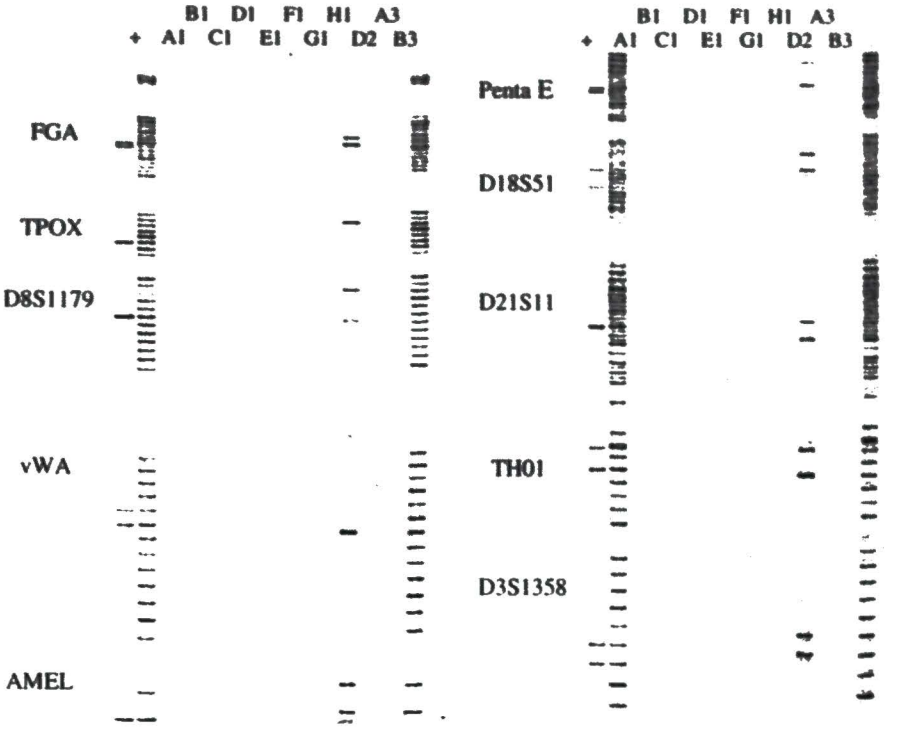

Figure 17. PowerPlex®16 BIO System polyacrylamide gel images for reagent blank samples from Zebra Stripe Test 2. Loci are indicated on the left. Image 1 shows the 598 nm scan. Image 2 shows the 505 scan. Key: + = positive control (9947A), all other lanes are reagent blanks except for D2 which contains P3.

plate wells after extraction. Both a RCP and a RCN were included in this study and produced the expected results.

Buccal Swabs and Blood Standards

All buccal swabs and blood standard samples were quantified, amplified, run on a 6% PAGE Plus™ vertical gel and analyzed. All these samples demonstrated positive quantification results with DNA concentrations ranging from 0.722 – 12.12 ng/µl. The data is shown in Figure 18. No samples required Microcon® Concentration. In order to conserve time, the polyacrylamide gel utilized previously for Zebra Stripe Test 2 was reused. After electrophoresis of the Zebra Stripe test samples, the positive and negative

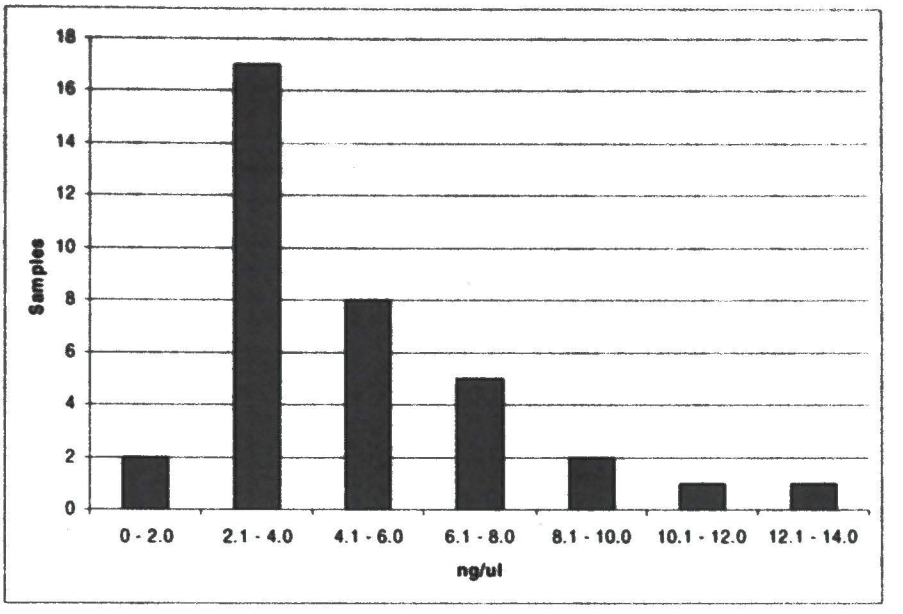

Figure 18. DNA concentrations obtained for the buccal swabs and blood standards.

electrodes were switched and the plate was run with the polarities reversed for 4 hours. This was done to ensure that any DNA present had migrated out of the gel. Eighteen (three replicates of six samples) of the oral standards were then run on this gel. The other twelve samples were run on a freshly prepared polyacrylamide gel. Interpretation of both gel files showed no indication of mixtures and complete profiles were obtained for all 36 samples. Profiles for 3 of the individuals (9 samples total) were known and all resulting allele calls were concordant with the known DNA profiles. Gel scans for approximately half of the buccal swabs and blood standards are provided in Figure 19.

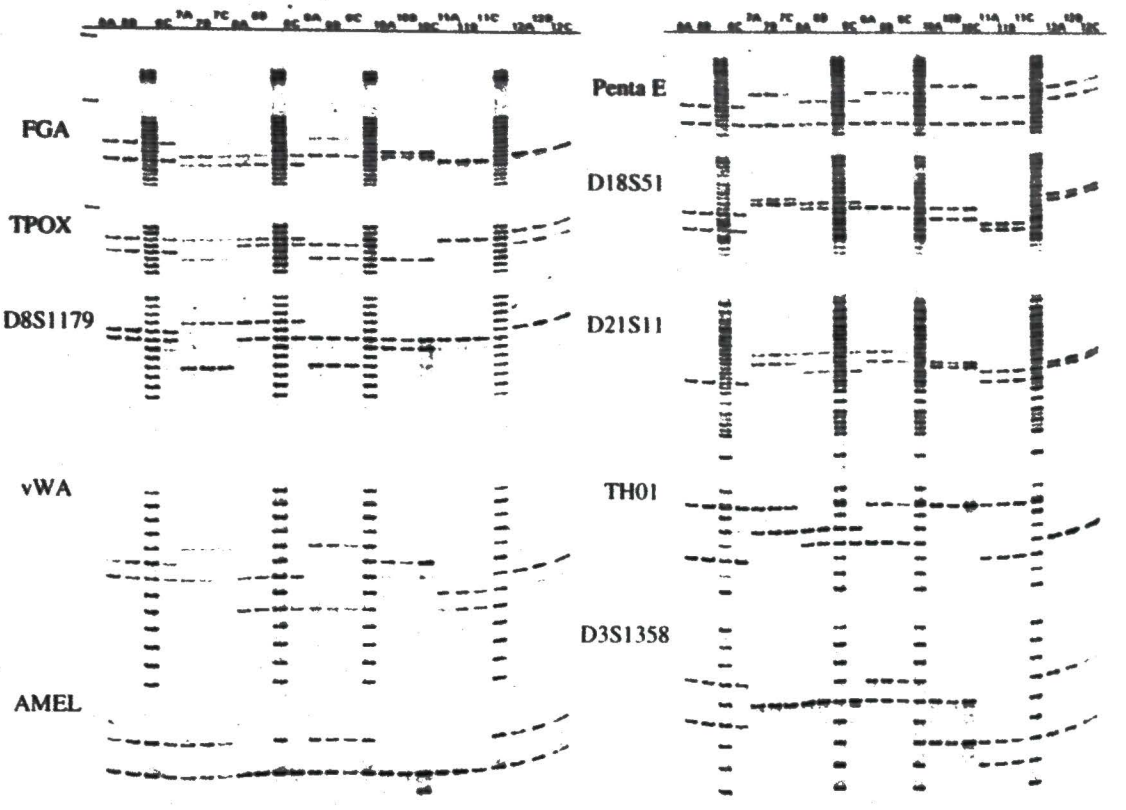

Figure 19. PowerPlex®16 BIO System polyacrylamide gel images for buccal swabs and blood standards. Loci are indicated on the left. Image 1 shows the 598 nm scan. Image 2 shows the 505 nm scan. Key: 6-12 represent the seven individuals included on this gel. A, B and C represent the three cuttings taken from each buccal or blood swab.

Mixtures

The mixture study was performed to see if interpretable mixed profiles could be obtained when the Slicprep™ 96 Device was used for extraction. Three different mixtures were utilized for this study, one of which was a mixture of three individuals. The 2-individual mixtures were prepared in the following ratios: 3:1 male:female and 1:3 male:female. To create the 3-individual mixture, a buccal swab from a female was soaked overnight in a 1:1 ratio of mixed whole blood from a male and female. The same

male and female contributed the blood samples to both the 2-individual and 3-individual mixtures. All nine mixture samples were quantified, amplified, run on a 6% PAGE Plus™ vertical gel and analyzed. The mixtures demonstrated positive quantification results with DNA quantities ranging from 0.737 – 5.46 ng/μl. The chart in Figure 20 summarizes the quantification results. Once again, no Microcon® concentration was necessary.

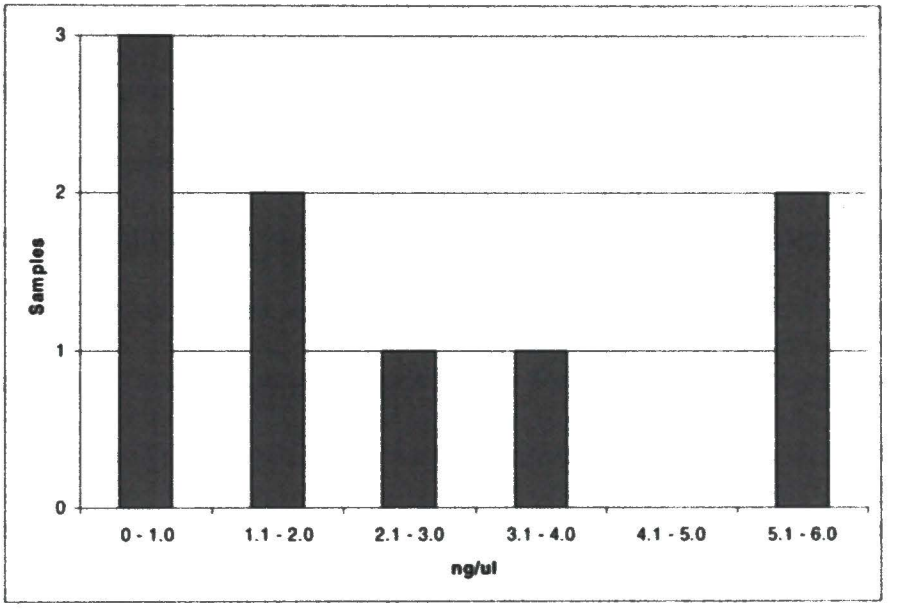

Figure 20. DNA concentrations for the mixture study.

Following quantification, all mixture samples were amplified, run on a 6% PAGE Plus™ vertical gel and then scanned on the FMBIO® II. These mixtures were interpreted according to the interpretation guidelines provided in PBSO's protocol (4). A mixture may be present if there are multiple bands (3 or more) present at one or more loci. If a weak band is present in the n-4 position at a particular locus, it could be a stutter product or the sample may contain a mixture of two or more DNA sources. In order to

make this determination, a ratio of band intensities (optical density values) was calculated for alleles present at each locus. This was done by dividing the optical density of the major band from that of the minor bands. The ratio was then converted to a percent and compared to the average stutter values PBSO obtained for each PowerPlex® 16 BIO locus during validation. If the percentage exceeded the average stutter percentage validated for that locus, it was designated as a true band. The DNA profiles of the individuals making up the mixtures were known, therefore a determination could be made as to whether or not a complete mixture profile was obtained.

No mixture profiles which contained all contributors to the mixture at every locus were observed for either the 2-individual or 3-individual mixtures. Scanned gel images for the 3:1 male:female mixture and the male and female who contributed to them are provided in Figure 21. Scanned gel images for the 3-individual mixture are shown in Figure 22. With the 3:1 male to female mixtures, allele dropout was minimal. For the first replicate, a full male DNA profile was evident and only one allele from the female contributor dropped out. This occurred at the D16S539 locus. Replicate 2 experienced allele dropout of the male contributor at two loci: Penta D and Penta E, both of which are high molecular weight loci. All alleles corresponding to the female were present in both this sample and replicate 3. However, one allele from the male contributor was missing at D21S11 for replicate 3. An imbalance in band intensity, based on optical density values, was observed for these mixtures as expected (Table 4). However, a major and minor contributor could not be assigned at every locus and the individual whose allele was designated as the minor allele varied from locus to locus. Minor alleles tended to be

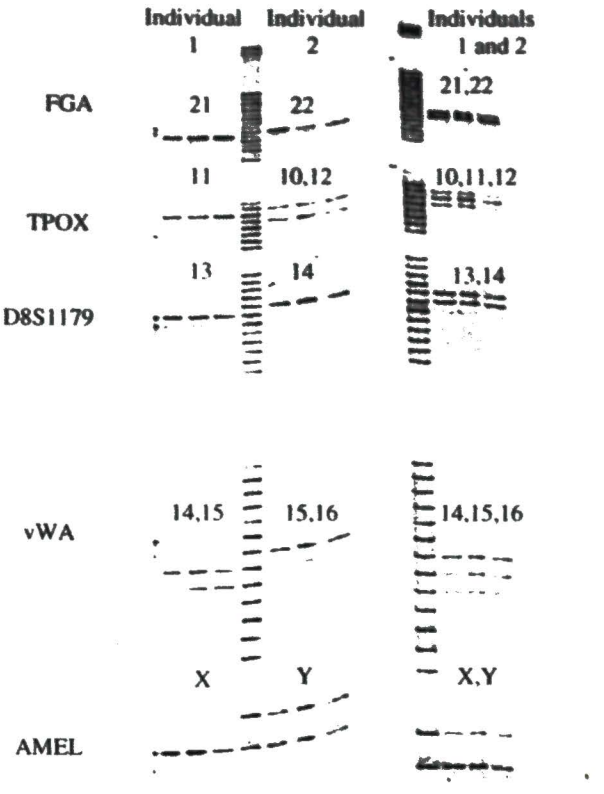

Figure 21. PowerPlex®16 BIO System polyacrylamide gel images for the 3:1 male:female mixture and the single source contributor profiles. Loci are indicated on the left. Both images show the 598 nm scan. Allele calls are provided.

observed only at loci where an individual was homozygous or the male and female shared 1 allele. The male and female contributors shared alleles at 6 of the 16 loci.

Allele dropout was much more evident with the 1:3 male to female mixtures. A complete female DNA profile was present in all three replicates. However, the male contributor experienced allele or locus dropout. This imbalance may be due to either a difference in the nucleated cell population of the male donor or an incomplete mixing of the two individual's blood prior to making the swab. The alleles from the male contributor which dropped out are listed in Table 5. For the first replicate, alleles from

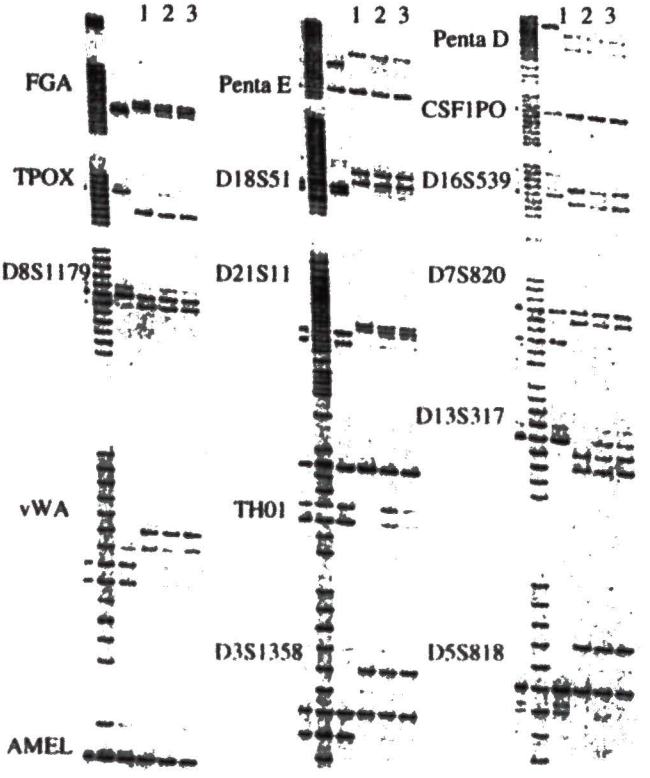

Figure 22. PowerPlex®16 BIO System polyacrylamide gel images for the 3-individual mixtures. Loci are indicated on the left. Image 1 shows the 598 nm scan, image 2 shows the 505 nm scan and image 3 shows the 577 nm scan.

the male dropped out at every locus, with the exception of D8S1179 and D5S818, two of the smaller-sized loci. The only other allele calls that matched the male profile were alleles that the female contributor had as well. Therefore, it is difficult to tell whether or not the male contributed to those alleles, but the optical density values do appear to be higher at those locations. No major or minor contributor profiles could be determined for replicate 1. However, a determination could be made for the second and third replicates, with the female being the major and the male being the minor contributor.

	Alleles Observed			Optical Density		
Locus	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
FGA	21	21	21	1265	1053	583
	22	22	22	1134	730	272
TPOX	10	10	10	819	695	93
	11	11	11	1104	1235	595
	12	12	12	679	174	70
D8S1179		12			365	
	13	13	13	3074	2033	930
	14	14	14	2360	2097	948
vWA	14	14	14	466	381	211
	15	15	15	622	418	319
	16	16	16	661	933	372
Amelogenin	X	X	X	2227	1856	944
	Y	Y	Y	552	636	235
Penta E	7	7	7	290	90	91
	12	AD	12	150	AD	27
	13	13	13	86	108	76
	15	15	15	263	88	61
D18S51	12	12	12	283	230	168
	13	13	13	239	131	170
	17	17	17	237	22*	79
	18	18	18	269	14*	107
D21S11	29	29	29	273	361	154
	30	30	30	479	569	279
	30.2	30.2	AD	275	137	AD
TH01	6	6	6	328	354	161
	7	7	7	1120	793	489
	9.3	9.3	9.3	483	490	278
D3S1358	13	13	13	295	328	170
	14	14	14	721	445	216
	17	17	17	252	312	131
Penta D	11	AD	11	146	AD	144
	13	13	13	283	114	73
	15	15	15	434	42	131
CSF1PO	10	10	10	501	109	61
	12	12	12	914	526	115
D16S539	11	11	11	221	425	183
	12	12	12	429	470	117
	13	13	13	335	237	137
D7S820	8	8	8	466	540	262
	10	10	10	586	386	167
	11	11	11	429	537	171
	13	13	13	429	253	108
D13S317	11	11	11	2241	1811	1005
	12	12	12	1123	556	234
D5S818	10	10	10	1370	1085	221
	11	11	11	2709	2160	1117

Table 4. Alleles observed for the 3:1 male to female mixture. Key: R1 = replicate 1, R2 = replicate 2, R3 = replicate 3, AD = allele dropout, * = bands confirmed visually.

Results obtained for the 3-individual mixture replicates were similar to those of the 1:3 male to female mixture. Allele dropout of one or two individuals was evident at 9 of the 16 loci. Of these individuals, the male contributor experienced more allele dropout than the female contributor. A full DNA profile for the female who contributed the oral standard was obtained in all three replicates and was the major profile observed. These results are summarized in Table 6.

Locus	Replicate 1	Replicate 2	Replicate 3
FGA	22	22	22
TPOX	12	12	12
D8S1179	-	-	-
vWA	16	-	-
Amelogenin	Y	-	-
Penta E	12,15	12,15	12,15
D18S51	17,18	17,18	-
D21S11	30.2	30.2	30.2
TH01	7	-	-
D3S1358	17	17	17
Penta D	11,13	11,13	11,13
CSF1PO	10	10	10
D16S539	12,13	12,13	-
D7S820	10,13	10,13	-
D13S317	12	-	-
D5S818	-	-	-

Table 5. Allele dropout observed for the 1:3 male to female mixture.
Key: Alleles listed in black are alleles lost from the male contributor.
Alleles listed in blue represent locus dropout of the male contributor;
- = all alleles from male contributor were present.

Locus	Individual 1	Individual 2	Individual 3	Replicate 1	Replicate 2	Replicate 3
FGA	22,23	21	22	22,23	21,22,23	21,22,23
TPOX	8	11	10,12	8	8	8
D8S1179	12,13	13	14	12,13,14	12,13,14	12,13,14
vWA	17	14,15	16	16,17	14,15,16,17	14,16,17
Amelogenin	X	X	X,Y	X	X,Y	X,Y
Penta E	7,16	7,13	12,15	7,16	7,16	7,16
D18S51	14,16	12,13	17,18	14,16	12,13,14,16	12,13,14,16
D21S11	30.2,31	29,30	30,30.2	30.2,31	29,30,30.2,31	29,30,30.2,31
TH01	9.3	6,9.3	7	9.3	6,7,9.3	6,7,9.3
D3S1358	14,16	13,14	14,17	14,16	14,16	14,16
Penta D	10,13	15	11,13	10,13	10,13,15	10,13,15
CSF1PO	12	12	10,12	12	12	12
D16S539	10,12	11,14	12,13	10,11,12	10,11,12	10,12
D7S820	10,11	8,11	10,13	10,11	10,11	10,11
D13S317	9,10	11	11,12	9,10	9,10,11,12	9,10,11,12
D5S818	11,13	11	10,11	11,13	11,13	11,13

Table 6. DNA profiles obtained for three replicates of the 3-individual mixture. The DNA profiles for the three individuals contributing to the mixture are also shown. Individual 1 provided the buccal swab. Individuals 2 and 3 were included in the 1:1 blood mixture. Alleles in blue were not present in any of the three mixture replicates.

Touch Evidence

Touch evidence samples included swabs of a keyboard, microwave, soda can, television remote, car steering wheel and a light switch. DNA quantities for these samples were low with 10 of 18 samples yielding negative quantification results (C_T values >36). The range of DNA concentrations observed was unknown undetermined – 0.052 ng/µl. Quantification data is summarized in Figure 23. The quantification results were expected, given the nature of touch evidence. Also, the ABgene plate was used instead of the Beckman Coulter Square Well plate for this study. As a result approximately 25 – 50 µl of sample/resin remained in the plate since the

BioMek® 2000 method was not defined for the ABgene plate. In addition, three cuttings were taken from each swab, so the number of epithelial cells present on each cutting was most likely only a few, if any.

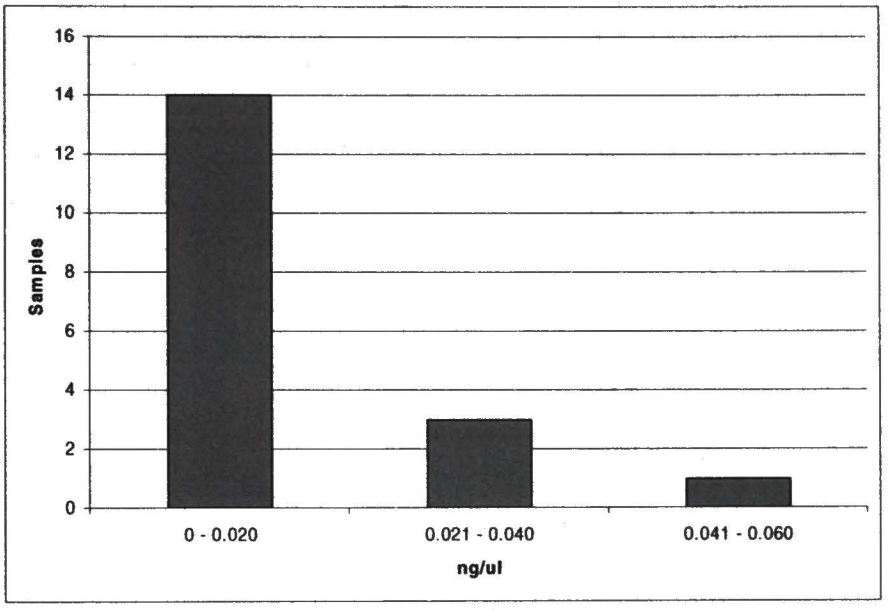

Figure 23. DNA concentrations for the touch evidence samples.

All touch evidence samples were Microcon® concentrated with the exception of the three for which unknown undetermined DNA quantities were obtained. The concentrated samples were then amplified and run out on a 3% Embitec gel to determine if any amplification product was present (data not shown). Only two samples yielded positive, but weak amplification results. Both were swabs from a keyboard. Therefore, only those two samples were analyzed further. Polyacrylamide gel electrophoresis and forensic detection revealed a weak, complex mixture for both samples (Figure 24).

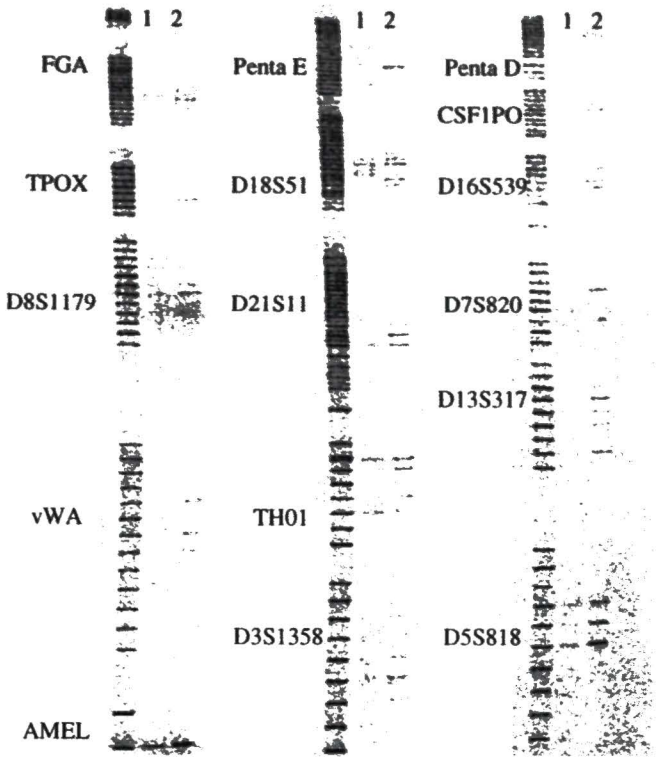

Figure 24. PowerPlex® 16 BIO System polyacrylamide gel images for the two keyboard samples. Loci are indicated on the left. Image 1 shows the 598 nm scan, image 2 shows the 505 nm scan and image 3 shows the 577 nm scan.

Due to time constraints, no further testing was done. Had the touch evidence samples been run using the Beckman Coulter plate with the Slicprep™ 96 Device rather than the ABgene plate, it is likely that DNA yields would have improved. Yet, the amount of DNA extracted still may not have been sufficient enough to obtain a DNA profile. Another experiment which should be conducted would involve varying the volume of DNA IQ™ Resin added during extraction. Currently, a small amount of resin (665 µl) is added compared to a large volume of Lysis Buffer (10.7 ml). Since DNA has an affinity for the resin, the more resin added the greater potential for DNA binding.

Reproducibility

Ten buccal swabs, two blood standards, three different mixtures and six touch evidence samples were utilized for this study. Three cuttings were made from each swab so that a total of 63 samples were tested from 21 different sources. The purpose of this study was to determine if reliable and consistent DNA profiles could be obtained using the Slicprep™ method. The success of the extraction method is based on a minimum of positive quantification results and a maximum of obtaining complete identical profiles for replicates from each sample source.

Positive quantification results (0.722 – 12.12 ng/μl) and complete DNA profiles were obtained for all replicates of the buccal swabs and blood standards. For amplification, ~ 1 ng of DNA was added to each reaction. The actual amount of DNA added to the amplification reaction ranged from 0.722 – 1.41 ng. Following vertical electrophoresis and detection, no mixtures were observed and all allele calls were consistent between replicates. Heterozygote balance at each heterozygous locus was calculated by dividing the optical density of the major band from the optical density of the minor band and multiplying by 100 (data not shown). A locus with a heterozygote balance of <70% was considered to be imbalanced. For the majority of the heterozygous loci, heterozygote balance was >70%. Some replicates (7 of 36) had no loci exhibiting imbalance. The highest number of loci exhibiting imbalance for any one replicates was four. Slight variations in heterozygote balance could have resulted from the varying amounts of template DNA added to the amplification reactions. No trend was observed as to a particular locus exhibiting more heterozygote imbalance than others.

Positive quantification results were obtained for all mixture replicates (0.737 – 5.46 ng/μl), but no complete mixture profiles were observed for either the 2-individual or 3-individual mixtures. The profiles for the 3:1 male to female mixture were fairly consistent between replicates with a total of four alleles dropping out. For the 1:3 male to female mixtures, the DNA profiles obtained at 9 loci were identical for all three replicates. At the other 7 loci, the number of alleles which dropped out varied between replicates (Table 5). More variability was seen with the 3-individual mixtures, since more alleles had to compete during amplification. Alleles from the individual who provided the oral swab were preferentially amplified compared to those from the male and female contributing to the 1:1 blood mixture. That was especially evident in replicate 1 (Table 6). The profiles obtained for replicates 2 and 3 were nearly identical with 2 additional alleles observed in replicate 2 than in replicate 3.

DNA quantities for the touch evidence samples were low with only 8 of 18 yielding positive results and only 2 samples yielding positive post amplification results. The DNA concentration range was unknown undetermined – 0.052 ng/μl. DNA profiles were obtained for only two samples, both of which were taken from a keyboard. A significant amount of variation was seen between these two replicates. Allele calls were made for all 16 loci for replicate 2, but only 7 loci for replicate 1. These results suggest that low quantity DNA samples are less likely to produce consistent, high quality profiles.

CHAPTER V

CONCLUSION

With the advances seen in forensic technology in the last several years, forensic laboratories now receive much more evidence and types of evidence than ever before. As a result, forensic laboratories are receiving more cases than they can process at any given time. The incorporation of the BioMek® 2000 Laboratory Automation Workstation into PBSO's Crime Laboratory has helped to reduce the number of backlogged cases since analysts can now focus more of their time on analysis instead of performing a lengthy DNA extraction. However, a number of manual preprocessing steps are still required before the samples can be placed on the robot for extraction. The Slicprep™ 96 Device is easy to use and reduces the number of manual preprocessing steps. Although in order for the device to be beneficial in the laboratory, it must also provide sufficient DNA concentrations with no contamination and consistent, accurate results.

During the first contamination study, issues with both the DNA sample used and the alignment of the robot occurred. Therefore, no further analysis was done after the quantification step. For the second contamination study, 176 samples were once again processed, this time with a new DNA source. Two of the reagent blank samples yielded low, but positive quantification results. No DNA profile was obtained for either sample and therefore, no evidence of contamination was observed. Buccal swabs and blood standards, mixed samples and touch evidence samples were also tested to determine the

applicability of the Slicprep™ 96 Device to the various types of evidence a forensic laboratory receives. Positive quantification results and complete DNA profiles were obtained for all buccal swabs and blood standards tested. The DNA profiles for three of the individuals (9 samples total) were known and the profiles obtained during this study produced the expected results. In addition, the profiles observed for each replicate were consistent. Positive quantification results were also obtained for all the mixture samples tested. However, no full DNA profiles resulted and the profiles were not consistent between replicates for either the 1:3 male to female mixture or the 3-individual mixture. A difference in the nucleated cell population of the male donor may have contributed to the large amount of allele dropout observed with the 1:3 male to female mixture. As expected the touch evidence samples produced the most inconsistent results. Only 8 of 18 samples yielded positive quantification results and of those eight samples, only two produced DNA profiles. The two samples for which a DNA profile was obtained were both from a keyboard. Yet one sample had many more alleles present than the other and some allele calls were inconsistent between the two. These results are not surprising given the nature of touch evidence samples, however, the use of the ABgene plate instead of the Beckman Coulter plate may have impacted the results. The Slicprep™ 96 Device is designed to fit a number of deep-well plates, but the plate must be properly defined for the BioMek® 2000 in order to produce optimal results.

Overall, the efficiency of the process was improved. Although the total extraction time with the Slicprep™ method was similar to PBSO's current method, less manual work by the analyst was required. When 88 samples are run, the amount of hands on

time required of an analyst using the current method is approximately 3 hours. The use of the Slicprep™ 96 Device reduces the number of manual hours to approximately 1.5. During the automated steps of extraction the analyst can walk away. In addition, the less manual time spent processing samples, the less chance for human error. A possible downside of this device is its cost. No cost comparison has been made between the Slicprep™ method and the current method utilized by PBSO, but the Slicprep™ method is most likely more expensive. It consumes slightly more DNA IQ™ Lysis Buffer and the Slicprep™ 96 Device itself is not cheap. However, any slight cost differential can be offset by the amount of manual preprocessing time saved.

Further analysis should involve repeating the experiment with the Beckman Coulter 96 Square Well plate in place of the ABgene plate or with the ABgene plate properly defined to see if there is any improvement, especially with touch evidence samples. Greater DNA yields are expected since little or no sample should remain in the deep-well plate if the BioMek® 2000 definitions are accurate for the plate being used. The amount of resin added to touch evidence samples should also be experimented with. Currently there is a tremendous volume of Lysis Buffer added compared to the resin. The addition of more resin could increase the potential for binding more DNA. Finally, all the mock forensic samples used for this study should be extracted with the current method so that a direct comparison can be made between the current method and the Slicprep™ method.

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