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Organic extraction from forensic samples has consistently produced high DNA yields. However, organic extraction is time-consuming and contains many steps where sample manipulation can occur. In this study, sensitivity of organic extraction method is compared to QIAamp DNA Investigator Kit, a silica based extraction utilizing carrier RNA. The results suggested that the QIAamp DNA Investigator Kit is just as sensitive as organic extraction method in obtaining partial and full DNA profiles. The QIAamp DNA Investigator Kit is less time-consuming than organic extraction and can be automated on the QIAcube. The study also studied the reliability of a post-PCR purification kit in obtaining a better DNA profile. The Qiagen MinElute PCR Purification Kit increased the number of loci detected in 64% of samples tested.

SENSITIVITY COMPARISON OF AN ORGANIC BASED DNA EXTRACTION METHOD TO A SILICA BASED DNA EXTRACTION SYSTEM UTILIZING CARRIER RNA AND EFFECTS OF A POST-PCR PURIFICATION PROCESS

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Chau Nguyen, B.S.

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CHAPTER 1

INTRODUCTION

Definition of the Problem

In the Acadiana Criminalistic Laboratory, the standard method of DNA extraction used is through the use of organic solvents, such as phenol/chloroform/isoamyl/alcohol (PCIA), with the addition of other reagents such as sodium dodecyl sulfate (SDS) and dithiothreitol (DTT). The use of PCIA can be problematic due to its toxicity and should be handled with caution. According to the material safety data sheet (MSDS) (1), PCIA can cause corneal damage or blindness if it came in contact with the eyes. PCIA can also cause skin burns and severe irritation of the upper respiratory tract if inhaled. Organic extractions can also be laborious and should be performed under a fume hood. For laboratories that have a limited number of fume hoods, DNA analysts would have to take turns in order to perform organic extractions which can slow down the flow of the entire laboratory and prolong the amount of cases being completed.

Another issue encountered in Acadiana Criminalistic Laboratory is typing DNA of limited amount. Some of these DNA samples can be considered low copy number (LCN) DNA samples. LCN samples have been defined as samples that contain less than 100 pg of DNA (2). The small starting amount of DNA in LCN samples make it difficult to obtain a full genetic profile using standard STR amplification method. The standard technique used in typing LCN DNA is increasing the number of amplification cycles (2).

However, the use of LCN DNA typing is controversial and has raised concerns about the increase in contamination and difficulty in data interpretation due to allele-drop out, allele drop-in and increased stutter products. In the United States, the use of LCN typing is not allowed as evidence in a trial and can only be used as an investigative tool. The use of LCN typing is not being used worldwide and is only accepted for evidential purposes in only three countries (3).

Scope of the Study

The QIAamp® DNA Investigator Kit (Qiagen Corporation, Valencia, CA) is a kit designed for DNA extraction from forensic and human-identity samples. QIAamp® DNA Investigator Kit utilizes QIAamp® MinElute® spin column technology in conjunction with a poly-A carrier RNA. The spin column contains a silica gel membrane that binds and purifies DNA (4). The QIAamp® DNA Investigator Kit is designed to ensure that there is no sample-to-sample cross-contamination and is capable of automation with the QIAcube® instrument (Qiagen Corporation), a small, enclosed robotic workstation that purifies DNA, RNA, or protein for up to 12 samples per run (5). By replacing the standard organic extraction method with the combination of the QIAamp® DNA Investigator Kit and the QIAcube® instrument, DNA yields from forensic and human-identification casework samples could be optimized. The addition of the QIAcube® instrument minimizes human contamination and increases the consistency of the extraction process.

The typing of LCN DNA samples can prove to be challenging regardless of the extraction method used. An alternative to the standard LCN DNA typing is through the use of post-PCR purification. The Qiagen® MinElute® PCR Purification Kit (Qiagen Corporation, Valencia, CA)

is designed to purify amplified product and could assist in the usage of LCN DNA samples by removing primers, salts and unincorporated deoxyribonucleotide triphosphate (dNTPs). The Qiagen® MinElute® PCR Purification Kit purifies amplified product by using silica-based MinElute spin columns and a series of specialized buffers to clean up PCR product from the amplification mixture (6). Once purified, the PCR product can be used in downstream assays such as transformation, restriction enzyme digest, ligation and sequencing. The Qiagen® MinElute® PCR Purification Kit is capable of automation with the QIAcube® instrument. The combination of the Qiagen® MinElute® PCR Purification Kit with the QIAcube® could be a valuable tool for purifying PCR product quickly and efficiently for analysis of forensic casework samples including LCN samples.

Specific Aims

The objective of this study was to: 1) compare the sensitivity of DNA extraction between QIAamp® DNA Investigator Kit and organic extraction; 2) compare the ability of QIAamp® DNA Investigator Kit and organic extractions method to obtain full and partial DNA profiles; and 3) investigate the reliability of Qiagen® MinElute® PCR Purification Kit.

CHAPTER 2

BACKGROUND

Ever since the development of DNA testing in 1985 by Sir Alec Jeffreys (7), the application of DNA technology in the forensic field has been implemented due to its accuracy and reliability. With the increasing demands of DNA testing, procedures such as DNA extraction and post PCR purification are being studied to cut time and cost while maintaining its efficiency and reliability. The quantity and quality of DNA recovered from an extraction method is vital and can affect obtaining a DNA profile. With increasing interest in obtaining a DNA profile from low copy number (LCN) samples, it is important to retain a high level of sensitivity during STR analysis and to keep problems such as increased stutter, allele drop-in and allele dropout to a minimum.

One way to help relieve the increasing demand of DNA analysis is by adding robotics to automate sample processing. With robotics, samples can be efficiently processed with a high sample throughput and rapid data generation. The use of robotics can also reduce human interaction with samples therefore helping to minimize human error, sample contamination, sample switching and improve the consistency of sample processing (8).

DNA extraction

Human DNA can be extracted from a wide range of forensic samples including blood, saliva, semen, hair, body fluid stains, nail clippings, bones and teeth. There are different techniques for DNA isolation such as organic extraction, Chelex® 100 extraction and silica-based extractions. DNA extraction is a crucial step in forensic DNA testing since all the subsequent steps rely heavily on the quality and quantity of DNA extracted. Currently, organic extraction is the standard DNA extraction method used in forensic laboratories today. There are certain samples, such as decomposed tissue and bone samples, where silica-based methods have been found to give better extraction yields (9, 10). Nevertheless, organic extraction method is still more widely used and consistently gives high DNA yields (11).

The standard organic extraction method usually involves an initial overnight incubation with a digest buffer and proteinase K, a proteolytic enzyme that degrades cellular proteins and histones. The digest buffer usually includes a detergent such as sodium dodecyl sulfate (SDS) that disrupts the nuclear membrane to expose the nucleic acid and ethylenediaminetetraacetic acid (EDTA), a chelating agent. The DNA digestion is followed by DNA extraction with phenol/chloroform/isoamyl/alcohol (PCIA). Phenol is partially soluble in water and chloroform is immiscible in water. By combining phenol with chloroform, chloroform will ensure that phenol will be separated from the aqueous phase. The phenol/chloroform solution denatures and dissolves proteins and the isoamyl alcohol stabilized chloroform making it less volatile. The DNA is left to dissolve into an aqueous solution. The DNA is concentrated using centrifugal filters or by ethanol precipitation (12). The overall organic extraction process is time-consuming and contains many steps where samples can be manipulated and DNA recovery can be lost (8). Phenol/chloroform methods also may extract PCR inhibitors along with DNA (13). Due to the

volatile nature of the phenol/chloroform solution, careful handling of the solvents must be taken into consideration and any usage of phenol/chloroform should be performed within a fume hood (1).

Another commonly used method of DNA extraction incorporates Chelex®100, an ion-exchange resin that has a high affinity for multivalent metal ions. The Chelex resin is made of styrene divinylbenzene copolymers that contain paired iminodiacetate ions (14). The classic Chelex extraction method starts with the preparation of a 5% Chelex solution. DNA samples were then mixed with 200 µl of 5% Chelex solution and incubated at 56°C for 30 minutes and boiled at 100°C for 8 minutes. The samples are then centrifuged to pellet the Chelex resins. The supernatant, containing the purified DNA, is removed and stored at 4°C. In order for the Chelex extraction to be successful, an alkaline environment and exposure to 100°C temperature will allow the lysis of the cell membrane and release of DNA (15). Singer-Sam et al (16) suggested that when boiling samples with Chelex resins, the resins prevents DNA from degrading by chelating metal ions that can act as means for DNA degradation at high temperatures. Therefore, by binding to metal ions and removing them during DNA extraction, the Chelex resin prevents damage to DNA and also prevents inhibition to Taq polymerase. Chelex has been used to extract DNA from a variety of forensic samples such as whole blood, bloodstains, seminal stains, saliva and hair samples (14). The Chelex-100 extraction method is attractive because it is simple to perform, rapid, and does not involve the use of toxic chemicals or multiple tubes which cuts down on operator contamination (17). However, the Chelex resin has been found to inhibit PCR; therefore, care must be taken not to allow any Chelex resin to carry over with the DNA extracts (16). A study by Sweet et al (15) showed that Chelex-100 extraction of DNA from saliva was

more effective than using phenol-chloroform. However, other studies showed that Chelex resin failed to isolate amplifiable DNA from old, degraded tissue and decomposing tissues (18, 19).

The last form of DNA extraction used in the forensic field is silica-based extractions. Silica-based extractions can vary; they can be found as silica membrane, silica magnetic beads, or as silica suspensions. Silica particles have been successful in DNA extraction due to its high binding ability to DNA and have been used in a variety of forensic samples (19). Silica-based extractions usually consist of 4 steps: cellular digest, DNA binding, DNA washing and DNA elution. DNA is digested with chaotropic agents such as guanidine hydrochloride. Chaotropic agents denature proteins and produce single-stranded DNA by disrupting hydrogen bonds in double-stranded DNA. With the addition of a high salt buffer containing chaotropic agents, cations form a bridge between the negatively charged silica particles and negatively charged phosphate backbone of DNA allowing the DNA to tightly bind to the silica particles (20). The silica-nucleic acid complex is washed using a buffer composed of chaotropic agents and ethanol. Purified DNA is eluted in a low salt environment or in purified water. The silica extraction method has been found to be very specific for DNA; therefore, PCR inhibitors are unlikely to be co-purified along with the DNA (21). A study by Hoff-Olsen et al. (18) showed that silica suspensions were more successful in extracting DNA from decomposed tissue than phenol-chloroform extraction, glass fiber filter extraction, Instagene Matrix extraction and Chelex extraction. The silica suspension extraction was less expensive than phenol-chloroform extraction and gave full short tandem repeat (STR) profiles in 90% of the cases as compared to phenol chloroform extraction which only gave 60% full STR profiles. The use of silica membrane was also effective in the DNA extraction from skeletal remains that are 3-15 years postmortem. A study by Davoren et al. (22) demonstrated that silica-based extraction was more

efficient than phenol/chloroform extraction on bone samples. The silica-based extraction was able to produce full profiles in 100% of the samples, whereas the phenol/chloroform extraction was only able to produce full profiles in 70% of the samples. A major advantage of silica-based extraction methods over phenol/chloroform and Chelex extraction is that the silica-based methods can be automated using robotics.

Qiagen Corporation (Valencia, CA) developed a DNA extraction kit called the QIAamp® DNA Investigator Kit that uses a silica-based spin column with the addition of carrier RNA. The QIAamp® DNA Investigator Kit is compatible for use on an automated instrument called the QIAcube® (Qiagen Corporation). The QIAamp DNA Investigator method consists of 4 steps.

- Lysis: Samples are lysed under denaturing conditions with guanidine chloride and proteinase K
- Binding: DNA binds to the silica membrane and contaminants flow through the membrane
- Wash: Two wash steps are conducted to remove any residual contaminants
- Elution: Pure, concentrated DNA is eluted from the silica membrane

The entire extraction process occurs within one spin column tube reducing the occurrence of sample switching and sample to sample contamination. The kit allows for adjustments of DNA concentration by allowing for variable elution volumes from 20 µl to 100 µl (4). The kit uses a digest buffer that contains guanidine chloride, a denaturant with chaotropic properties that digests cells and disrupts proteins. Guanidine chloride also enhances the binding of DNA to the silica membrane in the spin columns (8). The kit can be used for purification of DNA from a variety of forensic samples such as bone, teeth, blood, saliva, sexual assault samples, buccal swabs, FTA cards, body fluid stains, chewing gum, cigarette butts and nail clippings. The

purification process is quick, does not require the use of toxic chemicals and reduces cross contamination between samples. The purified DNA is free of inhibitors, proteins and nucleases (4).

The QIAamp® DNA Investigator Kit also includes the addition of carrier RNA for the enhancement of DNA extraction. DNA can bind to polypropylene tubes, such as microcentrifuge tubes and PCR amplification tubes, which retain DNA in the ranges from 0.25 to 5 ng/mm² (23). The addition of poly A carrier RNA before the DNA extraction process is believed to increase DNA recovery by preventing the binding to polypropylene tubes (24). There are two hypotheses about the mechanism of carrier RNA. The first hypothesis states that RNA blocks sites on the side of tubes, centrifugation device filter, and surface of silica beads which could hold on to sample DNA. The second hypothesis declared that carrier RNA might enhance DNA binding to the silica particles by competing for the remaining solvent water molecules that are not bound to chaotropic agents therefore allowing the target DNA from being irreversibly bound (24). In a study by Kishore et al (24), samples that included carrier RNA in the extraction process showed an increase in DNA yield in blood and semen samples as compared to samples that were extracted without carrier RNA. The sensitivity level was also increased in samples that were extracted with carrier RNA as opposed to samples that were not. By adding carrier RNA, the extraction DNA recovery of low-yield samples was comparable to extraction by phenol/chloroform and in some cases even better.

Low Copy Number Typing

LCN typing typically refers to typing samples containing less than 100 pg of DNA (2). LCN samples can be obtained from aged samples, degraded samples or trace DNA samples

which are small amounts of DNA transferred through skin contact (25). LCN typing can be very beneficial to a case, yet attempting LCN typing should be used with caution. Issues such as increased contamination and difficult data interpretation due to stochastic variation were seen when LCN samples were typed. Associating a LCN DNA profile to specific evidence can be difficult since contaminating DNA can be transferred to the LCN sample before, during and after the crime (2). Within the past year, the use of LCN typing was questioned and then suspended for almost a month in England and Wales following the ruling on the Omagh trail, a court case that involved the use of LCN DNA as evidence in a bombing case (3). The United States does not allow the use of LCN DNA as evidence and data obtained from LCN typing cannot be entered into the Combined DNA Index System (CODIS). There are considerations that should be made known if LCN typing is applied: 1) the kits used may not have been subjected to quality control for use with LCN samples, 2) results are often not reproducible, 3) cannot be applied to post-conviction analyses and old cases and 4) there is no minimum threshold in interpretation (26).

Typing LCN samples using standard STR amplification can result in partial or no profiles. In order to obtain better profiles, the sensitivity of PCR could be improved by adjusting the primer concentration and increasing the number of PCR cycles from 28 to 34. However, LCN DNA typing can show instances of increased stochastic effects such as stutter product formation, allele dropout, and allele drop-in (2). Other techniques that can be used with LCN samples and does not include increasing the amplification cycle number include reducing the PCR reaction volume during amplification, using whole genome amplification (WGA) (27), increasing injection time (26) and post-PCR purification (28).

Post-PCR purification is method that increases PCR sensitivity by purifying PCR product and does not require increasing amplification cycles. During capillary electrophoresis, the typical injection time of 5 seconds allows a limited amount of DNA to be introduced into the capillary. However, components such as primers, unincorporated deoxynucleotide triphosphates (dNTPs), salts and other anionic PCR reaction parts can also be injected into the capillary along with the amplified STRs (29). If these components are removed, there should be an increase in signal intensity during electrophoresis due to the increase in amount of amplicons injected (30). In addition, the signal to noise ratio is also greatly improved in post-PCR purified samples as compared to amplified samples that have not been further purified (28).

There are a few different methods in post-PCR purification. The first method employs the use of gel filtration device such as Sephadex columns that uses a porous matrix that removes salts and unincorporated nucleotide. A study by Hutchinson et al. (31) used a modified Sephadex filtration procedure to show that when PCR products were cleaned the signal-to-noise ratio was 3.5 times higher than unclean PCR product. Another method employed in PCR product purification is through the use of size exclusion kit. These kits, which can be assisted with vacuum filtration, allow the PCR product to become trapped on top of the column and unincorporated nucleotides and salts are filtered through the column. Washing and elution step are not required with use of size exclusion kits. The purified PCR products are resuspended via shaking and then collected (32). The last method is a bind-wash-elute procedure with a silica membrane used to purify amplified product. Unincorporated nucleotides, salts and primers are not bound to the silica membrane and are washed through with ethanol. All traces of ethanol must be removed from the purified PCR product in order to prevent any inhibition to downstream applications. Purified DNA is then eluted using a low salt buffer or purified water.

The method utilizing silica membrane and the bind-wash-elute method can be automated for the purification of amplified product (28).

Qiagen Corporation has developed a kit that can be used to purify amplified products called the Qiagen® MinElute® PCR Purification Kit. The purification kit uses the bind-wash-elute procedure that is capable of being automated. Qiagen® MinElute® PCR Purification Kit is suitable for purification of double-stranded PCR products that are between 70 bp and 4 kb and uses a silica membrane with special buffers that offers an optimal environment for the purification of PCR product (6). DNA is bound to the membrane using a high concentration of chaotropic salts such as guanidine chloride. The chaotropic salts alter the characteristics of water molecules which surround the DNA molecule; therefore allowing the DNA molecules to become more easily bound to the silica membrane (8). The binding of DNA to a silica membrane is also pH dependent. DNA adsorption is most efficient when the pH is ≤ 7.5. As the pH increases, the DNA binding to silica is drastically reduced. The kit supplies a binding buffer that contains a color pH indicator that allows for a quick visual determination for the most favorable pH for DNA adsorption.

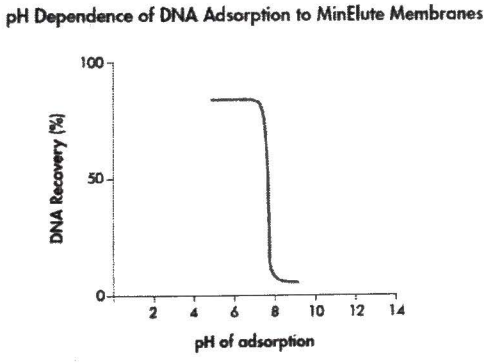

Figure 2.1: pH dependence of DNA adsorption to silica (6)

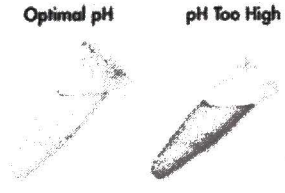

Figure 2.2: pH indicator buffer (6)

The wash buffer is an ethanol based buffer and the purified PCR product is eluted with a low-salt buffer or water. This whole process can be performed using a table-top microcentrifuge or a vacuum manifold. The purification procedure removes excess primers, nucleotides, enzymes, and other impurities from the DNA samples (6). The Qiagen® MinElute® PCR Purification Kit can be used with LCN sample without having to increase amplification cycles from 28 to 34 cycles (27). The purification kit can also be automated for use on the QIAcube® instrument.

Robotics

Many forensic laboratories have incorporated the use of robotics to efficiently process samples in order to enable high sample throughput. Currently, it is possible to fully automate DNA extraction, quantification, amplification, and electrophoresis. The use of robotics is appealing because of the automated procedures, minimal sample manipulation, ease of use and reduced analyst labor time (13). It also provides a consistency in processing a wide range of samples. Robotic procedures have been shown to be comparable to manual methods in DNA extraction and helped to reduce the opportunity for human error (24).

The Qiagen Corporation has developed an enclosed automated workstation called the QIAcube® that can be used in the purification of DNA, RNA and proteins. The QIAcube® consists of tube racks, places for reagents, robotic gripper arm, swing bucket rotor centrifuge and 12 sample holder which is also used to shake and heat the samples. The QIAcube® uses Qiagen spin-column technology and can perform steps from sample lysis to elution of highly pure nucleic acids or proteins. The automated workstation applies the same chemistry that is currently used in Qiagen extraction kits therefore the performance of the instrument is comparable to the manual method. The instrument operates using a silica based extraction

technique that is used in combination with reagents that are provided by the manufacturer. There is no need for an additional computer since it has over 100 pre-installed protocols and can accept customized protocols as well. The QIAcube® is capable of automating the QIAamp® DNA Investigator Kit and the Qiagen® MinElute® PCR Purification Kit. The QIAcube® is capable of processing anywhere from 2 to 12 samples at one time (5).

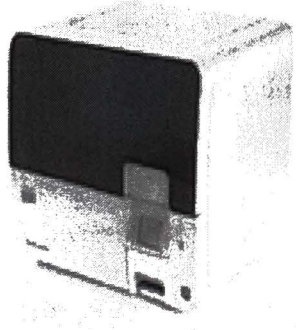

Figure 2.3: QIAcube® Automation Instrument (5)

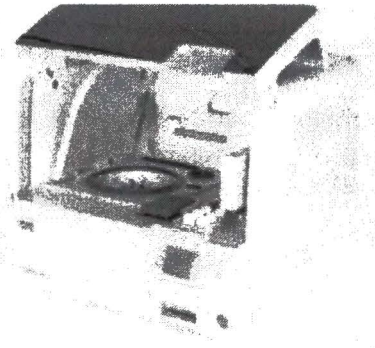

Figure 2.4: Inside of QIAcube® Automation Instrument (5)

CHAPTER 3

MATERIALS AND METHOD

Liquid blood, buccal cells and sperm cells were obtained from one male individual. The DNA technical leader prepared 11 dilutions that were set up in phosphate-buffered solution (PBS). Replicates of each samples were prepared, one for organic extractions and one for QIAamp® DNA Investigator Kit extractions (Table 3.1 and Table 3.2). Extraction blanks were also processed alongside the samples to monitor for possible human contamination and cross-contamination among the samples.

Organic extracted samples		
Liquid Blood	Buccal cell	Sperm cell
OB-A	OE-A	OS-A
OB-B	OE-B	OS-B
OB-C	OE-C	OS-C
OB-D	OE-D	OS-D
OB-E	OE-E	OS-E
OB-F	OE-F	OS-F
OB-G	OE-G	OS-G
OB-H	OE-H	OS-H
OB-I	OE-I	OS-I
OB-J	OE-J	OS-J
OB-K	OE-K	OS-K

Table 3.1: Setup of Organic Extracted Samples

QIAamp extracted samples		
Liquid Blood	Buccal cell	Sperm cell
QB-A	QE-A	QS-A
QB-B	QE-B	QS-B
QB-C	QE-C	QS-C
QB-D	QE-D	QS-D
QB-E	QE-E	QS-E
QB-F	QE-F	QS-F
QB-G	QE-G	QS-G
QB-H	QE-H	QS-H
QB-I	QE-I	QS-I
QB-J	QE-J	QS-J
QB-K	QE-K	QS-K

Table 3.2: Setup of QIAamp Extracted Samples

The validation study was a blind study (Table 3.3, Table 3.4 and Table 3.5). The sensitivity of the QIAamp® DNA Investigator Kit extraction was compared to the sensitivity of organic based DNA extraction method by analyzing 1:10, 1:50, 1:100, 1:500, 1:1000, 1:5000, 1:10000, 1:50000, 1:100000, 1:500000 and 1:1000000 dilutions of liquid blood, buccal cells and sperm cells. The samples were not duplicated to assess the reproducibility of the validation study.

Liquid Blood Dilutions	Organic Extraction	DNA Investigator
1:10	OB-J	QB-E
1:50	OB-I	QB-J
1:100	OB-F	QB-K
1:500	OB-B	QB-A
1:1000	OB-A	QB-D
1:5000	OB-E	QB-C
1:10000	OB-D	QB-G
1:50000	OB-K	QB-B
1:100000	OB-G	QB-F
1:500000	OB-H	QB-H
1:1000000	OB-C	QB-I

Table 3.3: Blood Cell Sample Key

Buccal Cell Dilutions	Organic Extraction	DNA Investigator
1:10	OE-J	QE-G
1:50	OE-I	QE-D
1:100	OE-B	QE-I
1:500	OE-H	QE-A
1:1000	OE-D	QE-E
1:5000	OE-K	QE-C
1:10000	OE-A	QE-J
1:50000	OE-G	QE-K
1:100000	OE-C	QE-B
1:500000	OE-F	QE-H
1:1000000	OE-E	QE-F

Table 3.4: Buccal Cell Sample Key

Sperm Cell Dilutions	Organic Extraction	DNA Investigator
1:10	OS-J	QS-A
1:50	OS-F	QS-K
1:100	OS-K	QS-H
1:500	OS-H	QS-B
1:1000	OS-C	QS-G
1:5000	OS-D	QS-C
1:10000	OS-G	QS-E
1:50000	OS-E	QS-F
1:100000	OS-I	QS-I
1:500000	OS-A	QS-J
1:1000000	OS-B	QS-D

Table 3.5: Sperm Cell Sample Key

The maximum number of samples extracted at any one time was 7 samples including an extraction blank. In order to protect the working personnel and to guard against sample contamination, a lab coat, gloves, eye protection and mouth mask were worn at all times during every stage of DNA testing. There were separate lab coats, eyewear and gloves that were designated for the post-amplification room.

Organic DNA Extraction Procedure

The phenol/chloroform/isoamyl/alcohol extraction procedure for the blood, buccal and sperm cells was performed according to the Acadiana Criminalistic Laboratory's protocol (Appendix A). The organic DNA extraction procedure for the blood and buccal cells was set up by adding 50 µl of the samples to 300 µl of digest buffer (1.0M Tris pH7.5, 20% SDS, 5.0M NaCl, 0.5M EDTA, 18MΩ H_2O) and 10 µl of proteinase K (provided at 10 mg/ml). The sperm samples were extracted by adding 50 µl of sample to 300 µl of digest buffer and 10 µl of proteinase K; additionally, 20 µl of 1 M dithiothreitol (DTT) was added. The samples were incubated at 56°C overnight. After incubation, the cell lysates were extracted with PCIA (25:24:1, v/v) (Invitrogen, Carlsbad, CA) and centrifuged at 10,000 rpm for 3 minutes. The extraction step with PCIA was repeated two more times. The cell lysates were mixed with butanol (Thermo Fisher Scientific, Waltham, MA) to remove any residual PCIA. DNA was concentrated by adding the DNA samples to Microcon ® YM-100 concentrators (Millipore Corporation, Billerica, MA). The concentrators were first primed by adding 100 µl of TE^{-4} (1M Tris, pH 8.0, 0.5M EDTA, 18MΩ H_2O) to the membrane of the concentrators. DNA samples were washed twice with TE^{-4} and centrifuged at 5000 rpm for 10 minutes. DNA was then eluted in a volume of 45 µl in TE^{-4} by centrifuging the sample at 5000 rpm for 5 minutes. DNA

extracts were transferred to a 96-well Greiner plate (Greiner Bio-One, Monroe, NC) and sealed with X-pierce cross-cut pierceable film (USA Scientific, Ocala, FL) to prevent sample evaporation. The samples were stored at -20°C.

QIAamp DNA Investigator Kit Extraction

Preparation of Buffers and Reagents

The QIAamp® DNA Investigator Kit provided all the buffers, proteinase K, carrier RNA and QIAamp® MinElute® column needed to perform the extraction. The kit did not provide DTT needed in the sperm sample extraction and ethanol needed for reconstitution of the buffers and for the extraction procedure. Both ethanol and DTT were provided by the Acadiana Criminalistic Laboratory. The buffers in the kit consisted of buffers ATL, AL, AW1, AW2 and ATE. Certain reagents and buffers were reconstituted before use. The lyophilized carrier RNA contained 310 µg and was reconstituted in 310 µl Buffer ATE to obtain a final concentration of 1 µg/µl. The dissolved carrier RNA was distributed into 10 µl aliquots and stored at -20°C. The proteinase K solution (600 mAU/ml, Qiagen) was divided into 100 µl aliquots and stored at 4°C. Buffer AW1 was reconstituted with 25 ml ethanol and stored at room temperature (15-25°C). Buffer AW2 was reconstituted with 30 ml ethanol and also stored at room temperature. Buffer ATL, Buffer AL and Buffer ATE were all stored at room temperature. Before use of Buffer AL in blood and buccal cell extractions, 1 µl of dissolved carrier RNA was added to 100 µl Buffer AL for a final concentration of 1µg carrier RNA/ 100 µl Buffer AL. For the sperm cell samples, 1 µl of dissolved carrier RNA was added to 300 µl Buffer AL for a final concentration of 1 µg carrier RNA/ 300 µl Buffer AL.

Preparation of tubes for QIAamp extraction

Tubes needed for the extraction were labeled with a sample identifier. One set of blood, buccal and sperm samples contained 11 diluted samples and 2 extraction blanks each for a total of 39 samples. For each 39 samples, 1 set of QIAamp® MinElute® column was prepared along with 4 sets of 2 ml collection tubes for each sample. Two 1.5 ml microcentrifuge tubes were prepared for each blood and buccal sample and three 1.5 ml microcentrifuge tubes were prepared for each sperm sample. All tubes were all sterilized under the UV hood for 30 minutes to eliminate any presence of DNA (33).

Qiagen Extraction procedure

Blood and buccal samples were processed according to QIAamp® DNA Investigator Kit protocols. Blood and buccal sample extractions were started by adding 50 µl of blood or buccal sample to a 1.5 ml microcentrifuge tube. A volume of 50 µl of Buffer ATL, 10 µl proteinase K (600mAU/ml) and 100 µl Buffer AL including carrier RNA (1 µg/100µl) were added to each samples. The blood and buccal samples were incubated for 10 minutes at 56°C with vortexing every 3 minutes. After incubation, 50 µl absolute ethanol was added to blood and buccal samples and mixed by vortexing for 15 seconds and allowed to incubate at room temperature for 3 minutes. Blood and buccal samples were then pipetted into the QIAamp® MinElute® columns and centrifuged at 8000 rpm for 1 minute. Samples were washed with 500 µl Buffer AW1 and centrifuged at 8000 rpm for 1 minute and were washed again with 500 µl Buffer AW2 and centrifuged at 8000 rpm for 1 minute. A volume of 700 µl of absolute ethanol was added to the samples and centrifuged at 8000 rpm for 1 minute. The ethanol was discarded and samples were centrifuged at 14,000 rpm for 3 minutes to dry the column membrane completely. The column was then placed in a clean 1.5 ml microcentrifuge tube and allowed to incubate for 10 minutes at

room temperature. Elution Buffer ATE was added at 50 µl to the samples and allowed to incubate for 5 minutes at room temperature. The blood and buccal DNA were eluted by centrifuging the sample at 14,000 rpm for 1 minute. The eluted DNA was stored in a 96-well Greiner plate (Greiner Bio-One, Monroe, NC) and sealed with X-Pierce cross-cut pierceable film (USA Scientific, Ocala, FL). The extracted DNA samples were stored at -20°C.

The protocol for sperm cell extraction using the QIAamp® DNA Investigator Kit was similar to the protocol for blood and buccal samples (Table 3.6). DNA extraction of sperm samples started by digesting 50 µl of sperm sample with 250 µl of lysis Buffer ATL, 10 µl proteinase K (600mAU/ml) and 10 µl DTT (1M) and incubated for one hour at 56°C. After incubation, 300 µl Buffer AL including dissolved carrier RNA (1 µg/300 µl) was added to the sperm samples and further incubated at 70°C for 10 minutes. After the second incubation 150 µl of absolute ethanol was added to the sperm cell lysates. The sperm cell lysates were transferred to the QIAamp® MinElute® column and were washed with Buffer AW1 and Buffer AW2. Sperm DNA was eluted in Buffer ATE and stored in a sealed 96-well plate at -20°C.

QIAamp® DNA Investigator Kit Protocols	
Blood and Buccal Samples	50 µl Buffer ATL 10 µl Proteinase K (600 mAU/ml) 100 µl Buffer AL with carrier RNA (1 µg/100 µl) 50 µl Ethanol 500 µl Buffer AW1 700 µl Buffer AW2 700 µl Ethanol 45 µl Buffer ATE
Sperm Samples	250 µl Buffer ATL 10 µl DTT (1 M) 10 µl Proteinase K (600 mAU/ml) 300 µl Buffer AL with carrier RNA (1 µg/300 µl) 150 µl Ethanol 500 µl Buffer AW1 700 µl Buffer AW2 700 µl Ethanol 45 µl Buffer ATE

Table 3.6: Set up of QIAamp® DNA Investigator Kit Extraction

Modifications to QIAamp® DNA Investigator Kit procedure

Re-extracted blood and buccal samples had an increase in digest time from 10 minutes to 60 minutes at 56°C and re-extracted sperm samples had an increase in digest time from 1 hour to overnight at 56°C. The amount of DTT added to the sperm sample digest was increased from 10 µl to 20 µl. The rest of the extraction process was performed according to the QIAamp® DNA Investigator Kit protocols.

DNA Quantification

The DNA quantification step is used to determine the amount of DNA present in the sample and was performed according to the Acadiana Criminalistic Laboratory's protocol (Appendix B). The Biomek® 2000 robot (Beckman Coulter, Fullerton, CA), Bioworks™ Software (Beckman Coulter) and the Quantifiler® Human DNA Quantification Kit (Applied

Biosystems, Foster City, CA) were used to set up the reaction plate for real-time PCR quantification. The ABI Prism® 7000 Real-time PCR instrument (Applied Biosystems) and the ABI Prism 7000® Sequence Detection System (SDS) software (Applied Biosystems) were used to amplify and quantify the DNA. The Biomek® 2000 workstation was set up by starting the Bioworks™ Software and following the directions from the software. A MicroAmp™ 8-tube strip (Applied Biosystems) used to prepare the standards and a MicroAmp™ Optical 96-well Reaction Plate (Applied Biosystems) was placed onto the workstation. Necessary barrier tips and DNA samples in the 96 well Greiner plate were added to the workstation in the relevant positions.

Preparation of reagents

The Quantifiler® Human DNA Quantification Kit supplied the DNA standard, primer mix and PCR reaction mix needed to set up the quantification. The standard used were the Quantifiler® Human DNA Standard (Applied Biosystems) and serial dilutions were set up to 50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068 and 0.023ng/µl in TE^{-4} buffer (1M Tris, pH 8.0, 0.5M EDTA, 18MΩ H_2O). A master mix of 10.5 µl Quantifiler® Human Primer Mix (Applied Biosystems) per sample and 12.5 µl Quantifiler® PCR Reaction Mix (Applied Biosystems) per sample was made and added to the reservoir on the workstation. The Biomek® 2000 robot aliquoted 23 µl of the master mix to each reaction well in the MicroAmp™ Optical 96-well Reaction Plate. DNA from each standard and sample DNA extracts was added to the reaction wells at 2 µl for a total volume of 25 µl. The DNA standards were set up in duplicates. The 96-well Optical Reaction Plate was sealed with the MicroAmp™ Optical Adhesive Cover (Applied Biosystems) using the spatula to secure the seal. The reaction plate was centrifuged at 3000 rpm for 20 seconds to

remove any bubbles in the samples. A compression pad was placed over the Optical Adhesive Cover with the holes positioned directly over the reaction wells. The 96-well Optical Reaction Plate was then placed into the thermal block of the ABI Prism® 7000 Sequence Detection System (Applied Biosystems). The Sequence Detection System software was programmed for a 25 µl reaction volume with cycling parameters that included 95°C incubation for 10 minutes followed by 40 cycles at 95°C for 15 seconds followed by 60°C for 1 minute. After the amplification was finished, the data was analyzed by the Sequence Detection System software to produce a standard curve based on the standard serial dilutions. The DNA amounts were determined based upon the standard curve.

DNA Amplification

The DNA amplification process was performed according to the Acadiana Criminalistic Laboratory's protocol (Appendix C). PCR amplification was performed using the AmpF*l*STR® Identifiler® PCR Amplification Kit (Applied Biosystems) on the GeneAmp® PCR System 9700 (Applied Biosystems). The Identifiler® System was designed to co-amplify 15 STR loci and Amelogenin. The 15 STR loci amplified included CSF1PO, D7S820, D8S1179, D21S11, D2S1338, D3S1358, D13S317, D16S539, THO1, D18S51, D19S433, TPOX, vWA, D5S818 and FGA. Samples were diluted to 0.1ng/ul with TE^{-4} buffer (1M Tris, pH 8.0, 0.5M EDTA, 18MΩ H_2O). If samples contained < 0.1ng/µl of DNA, the samples were used in amplification without diluting. A MicroAmp™ Optical 96-well Reaction Plate was sterilized and labeled accordingly. The PCR Master Mix was prepared for the number of reactions plus 2 by calculating the required amount of each component of the Master Mix. The components of each reaction consisted of 10.5 µl of PCR Reaction Mix, 5.5 µl Primer Set and 0.5 µl AmpliTaq Gold™ polymerase (5u/µl)

(Applied Biosystems). The Master Mix was added to each reaction well at 15 µl each and 10µl of 0.1ng DNA template was also added to the relevant reaction well. A positive control (AmpF*l*STR® Control DNA 9947A, 0.1 ng/µl) and negative control (TE^{-4}) was also included in the amplification and were performed in duplicates. The MicroAmp™ Optical 96-well Reaction Plate was sealed with an adhesive PCR foil (Thermo Scientific, Waltham, MA) that was used to prevent evaporation during thermal cycling and storage. A spatula was used to seal between each row and column on the reaction plate along with the edges of the reaction plate. The 96-well Optical Reaction Plate was placed within the tray set in the GeneAmp® PCR System 9600 (Applied Biosystems) and an insulating cover was placed over the reaction plate. The amplification program was set for a 25 µl reaction. The cycle parameters included an initial hotstart activation at 95°C for 11 minutes followed by 28 cycles of DNA denaturation at 94°C for 1 minute, primer annealing at 59°C for 1 minute, and an extension at 72°C for 1 minute. Afterwards, there was a final extension at 60°C for 60 minutes and then a 4°C hold indefinitely. The samples were stored at 4°C in the post amplification room.

Qiagen® MinElute® PCR Purification Kit

The Qiagen® MinElute® PCR Purification Kit provided all of the buffers (Buffer PBI, Buffer PE and Buffer EB) used in the purification process of amplified product. Before PCR purification took place, Buffer PE had to be reconstituted with absolute ethanol and a pH check was done on Buffer PBI. If the pH was too high, the pH was adjusted by adding 10µl of 3M sodium acetate, pH 5.0.

Two 1.5 ml microcentrifuge tubes and one set of MinElute columns were labeled with a sample identifier and set up for each amplified product. The PCR product was pipetted into a 1.5

ml microcentrifuge and mixed with 5 volumes of Buffer PBI to 1 volume of PCR product. The color of the solution was checked. A yellow solution would indicate that the reaction was at optimal pH. If the solution was an orange or violet color, 10 µl of 3M sodium acetate, pH5.0 was added to the solution. The amplified products were then briefly centrifuged to remove drops from inside the lid and all of the solution mixture was applied to a MinElute column within a 2 ml collection tube. The amplified products were centrifuged at 10,000 rpm for 3 minutes and 30 seconds and the flow-through was discarded. To wash the PCR products, 750 µl Buffer PE was added to the column and then centrifuged at 10,000 rpm for 3 minutes and 30 seconds. The flow-through was discarded and the column was centrifuged at 10,000 rpm for 3 minutes and 30 seconds to remove any residual ethanol still present in the column. Elution Buffer EB (10mM Tris-Cl, pH 8.5) was added at 10 µl to the center of the column membrane and incubated at room temperature for 1 minute. The PCR products were centrifuged at 10,000 rpm for 3 minutes and 30 seconds to elute the purified PCR product into a 1.5 ml microcentrifuge tube. The purified PCR products were stored at 4°C in the post amplification room.

Capillary Electrophoresis and STR Analysis

Capillary electrophoresis and STR analysis on the amplified products was performed according to the Acadiana Criminalistic Laboratory's protocol (Appendix D) and interpretation guidelines. To prepare amplified product for electrophoresis, 24.5 µl HiDi™ Formamide (Applied Biosystems) and 0.5 µl of the GeneScan™ 500 LIZ™ Size Standard (Applied Biosystems) provided in the AmpF*l*STR® Identifiler® Kit was mixed for each sample. The formamide mix was aliquoted into 0.2 ml MicroAmp® Reaction Tubes (Applied Biosystems) and 1.5 µl of PCR product or allelic ladder was added to the 0.2 ml MicroAmp® Reaction Tube.

The allelic ladder was provided in the AmpF*l*STR® Identifiler® Kit and was duplicated in the setup for analysis. A septum was placed over all the samples and the samples were denatured at 95°C for 3 minutes and then rapid chilled for 3 minutes on ice. The samples were placed into a 96 well retainer clip ready to be placed into the genetic analyzer instrument.

In setting up the ABI 310® Genetic Analyzer (Applied Biosystems), 15 ml of 1X capillary electrophoresis buffer (Applied Biosystems) was prepared and the interior components of the ABI 310® Genetic Analyzer, such as the pump block, buffer reservoir, buffer vials and waste vials, were assembled according to the manufacturer's instructions. A capillary was installed into the ABI 310® Genetic Analyzer and DNA fragments were electrophoresed in POP-4 polymer (Applied Biosystems).

The ABI 310® Genetic Analyzer generated electropherograms that were analyzed using GeneMapper® ID version 3.2 software (Applied Biosystems). The alleles were assigned to each peak in a sample by referencing an allelic ladder. Only allele peaks that met a minimum threshold of 75 Relative Fluorescence Unit (RFU) were considered true peaks.

Data Analysis

DNA quantification results from initial DNA extractions with organic extraction method and QIAamp® DNA Investigator Kit were compared to determine which extraction method was more sensitive. DNA yields from initial QIAamp extractions and QIAamp re-extractions were evaluated to determine if a longer digest time had any effect on the amount of DNA extracted. Data obtained from STR analysis was used to determine the amount of complete loci detected in a DNA sample and to classify the samples as providing a full, partial or no profile. STR profiles from organic extraction method and QIAamp® DNA Investigator Kit were compared to establish

which method was able to detect more loci. The initial and second extractions with QIAamp® DNA Investigator Kit were also compared to determine if different digest times affected the amount of detectable loci in a profile. The Qiagen® MinElute® PCR Purification Kit was utilized in an attempt to improve the sensitivity of STR typing. A comparison in the amount of loci detected from STR analysis was determined in PCR products before purification and after purification.

CHAPTER 4

RESULTS

DNA Extraction Recovery

The analysis of the standard curve for the initial DNA extractions showed that the DNA yields were underestimated. The standard curve results showed the R^2 value measured at 0.925479 with a slope of -2.139641 and Y-intercept of 27.137102. When comparing the DNA recovery yields in blood and sperm samples, the organic extraction method consistently extracted more DNA than the QIAamp® DNA Investigator Kit (Table 4.1 and Table 4.3). There was little difference in the amount of DNA recovered from buccal samples extracted by organic extraction method and QIAamp® DNA Investigator Kit (Table 4.2). It was noted that three buccal samples extracted with organic extraction method had microcentrifuge tubes that leaked during phenol/chloroform extraction step (Table 4.2). During the set up of the reaction plate for the quantification procedure, it was noted that sample QS-K was not added to the reaction plate; therefore, the quantification result showed that there was no DNA in the sample (Table 4.3).

Dilutions	Method			
	Organic		DNA Investigator	
	sample	DNA yield (ng/µl)	sample	DNA yield (ng/µl)
1:10	OB-J	1.010	QB-E	0.354
1:50	OB-I	0.199	QB-J	0.014
1:100	OB-F	0.113	QB-K	0.009
1:500	OB-B	0.004	QB-A	0.002
1:1000	OB-A	0.003	QB-D	0.000
1:5000	OB-E	0.001	QB-C	0.000
1:10000	OB-D	0.000	QB-G	0.000
1:50000	OB-K	0.000	QB-B	0.000
1:100000	OB-G	0.000	QB-F	0.000
1:500000	OB-H	0.000	QB-H	0.000
1:1000000	OB-C	0.000	QB-I	0.000

Table 4.1: DNA recovery from blood samples using organic extraction method and QIAamp® DNA Investigator Kit

Dilutions	Method			
	Organic		DNA Investigator	
	sample	DNA yield (ng/µl)	sample	DNA yield (ng/µl)
1:10	OE-J	6.240	QE-G	6.920
1:50	OE-I	0.110	QE-D	0.069
1:100	OE-B	0.046*	QE-I	0.208
1:500	OE-H	0.003	QE-A	0.004
1:1000	OE-D	0.003	QE-E	0.001
1:5000	OE-K	0.001	QE-C	0.000
1:10000	OE-A	0.000*	QE-J	0.000
1:50000	OE-G	0.000	QE-K	0.000
1:100000	OE-C	0.000*	QE-B	0.000
1:500000	OE-F	0.000	QE-H	0.000
1:1000000	OE-E	0.000	QE-F	0.000

* tube leaked

Table 4.2: DNA recovery from buccal samples using organic extraction method and QIAamp® DNA Investigator Kit

	Method			
	Organic		DNA Investigator	
Dilutions	sample	DNA yield (ng/µl)	sample	DNA yield (ng/µl)
1:10	OS-J	233.280	QS-A	41.810
1:50	OS-F	15.440	QS-K	0.000**
1:100	OS-K	2.190	QS-H	0.683
1:500	OS-H	0.112	QS-B	0.052
1:1000	OS-C	0.116	QS-G	0.068
1:5000	OS-D	0.011	QS-C	0.004
1:10000	OS-G	0.003	QS-E	0.002
1:50000	OS-E	0.002	QS-F	0.000
1:100000	OS-I	0.000	QS-I	0.000
1:500000	OS-A	0.000	QS-J	0.000
1:1000000	OS-B	0.000	QS-D	0.000
** sample was not added during quantification setup				

Table 4.3: DNA recovery from sperm samples using organic extraction method and QIAamp® DNA Investigator Kit

DNA Recovery from Re-extracted Samples

The analysis from the standard curve showed that the DNA yields obtained from the re-extracted samples were accurate. The R^2 value measured at 0.996446 with a slope of -3.089699 and Y-intercept of 28.202583. Blood samples that were re-extracted using a longer digest time with the QIAamp® DNA Investigator Kit showed DNA yields that tripled the initial DNA extraction yields (Table 4.4). Two of the buccal samples (OE-B, OE-A) that had previous leaked were re-extracted with organic extraction method and the results indicated that there was an increase in the DNA yield as was expected (Table 4.5). DNA yields from re-extracted sperm samples using the QIAamp® DNA Investigator Kit showed a higher yield than the initial sperm sample extractions at all dilutions except 1:10 dilution (Table 4.6).

Dilutions	Method: DNA Investigator — sample	Initial Extraction: DNA yield (ng/µl)	Second Extraction: DNA yield (ng/µ/)
1:10	QB-E	0.354	1.370
1:50	QB-J	0.014	0.214
1:100	QB-K	0.009	0.067
1:500	QB-A	0.002	0.013
1:1000	QB-D	0.000	0.010
1:5000	QB-C	0.000	0.005
1:10000	QB-G	0.000	0.002
1:50000	QB-B	0.000	ND
1:100000	QB-F	0.000	ND
1:500000	QB-H	0.000	ND
1:1000000	QB-I	0.000	ND

ND=not done

Table 4.4: QIAamp® DNA Investigator Kit: DNA yields of re-extracted blood samples

Dilutions	Method: Organic — sample	Initial Extraction: DNA yield (ng/µl)	Second Extraction: DNA yield (ng/µ/)
1:10	OE-J	6.240	ND
1:50	OE-I	0.110	ND
1:100	OE-B	0.046*	0.289
1:500	OE-H	0.003	ND
1:1000	OE-D	0.003	ND
1:5000	OE-K	0.001	ND
1:10000	OE-A	0.000*	0.002
1:50000	OE-G	0.000	ND
1:100000	OE-C	0.000*	ND
1:500000	OE-F	0.000	ND
1:1000000	OE-E	0.000	ND

* tube leaked, ND=not done

Table 4.5: Organic Extraction: DNA yield of re-extracted buccal samples

	Method		
	DNA Investigator		
Dilutions	sample	Initial Extraction: DNA yield (ng/µl)	Second Extraction: DNA yield (ng/µ/)
1:10	QS-A	41.810	21.440
1:50	QS-K	0.000**	4.590
1:100	QS-H	0.683	1.990
1:500	QS-B	0.052	0.265
1:1000	QS-G	0.068	0.258
1:5000	QS-C	0.004	0.027
1:10000	QS-E	0.002	0.012
1:50000	QS-F	0.000	0.003
1:100000	QS-I	0.000	0.003
1:500000	QS-J	0.000	0.002
1:1000000	QS-D	0.000	0.002
**sample was not added during quantification step, ND=not done			

Table 4.6: QIAamp® DNA Investigator Kit: DNA yield of re-extracted sperm samples

STR Analysis

All samples analyzed had correct allele calls and the positive and negative controls did not show any presence of contamination. The results from STR analysis showed little difference between organic extraction method and QIAamp® DNA Investigator Kit in the blood, buccal and sperm samples (Table 4.7, Table 4.8, and Table 4.9).

Blood samples extracted with the organic extraction method produced 4 full profiles and 4 partial profiles while the QIAamp blood extraction produced 5 full profiles and 2 partial profiles. Full profiles from blood samples were obtained at dilutions of 1:1000 and higher. Blood sample OB-I gave a partial profile when a full profile was expected. The real-time PCR amplification results did not show any presence of PCR inhibition. Buccal samples that were extracted by organic extraction produced 4 full profiles and 2 partial profiles. The QIAamp® DNA Investigator Kit produced 5 full profiles and 2 partial profiles for buccal samples. Full profiles were obtainable at a minimum dilution of 1:1000. Buccal sample OE-H failed to have

any allele peaks detected. The sample was run on another genetic analyzer and the result was the same. There was no inhibition detected during real-time PCR for sample OE-H. Sperm samples that were extracted by organic extraction produced 7 full profiles and 4 partial profiles while the same samples extracted with QIAamp® DNA Investigator Kit produced 8 full profiles and 1 partial profile. Full profiles were obtained at 1:10,000 dilution with organic extraction method and 1:100,000 with QIAamp DNA Investigator Kit.

	Method			
	Organic		DNA Investigator	
Dilutions	sample	# loci detected	sample	# loci detected
1:10	OB-J	f	QB-E	f
1:50	OB-I	14	QB-J	f
1:100	OB-F	f	QB-K	f
1:500	OB-B	f	QB-A	f
1:1000	OB-A	f	QB-D	f
1:5000	OB-E	10	QB-C	5
1:10000	OB-D	6	QB-G	2
1:50000	OB-K	1	QB-B	n
1:100000	OB-G	n	QB-F	n
1:500000	OB-H	n	QB-H	n
1:1000000	OB-C	n	QB-I	n

x/16 = # of complete loci, f=full profile, n=no profile

Table 4.7: Number of complete loci detected in blood samples using organic extraction method and QIAamp® DNA Investigator Kit

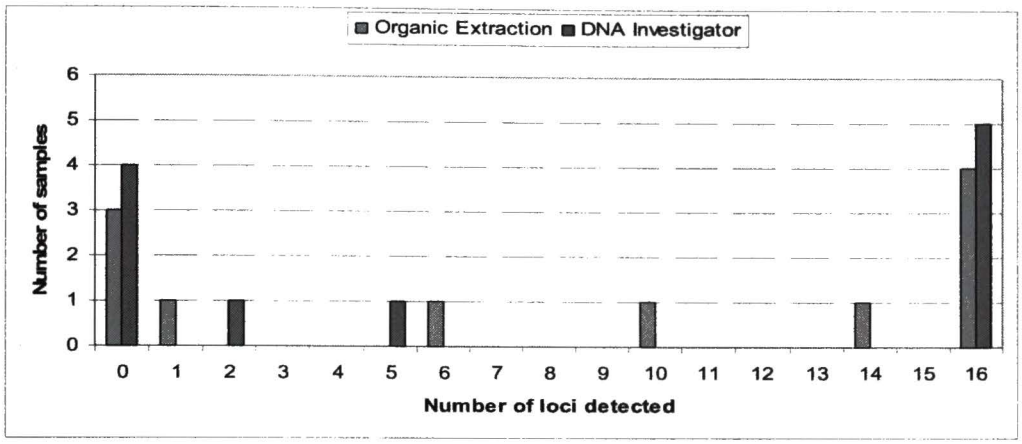

Figure 4.1: Bar graph illustrating number of loci detected from blood samples through organic extraction and QIAamp® DNA Investigator Kit

	Method			
	Organic		DNA Investigator	
Dilutions	sample	# loci detected	sample	# loci detected
1:10	OE-J	f	QE-G	f
1:50	OE-I	f	QE-D	f
1:100	OE-B	f	QE-I	f
1:500	OE-H	n	QE-A	f
1:1000	OE-D	f	QE-E	f
1:5000	OE-K	8	QE-C	9
1:10000	OE-A	5	QE-J	9
1:50000	OE-G	n	QE-K	n
1:100000	OE-C	n	QE-B	n
1:500000	OE-F	n	QE-H	n
1:1000000	OE-E	n	QE-F	n

x/16 = # of complete loci, f=full profile, n=no profile

Table 4.8: Number of complete loci detected in buccal samples using organic extraction method and QIAamp® DNA Investigator Kit

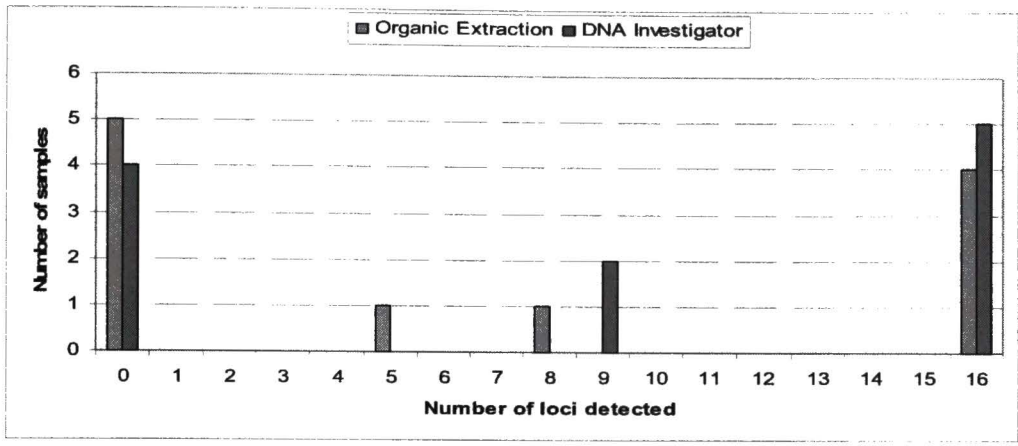

Figure 4.2: Bar graph illustrating profiles obtained from buccal samples through organic extraction and QIAamp® DNA Investigator Kit

	Method			
	Organic		DNA Investigator	
Dilutions	sample	# loci detected	sample	# loci detected
1:10	OS-J	f	QS-A	f
1:50	OS-F	f	QS-K	f
1:100	OS-K	f	QS-H	f
1:500	OS-H	f	QS-B	f
1:1000	OS-C	f	QS-G	f
1:5000	OS-D	f	QS-C	f
1:10000	OS-G	f	QS-E	f
1:50000	OS-E	13	QS-F	8
1:100000	OS-I	11	QS-I	f
1:500000	OS-A	1	QS-J	n
1:1000000	OS-B	1	QS-D	n

x/16 = # of complete loci, f=full profile, n=no profile

Table 4.9: Number of complete loci detected in sperm samples using organic extraction method and QIAamp® DNA Investigator Kit

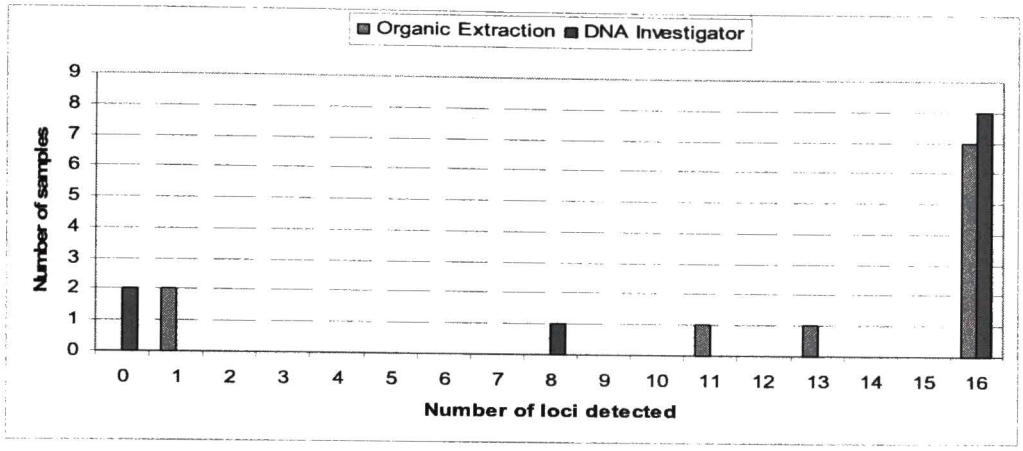

Figure 4.3: Bar graph illustrating profiles obtained from sperm samples through organic extraction and QIAamp® DNA Investigator Kit

STR Analysis of Re-extracted Samples

The DNA profiles obtained from initial extractions were compared to the DNA profiles obtained from re-extracted samples (Table 4.10, Table 4.11, and Table 4.12). Only a few re-extracted samples proceeded to STR analysis; therefore, it was difficult to make a valid comparison based on the limited amount of data acquired from the re-extracted samples. Re-extracted blood samples QB-C and QB-G showed a slight increase in the amount of loci detected. There was no change in the amount of loci detected from the initial extraction and re-extraction in the buccal sample. Two of the re-extracted sperm samples showed a decrease in the amount of loci detected when compared to the STR result from the initial extraction.

		DNA Investigator	
		Initial Extraction	Second Extraction
Dilutions	sample	# loci detected	# loci detected
1:10	QB-E	f	ND
1:50	QB-J	f	ND
1:100	QB-K	f	ND
1:500	QB-A	f	ND
1:1000	QB-D	f	ND
1:5000	QB-C	5	8
1:10000	QB-G	2	1
1:50000	QB-B	n	n
1:100000	QB-F	n	ND
1:500000	QB-H	n	ND
1:1000000	QB-I	n	ND
x/16 = # of complete loci, f=full profile, n=no profile ND=not done			

Table 4.10: QIAamp® DNA Investigator Kit:
Comparison of amount of loci detected in blood samples

		Organic Extraction	
		Initial Extraction	Second Extraction
Dilutions	sample	# loci detected	# loci detected
1:10	OE-J	f	ND
1:50	OE-I	f	ND
1:100	OE-B	f	ND
1:500	OE-H	n	ND
1:1000	OE-D	f	ND
1:5000	OE-K	8	ND
1:10000	OE-A	5	5
1:50000	OE-G	n	ND
1:100000	OE-C	n	ND
1:500000	OE-F	n	ND
1:1000000	OE-E	n	ND
x/16 = # of complete loci, f=full profile, n=no profile ND=not done			

Table 4.11: Organic Extraction Method: Comparison of amount of loci detected in buccal samples

		DNA Investigator	
		Initial Extraction	Second Extraction
Dilutions	sample	# loci detected	# loci detected
1:10	QS-A	f	ND
1:50	QS-K	f	ND
1:100	QS-H	f	ND
1:500	QS-B	f	ND
1:1000	QS-G	f	ND
1:5000	QS-C	f	ND
1:10000	QS-E	f	ND
1:50000	QS-F	8	6
1:100000	QS-I	f	6
1:500000	QS-J	n	n
1:1000000	QS-D	n	n
x/16 = # of complete loci, f=full profile, n=no profile ND=not done			

Table 4.12: QIAamp® DNA Investigator Kit: Comparison of amount of loci detected in sperm samples

Post-PCR Purification

In comparing the post-PCR purified samples to the unpurified PCR samples, a fourfold increase in fluorescence signal intensity and less noisy baseline was seen in post-PCR purified samples. Increased stutter and allele dropout were not observed in any post-PCR purified samples. Amplification artifacts were detected in a few samples.

Out of 12 blood samples subjected to the Qiagen® Min-Elute® PCR Purification Kit, 5 samples showed an increase in the number of loci detected. The organic extraction method had 2 samples that had increased amounts of loci after PCR purification while QIAamp® DNA Investigator Kit showed 3 samples that exhibited an increase in amount of loci detected. Amplification artifacts were observed in the blood samples OB-E and OB-K after purification with the Qiagen® MinElute® PCR Purification (Table 4.13).

Dilutions	Method					
	Organic			DNA Investigator		
	sample	# loci detected	# loci detected after purification	sample	# loci detected	# loci detected after purification
1:10	OB-J	f	ND	QB-E	f	ND
1:50	OB-I	14	ND	QB-J	f	ND
1:100	OB-F	f	ND	QB-K	f	ND
1:500	OB-B	f	ND	QB-A	f	ND
1:1000	OB-A	f	ND	QB-D	f	ND
1:5000	OB-E	10	f**	QB-C	5	10
1:10000	OB-D	6	6	QB-G	2	7
1:50000	OB-K	1	1**	QB-B	n	n
1:100000	OB-G	n	1	QB-F	n	1
1:500000	OB-H	n	n	QB-H	n	n
1:1000000	OB-C	n	n	QB-I	n	n
x/16 = # of complete loci, f=full profile, n=no profile, ND=not done ** amplification artifacts detected						

Table 4.13: Comparison of amount of loci detected in blood samples before and after PCR purification

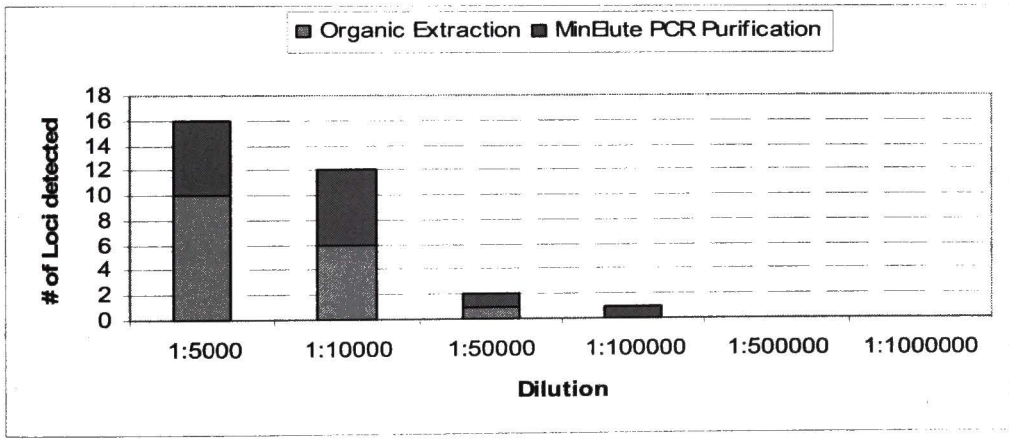

Figure 4.4

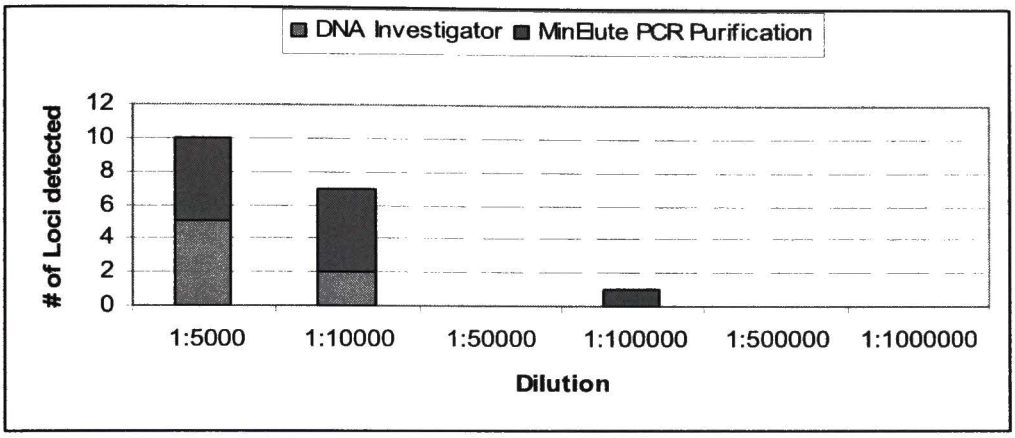

Figure 4.5

Bar graph illustrating the increase in complete loci obtained from blood samples extracted using organic extraction (Figure 4.4) and QIAamp® DNA Investigator Kit (Figure 4.5) after purification with Qiagen® MinElute® PCR Purification

Within the 12 buccal samples that were purified with the Qiagen® MinElute® PCR Purification Kit, 7 samples showed an increase in the number of loci detected. The organic extraction method had 3 samples with an increase in the amount of loci detected and QIAamp® DNA Investigator Kit method had 4 samples that showed an increase in the amount of loci detected. Amplification artifacts were observed in the OE-F buccal sample purified with the Qiagen® MinElute® PCR Purification Kit (Table 4.14).

Dilutions	Method					
	Organic			DNA Investigator		
	sample	# loci detected	# loci detected after purification	sample	# loci detected	# loci detected after purification
1:10	OE-J	f	ND	QE-G	f	ND
1:50	OE-I	f	ND	QE-D	f	ND
1:100	OE-B	f	ND	QE-I	f	ND
1:500	OE-H	n	ND	QE-A	f	ND
1:1000	OE-D	f	ND	QE-E	f	ND
1:5000	OE-K	8	15	QE-C	9	15
1:10000	OE-A	5	10	QE-J	9	12
1:50000	OE-G	n	n	QE-K	n	4
1:100000	OE-C	n	2	QE-B	n	2
1:500000	OE-F	n	n**	QE-H	n	n
1:1000000	OE-E	n	n	QE-F	n	n
x/16 = # of complete loci, f=full profile, n=no profile, ND=not done ** amplification artifacts detected						

Table 4.14: Comparison of amount of loci detected in buccal samples before and after PCR purification

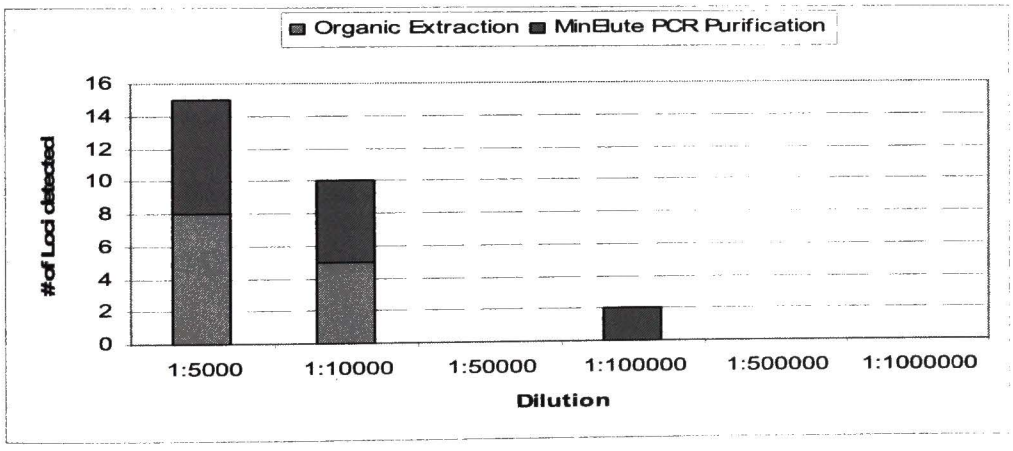

Figure 4.6

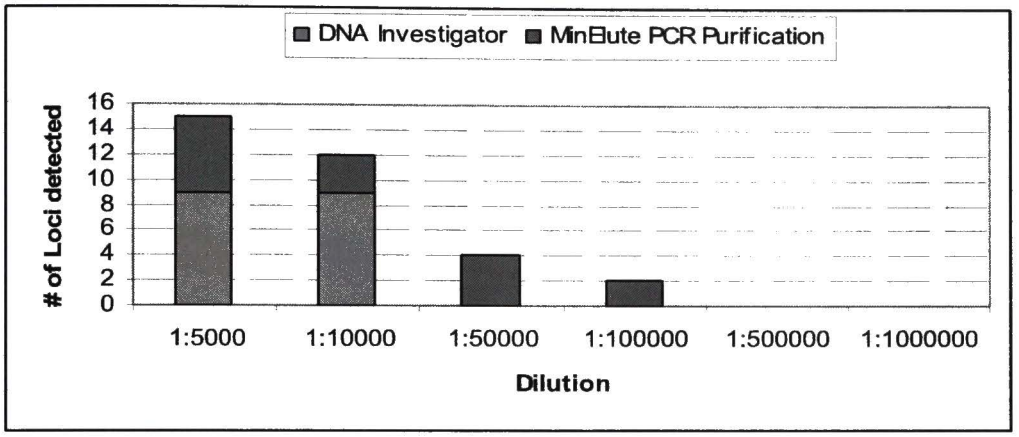

Figure 4.7

Bar graph illustrating the increase in complete loci obtained from buccal samples extracted using organic extraction (Figure 4.6) and QIAamp® DNA Investigator Kit (Figure 4.7) after purification with Qiagen® MinElute® PCR Purification

The Qiagen® MinElute® PCR Purification Kit was able to improve the amount of loci detected in 6 of the 7 sperm samples. The organic extraction method and QIAamp® DNA Investigator Kit both had 3 samples that showed an increase in loci detected after PCR purification (Table 4.15). There were no amplification artifacts detected in the sperm samples purified after PCR.

Dilutions	Method					
	Organic			DNA Investigator		
	sample	# loci detected	# loci detected after purification	sample	# loci detected	# loci detected after purification
1:10	OS-J	f	ND	QS-A	f	ND
1:50	OS-F	f	ND	QS-K	f	ND
1:100	OS-K	f	ND	QS-H	f	ND
1:500	OS-H	f	ND	QS-B	f	ND
1:1000	OS-C	f	ND	QS-G	f	ND
1:5000	OS-D	f	ND	QS-C	f	ND
1:10000	OS-G	f	ND	QS-E	f	ND
1:50000	OS-E	13	f	QS-F	8	14
1:100000	OS-I	11	11	QS-I	f	ND
1:500000	OS-A	1	2	QS-J	n	3
1:1000000	OS-B	1	5	QS-D	n	2
x/16 = # of complete loci, f=full profile, n=no profile, ND=not done						

Table 4.15: Comparison of amount of loci detected in sperm samples before and after PCR purification

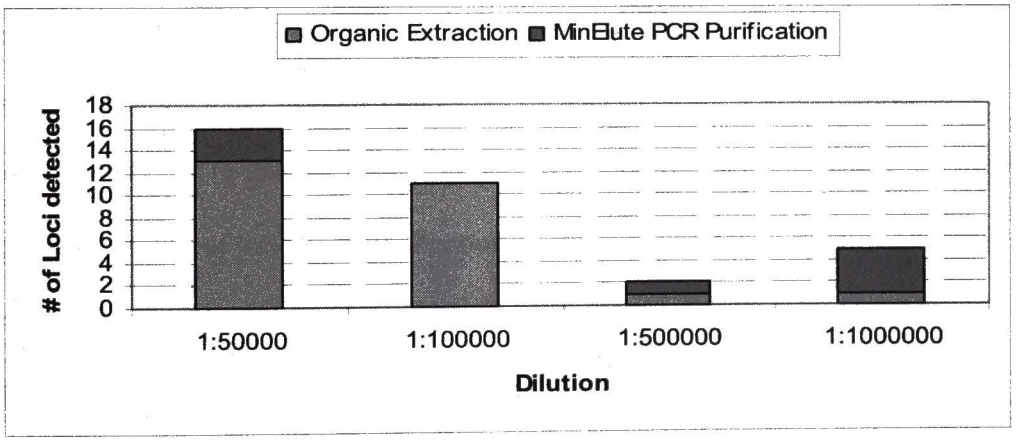

Figure 4.8

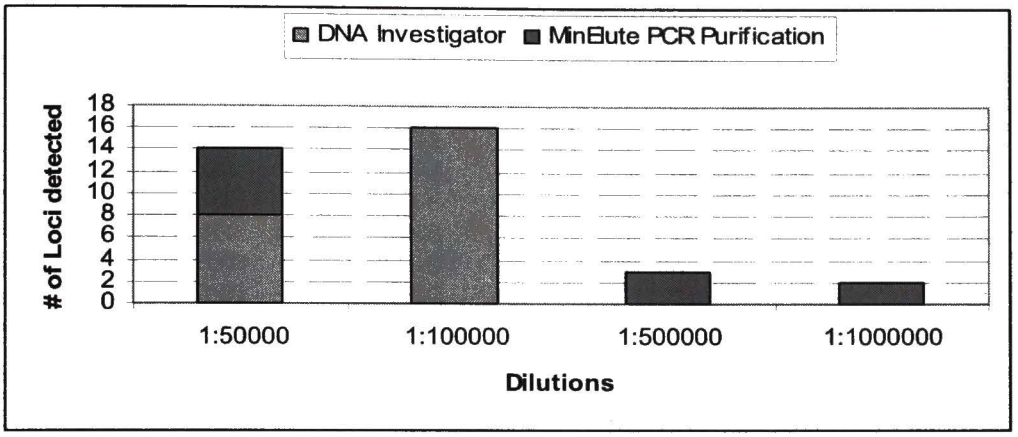

Figure 4.9

Bar graph illustrating the increase in complete loci obtained from sperm sample extracted using organic extraction (Figure 4.8) and QIAamp® DNA Investigator Kit (Figure 4.9) after purification with Qiagen® MinElute® PCR Purification

Post-PCR Purification of Re-extracted Samples

Out of the eight re-extracted samples subjected to post-PCR purification, 7 showed an increase in the amount of loci detected after purification with the Qiagen® MinElute® PCR Purification Kit. The blood samples all showed an increase in the amount of loci detected after purifying the amplification product. One of the blood samples did show amplification artifact (Table 4.16). The buccal sample that was post-PCR purified showed a decrease in the amount of loci detected from 5 loci to 4 loci (Table 4.17). The sperm cell samples all showed an increase in the amount of loci identified in the post PCR purified samples. However, three of the 4 sperm samples did show the presence of amplification artifacts (Table 4.18).

Dilutions	DNA Investigator Second Extraction sample	# loci detected	# loci detected after purification
1:10	QB-E	ND	ND
1:50	QB-J	ND	ND
1:100	QB-K	ND	ND
1:500	QB-A	ND	ND
1:1000	QB-D	ND	ND
1:5000	QB-C	8	10
1:10000	QB-G	1	6
1:50000	QB-B	n	1**
1:100000	QB-F	ND	ND
1:500000	QB-H	ND	ND
1:1000000	QB-I	ND	ND
x/16 = # of complete loci, f=full profile, n=no profile, ND=not done ** amplification artifacts detected			

Table 4.16: Comparison of amount of loci detected in re-extracted blood samples before and after PCR purification

Dilutions	Organic Second Extraction sample	# loci detected	# loci detected after purification
1:10	OE-J	ND	ND
1:50	OE-I	ND	ND
1:100	OE-B	ND	ND
1:500	OE-H	ND	ND
1:1000	OE-D	ND	ND
1:5000	OE-K	ND	ND
1:10000	OE-A	5	4
1:50000	OE-G	ND	ND
1:100000	OE-C	ND	ND
1:500000	OE-F	ND	ND
1:1000000	OE-E	ND	ND
x/16 = # of complete loci, f=full profile, n=no profile, ND=not done			

Table 4.17: Comparison of amount of loci detected in re-extracted buccal sample before and after PCR purification

Dilutions	DNA Investigator Second Extraction sample	# loci detected	# loci detected after purification
1:10	QS-A	ND	ND
1:50	QS-K	ND	ND
1:100	QS-H	ND	ND
1:500	QS-B	ND	ND
1:1000	QS-G	ND	ND
1:5000	QS-C	ND	ND
1:10000	QS-E	ND	ND
1:50000	QS-F	6	12**
1:100000	QS-I	6	14**
1:500000	QS-J	n	1
1:1000000	QS-D	n	1**
x/16 = # of complete loci, f=full profile, n=no profile, ND=not done ** amplification artifacts detected			

Table 4.18: Comparison of amount of loci detected in re-extracted sperm samples before and after PCR purification

CHAPTER 5

DISCUSSION

By using three different types of samples at various dilutions, the validation study was able to evaluate the sensitivity of the QIAamp® DNA Investigator Kit in extracting DNA and the performance of Qiagen® MinElute® PCR Purification Kit in purifying PCR product. The results from the study showed that the silica-binding DNA extraction method provided by QIAamp® DNA Investigator Kit was effective in extracting DNA from liquid blood, buccal cells and sperm cells and was able to provide full profiles for all three types of biological samples at dilutions of 1:1000 or higher. Organic extraction method was more efficient as extracting DNA from blood and sperm samples. There are possible explanations why the QIAamp® DNA Investigator Kit did not extract as much DNA as the organic extraction method. First, the organic extraction method included an overnight digest, whereas, the QIAamp extracted samples were digested for 10 minutes for blood and buccal samples or 1 hour for sperm samples. The shortened digest time might not allow enough time for the detergents to lyse cells. Secondly, the sperm samples that were extracted by organic extraction methods were digested in digest buffer with 20 µl of DTT while the QIAamp extracted sperm samples only had 10 µl of DTT added to digest buffer ATL. More DTT in a digest buffer could possibly improve the lysis of sperm cells allowing for more DNA to be extracted.

The blood and sperm samples that were re-extracted addressed the issues of a longer digest time and the addition of more DTT in sperm samples. It was difficult to compare the DNA yields between the initial DNA extractions to the re-extracted samples since the quantification results from the initial extraction were underestimated. DNA samples from the initial extraction should have been quantified again to obtain a better standard curve; therefore, a conclusion could have been reached to determine if the longer digest time and increase in amount of DTT would have resulted in higher DNA yields.

Even though QIAamp® DNA Investigator Kit produced DNA yields that were less for the blood and sperm samples, the amount of full and partial profiles obtained by the QIAamp kit was comparable to the amount of full and partial profiles obtained by organic extraction method. The QIAamp® DNA Investigator Kit does have its advantages. In the case of the Acadiana Criminalistic Laboratory, including the QIAamp® DNA Investigator Kit as part of their DNA extraction techniques would cut down on analyst's labor time and allow multiple analysts to perform DNA extraction at the same time. The need to take turns to use the fume hood for organic extractions would be eliminated. The QIAamp extraction process also reduces the occurrence of sample cross contamination by having the extraction procedure occur within one spin column tube. With the addition of the QIAcube® instrument, the QIAamp® DNA Investigator Kit can be automated which would reduce sample switching, contamination from the analyst and other samples, and improve the consistency of the extraction process.

The additional step of purifying the post amplified product using the Qiagen® MinElute® PCR Purification Kit established that the kit can be used to acquire more loci in a DNA profile without having to increase the number of amplification cycles. Amplified products that were further purified by the Qiagen® MinElute® PCR Purification Kit can increase the amount of full

and partial profiles obtained. The Qiagen® MinElute® PCR Purification kit was able to increase the number of loci detected in 64% of the post-PCR purified samples and produce partial profiles at a 1:1000000 dilution in sperm samples. Amplification artifacts were detected in a few samples that used the post PCR purification method. Since the method increases fluorescence signal intensity and produces a smoother baseline, any contaminating DNA that was amplified can show up as peaks in an electropherogram. These artifacts could also have resulted from the use of microcentrifuge tubes that were not sterilized before use or from the human contamination. Since the post-PCR purification process was not duplicated, it cannot be determined if these artifacts are reproducible.

Testing of the QIAamp® DNA Investigator Kit and the Qiagen® MinElute® PCR Purification Kit should have been performed in duplicates in order to assess the reproducibility of the two kits. Testing for reproducibility would have given more accurate results and a more solid conclusion on the reliability of the kits. Future testing of QIAamp® DNA Investigator Kit may consider DNA extractions from samples such as buccal swabs, vaginal swabs and blood stains.

CHAPTER 6

CONCLUSION

The use of the QIAamp® DNA Investigator Kit cannot completely replace the use of organic extraction but could be used as another technique in DNA extraction. The QIAamp® DNA Investigator Kit was not able to produce better DNA extraction yields than the standard organic method in the blood and sperm cell samples. However, the results from STR analysis did show that the QIAamp® DNA Investigator Kit was able perform as well as the organic extraction method. With features such as shorter extraction time and ease of use, the QIAamp® DNA Investigator Kit can appear more attractive than organic extraction. QIAamp® DNA Investigator Kit can be automated on the QIAcube® instrument which can greatly increase extraction consistency and sample output while minimizing contamination.

The Qiagen® MinElute® PCR Purification Kit has shown that the number of loci in a DNA profile can be increased without having to increase the number of amplification cycles which reduces problems such as increased stutter, allele dropout and allele drop-in. Even though amplification artifacts were detected in a few of the samples that utilized the Qiagen® MinElute® PCR Purification kit, these artifacts can be minimized by incorporating a strong guideline to prevent or minimize contamination. The Qiagen® MinElute® PCR Purification kit was easy to

use and can be performed in under 20 minutes. This kit can also be automated on the QIAcube® robot to help reduce sample manipulation and increase sample productivity.

Both the QIAamp® DNA Investigator Kit and Qiagen® MinElute® PCR Purification Kit can be used on a variety of forensic casework samples at various dilutions. Both kits have been shown to be advantageous for use in a forensic laboratory. In addition, the potential for automation also makes both of these kits attractive and helpful in increasing sample throughput and decreasing labor time for analysts.

APPENDIX A

Organic Extraction of DNA from Blood, Saliva, Contact DNA, Bone samples and other body tissue

Purpose of Procedure: This procedure is used to separate the DNA from other cellular components for genetic profiling.

Procedure

NOTE: All reference samples must be extracted separately and, whenever possible, after evidentiary samples. Evidentiary samples of vastly different amounts (i.e. possible contact DNA vs. bloodstain) must also be extracted separately. Evidentiary samples collected from different scenes or from different individuals must also be extracted separately. Limit extraction sets to five samples and an extraction blank.

A. Preparation of worksheets/tubes for organic extractions

1. Be sure that the case flow/summary sheet reflects the samples extracted, the date of extraction, and the type of extraction protocol used.
2. Put on lab coat, mask, and gloves.
3. Working on a clean surface covered with Bench coat paper, prepare a microcentrifuge tube for each sample. These tubes may be marked with the sample identifier using a permanent marker or an adhesive label. This is the tube where the sample cutting goes.
4. Prepare 3 sets of 1.5 ml microcentrifuge tubes for each sample. Each should be labeled appropriately.
5. Prepare one set of Microcon tubes with blue Micron filters for each sample. Each Microcon tube and filter should be labeled with the sample identifier.
6. Prepare 1 set of Microcon tubes as recovery tubes for the extracted samples. Each should be labeled with the sample identifier.
7. Sterilize all tubes under UV hood for at least 30 minutes.
8. Verify or set the water bath temperature or incubator to 56°C.

B. Organic extraction protocols (contact samples, blood, tissue, saliva, etc.)

NOTE: All liquid transfer of samples involving the organic chemicals must be performed in the chemical hood. While using phenol/chloroform/isoamyl alcohol (PCIA) or butanol, the hood fan must be on and the sash must be down. A lab coat,gloves,eye protection (either safety glasses or a suitable facial barrier), and a mask must be worn throughout this procedure, changing gloves when necessary and especially if wet with PCIA or DNA.

1. Clean the lab bench and cover the surface with a fresh piece of bench paper. Gather all utensils (e.g., scalpel, scissors, forceps, weigh paper, 10% chlorox, deionized water, alcohol burner, and tape dispenser) needed for sample cutting.
2. Using one sterile microcentrifuge tube for each sample, add the following to each tube:

   7.5-10 µl proteinase K (10 mg/ml) solution

300 µl digest buffer

Mix the proteinase K and the digest buffer, then close the tubes.

3. Before cutting a new sample, be sure to sterilize the forceps and cutting utensils by a 10% Chlorox rinse, followed by a deionized water rinse, and briefly (~1 second) holding the tip of the utensil in the flame of the alcohol burner.
4. Lay a new piece of weigh paper on the bench and place sample on the weigh paper. Carefully cut the stain (~3x3 mm when available), making new cut marks when possible, and place in the appropriately marked tube. If the staining is light a larger area may be taken. If the sample is on a swab, use ½ to one swab or the stained area if the staining is uneven. In the case of loose flakes, use at the discretion of the analyst. In the case of cigarette butts, remove ~0.5cm of the paper on the filter end of the cigarette. For bone samples, use approximately 100 mg of crushed bone. Close the tube after the addition of the sample.
5. Repeat steps 3-4 for each sample to be analyzed.
6. Place the samples in a microcentrifuge rack and place in the 56°C water bath or incubator overnight. The rack may be weighted down if necessary.
7. Remove the samples from the water bath or incubator and pulse spin in a microcentrifuge to force the condensate into the bottom of the tube. **Analysis can be paused at this point by placing the samples in the -20°C freezer. If stored at -20°C, then the samples should be warmed at 56°C for several minutes before starting phenol/chloroform extraction. From this point, once started, the extractions must be carried through to the Microcon purification.**
8. If necessary, gather a spin-ease basket and tube for each sample. Using sterile forceps, transfer the cutting (if applicable) into a basket insert. Place the basket insert into the respective spin-ease tube. Cap the spin-ease tube. Place the tubes in a microcentrifuge and spin for ~5 minutes at ~5,000 or greater rpm. If a spin-ease tube is not necessary, proceed directly to step 10.
9. Remove the tubes from the centrifuge and return the samples to the chemical hood. Remove and discard the basket insert with cutting into the biohazardous waste container. Transfer the supernatant from each spin-ease tube to the appropriate sample tube.
10. To the appropriate microcentrifuge tubes add:

    300 µl PCIA

    Vortex (low/med speed) the mixture briefly (at least 15 seconds) to attain a milky emulsion. Spin the tubes in a microcentrifuge for ~3 minutes at ~10,000 rpm.
11. Transfer the aqueous layer (top layer) to a fresh 1.5 ml microcentrifuge tube. Discard the old tube, capped, with the PCIA (bottom layer) in the biohazard waste.
12. Repeat steps 10-11 two more times for a total of three PCIA washes. There should be one set of 1.5 ml microcentrifuge tubes left. *Optional: fourth extraction for problem samples.*
13. Transfer the aqueous layer (top layer) to the last set of 1.5 ml microcentrifuge tubes.
14. To each of the tubes add:

    300 µl of water saturated butanol

    Vortex (med speed) the mixture briefly (15 seconds) to attain a milky emulsion. Spin the tubes in a microcentrifuge for ~3 minutes at ~10,000 rpm.

15. To the microcon 100 concentrators (blue filters in the microcon assemblies from part A, step 4) add 100 µl TE^{-4}. TE^{-4} must be added before the sample to avoid possible loss of membrane integrity from excess butanol or PCIA.
16. Transfer the aqueous layer (bottom layer) from the tube in step 14 to the concentrator. Avoid pipetting organic solvent (top layer) from the tube into the concentrator.
17. Place the cap on the concentrators and spin in a microcentrifuge for ~10 minutes at ~5,000 rpm. Remove from the centrifuge.
18. Carefully remove the concentrator unit from the assembly and discard the fluid from the bottom of the microcon tube into the biohazard waste container.
19. Return the concentrator to the microcon tube. Add 200 µl TE to the concentrator. Replace the cap and spin in a microcentrifuge for ~10 minutes at ~5,000 rpm. Remove from the centrifuge.
20. Remove the concentrator and discard the old microcon tube. Add a measured volume of TE that is between 30 µl and 200 µl to the concentrator. (Guideline: ~30 µl for small question samples; ~75-100 µl for semen or blood samples; ~150-200 µl for reference saliva samples.
21. Carefully invert the concentrator onto the appropriate microcon with the adhesive label markings. The tubes will not cap with the inverted filter.
22. Spin the assembly in a microcentrifuge for ~5 minutes at 5,000 rpm. Remove from the microcentrifuge.
23. Discard the concentrator. Cap the microcon tube with the extracted sample.
24. Estimate the quantity of DNA in the samples by real time PCR. After quantification, the samples can be amplified.

Store the samples at 4°C (short term) or frozen (long term). Prior to use of samples after storage, they should be vortexed, and spun in a microcentrifuge for ~5 seconds.

C. Variations of organic extractions

NOTE: DNA extracts from hair should be separated from blood and saliva extractions due to the limited quantity of DNA in hair roots.

1. Hair (optional steps)
    1. While holding the hair with sterile forceps, rinse it thoroughly in 100% ethanol. Follow the ethanol rinse with a thorough rinse in sterile dH2O.
    2. Place at least 1 cm of the hair root end into a labeled 2.2 ml spin-ease tube.
    3. Follow the same protocol as for regular organic extractions except add 20 µL 1 M DTT at part B, Step 5.

D. Preparation of worksheets/tubes for vaginal/seminal stains

1. Be sure that the case flow/summary sheet reflects the samples extracted, the date of extraction, and the type of extraction protocol used.
2. Working on a clean surface covered with bench coat paper, prepare a spin basket for each sample, and three 1.5 ml microcentrifuge tubes for each sample. These tubes may be marked with the sample identifier using a waterproof marker or an adhesive label.

3. Prepare one 1.5 ml microcentrifuge tube for each sample. Mark these with the case number, sample EX, and date. Mark these tubes with an "S" designation. These tubes are for the sperm fraction of the extract.
4. Prepare one 1.5 ml microcentrifuge tube for each sample. Mark these with an "E" designation. These will be for the epithelial (epi) fraction of the extract.
5. Prepare one 1.5 ml microcentrifuge tube and spin basket for each sample. This will hold the supernatant that can be tested for acid phosphatase and p30.
6. Prepare three 1.5 ml microcentrifuge tubes for each sample in the sperm and epi sets. Each should be labeled with the sample identifier including epi and sperm designations. Expose all the tubes to UV light for at least 30 minutes prior to use.
7. Prepare one set of Microcon tubes with blue Micron filters for each sample. Each tube and filter should be labeled with the sample identifier including epi and sperm designations.
8. Prepare one set of Microcon tubes as recovery tubes for the extracted samples. Each should be labeled with the sample identifier including epi and sperm designations.
9. Verify or set the water bath or incubator temperature to 56°C.

E. Differential extraction of vaginal/seminal stains

1. Place sample in the first microcentrifuge tube labeled "S".
2. Add 300 µl of PBS to the evidence stain or swab in each tube. Close the tubes and let them sit at room temperature for 30-60 minutes. Agitate the tubes occasionally during this step. Prepare a microscope slide for the epithelial and sperm fractions of each sample. Verify that there is one prepared 1.5 ml microcentrifuge tube for each sample (tubes from step 3 above).
3. Briefly centrifuge the tubes to collect the liquid at the bottom of the tube. Using a new wooden applicator stick or sterilized forceps for each sample, transfer the material to tube with the spin basket. Spin the spin basket tube in a microcentrifuge at maximum speed for approximately five minutes.
3. Remove basket insert (DO NOT DISCARD) and pipette all of the liquid back into the original tube labeled "S". Centrifuge the "S" tube at maximum speed for approximately five minutes.
4. Remove all but approximately 50 µl of the supernatant and pipette into the microcentrifuge tube containing the spin basket. Be careful not to disturb the cell pellet. The supernatant can be tested for AP and p30. This supernatant with the sample and spin basket can be stored at -20 degrees C.
5. In the tube labeled "S", re-suspend the pelleted material with a pipette tip and place 1 – 2.5 µl on the epithelial area of a prepared microscope slide.
6. To the "S" tube add:
   300-450 µl Digest Buffer
   5-10 µl proteinase K
   Mix the proteinase K and digest buffer
7. Incubate at 56°C for 2 hours.
8. Spin "S" tube at maximum speed for five minutes. While being very careful not to disturb the pelleted material, remove the supernatant fluid from the extract and place it into the corresponding "E" labeled tube. THIS SUPERNATANT IS THE EPITHELIAL FRACTION. ANALYSIS OF THE EPITHELIAL FRACTION

RESUMES AT STEP 15. THE PELLET REMAINING IN THE TUBE IS THE CELL PELLET (SPERM FRACTION).

9. Wash the cell pellet by resuspending it in 1000 µl Tris/EDTA/NaCl, vortexing the suspension briefly, and spinning the tube in a microcentrifuge at maximum speed for ~5 minutes. Remove and discard the supernatant fluid, being careful not to disturb the cell pellet.
10. Repeat step 10 two additional times for a total of three washes to the cell pellet.
11. After the final wash, remove all but approximately 50 microliters of the supernatant.
12. Re-suspend the cell pellet with a pipette tip and place 1 – 2.5 µl in the sperm area of the appropriate microscope slide. Dry the slide in the incubator at 56 degrees C for approximately ten minutes. Stain and view slide. Record results.

    If epithelial cells are still visible the analyst may, at his or her discretion, add 350 µl digest buffer and 7.5 µl proteinase K to the "S" tube. Vortex. Spin in a centrifuge for 2 seconds. Place sample a 56°C water bath for 1 hour. Repeat protocol from steps 8-12. (Supernatant may be added to previously collected supernatant (epi fraction) for the sample.)
13. To the tube containing the washed pellet, add:

    300-350 µl Digest Buffer
    20 µl 1M DTT
    10 µl proteinase K

    Close the tube caps and vortex for ~1 second and spin in a microcentrifuge for ~2 seconds to force all the fluid to the bottom of the tubes.
14. Incubate at 56°C for 2-4 hours or overnight.

After digestion all samples can be stored at -20 ℃ until phenol/chloroform extraction is started. If stored at -20 ℃, then the samples should be warmed at 56 ℃ for several minutes before starting phenol/chloroform extraction. Once started, the extractions must be carried through to the Microcon purification.

(NOTE: From this point on, the steps are done in duplicate, once for the epi fraction and once for the sperm fraction. The QIAamp extraction procedure for epithelial fractions can also be used for the epithelial fractions in place of the PCIA washes)

15. To the tube containing the sperm fraction (cell pellet) and to the tube containing the epi fraction, add:

    300 µl phenol/chloroform/isoamyl alcohol

    Vortex (low speed) the mixture briefly to attain a milky emulsion. Spin the tube in a microcentrifuge at maximum speed for ~3 minutes.
16. Transfer the aqueous layer (top layer) to a fresh 1.5 ml microcentrifuge tube. Discard the old tube, capped, with the PCIA (bottom layer) in the biohazard waste.
17. Repeat steps 13-14 two more times for a total of three PCIA washes. There should be one set of colored 1.5 ml microcentrifuge tubes left. *Optional: fourth extraction for problem samples.*
18. Transfer the aqueous layer (top layer) to the last set of 1.5 ml microcentrifuge tubes.
19. To each of the tubes add:

    300 µl of water saturated butanol

Vortex (med speed) the mixture briefly (~15 seconds) to attain a milky emulsion. Spin the tubes in a microcentrifuge for ~3 minutes at maximum speed.

20. To the microcon 100 concentrators (blue filters in the microcon assemblies) add 100 µl TE^{-4}. TE^{-4} must be added before the sample to avoid possible loss of membrane integrity from excess butanol or PCIA.
21. Transfer the aqueous layer (bottom layer) from the tube in step 14 to the concentrator. Avoid pipetting organic solvent (top layer) from the tube into the concentrator.
22. Place the cap on the concentrators and spin in a microcentrifuge for ~10 minutes at ~5,000 rpm. Remove from the centrifuge.
23. Carefully remove the concentrator unit from the assembly and discard the fluid from the bottom of the microcon tube into the biohazard waste container.
24. Return the concentrator to the microcon tube. Add 200 µl TE^{-4} to the concentrator. Replace the cap and spin in a microcentrifuge for ~10 minutes at ~5,000 rpm. Remove from the centrifuge.
25. Remove the concentrator and discard the old microcon tube. Add a measured volume of TE^{-4} that is between 30 µl and 200 µl to the concentrator. (Guideline: ~30 µl for small question samples; ~75-100 µl for semen or blood samples; ~150-200 µl for reference saliva samples or vaginal fractions)
26. Carefully invert the concentrator onto the appropriate microcon with the adhesive label markings. The tubes will not cap with the inverted filter.
27. Spin the assembly in a microcentrifuge for ~5 minutes at ~5,000 rpm. Remove from the microcentrifuge.
28. Discard the concentrator. Cap the microcon tube with the extracted sample.
29. Extracts can also be transferred to a Greiner plate and then sealed with an adhesive cover. An extraction plate record will be kept indicating the well location of each extract.
29. Estimate the quantity of DNA in the samples by real-time PCR. After quantification, the samples can be amplified.

Store the samples at 4°C (short term) or frozen (long term). Prior to use of samples after storage, they should be vortexed, and spun in a microcentrifuge for ~5 seconds.

APPENDIX B

Sample Set-up for Real-Time PCR Quantification Using the Biomek2000 Robot

I. PURPOSE

A. To use the Biomek2000 robot to set up the reaction plate for real-time PCR quantification.

B. The Biomek2000 is used to set up the serial dilutions of the DNA standard, aliquot the master mix, and then dispense the standards and sample extracts into the 96-well reaction plate.

II. MATERIALS

Items listed in the Real-Time PCR Quantification of Human DNA procedure
Biomek2000 automated workstation
Pipet tips for Biomek2000
Quarter module reagent reservoir
Black support base
8 X 0.2ml PCR tube strip
Centrifuge for 96-well plates
Greiner plates containing DNA sample extracts

III. PROCEDURE

A. Set-up of Biomek2000 workstation

1. Open the Bioworks folder located on the desktop of the Biomek2000 workstation.
2. Open the Lab Book Manager and set "ACL Methods" as the current lab book.
3. Open the Edit file in the Bioworks folder and choose the "Quant set-up_2 plate" method.
4. Set up the Biomek2000 work surface as depicted in the diagram on the computer screen.

B. Preparation of reagents

1. Defrost the Quantifiler human DNA standard, reaction mix, and primer mix as described in the Real-time PCR procedure.
2. Add the calculated amounts of reaction mix and primer mix to the sterile reservoir 1 as described in the comments (in green) on the computer screen.
3. Add 1 ml of TE-4 buffer to the sterile reservoir 2.
4. Pipette 10 µL of the Quantifiler human DNA standard into the first tube of the tube strip.

C. Preparation of sample

1. Prepare the Quantifiler worksheet as described in the Real-Time PCR Quantification of Human DNA procedure or import the sample information as described in the Set-up of Worksheet for Real-Time PCR Quantification using Excel procedure.
2. Defrost and centrifuge Greiner plates containing the samples that are to be quantified.
3. Place the plates in the designated areas on the Biomek2000 work surface.

D. Running the method

1. If necessary, edit the method to correspond to the number of pipet tips used and the number of wells to be used on the reaction plate. See comments (in green).
2. Save and run the method.
3. When the run has completed, seal the reaction plate with the Optical Adhesive Cover using the spatula to get a better seal.
4. Centrifuge the plate at 3000rpm for approximately 20 seconds in a tabletop centrifuge to remove any bubbles.
5. Place the compression pad over the Optical Adhesive Cover with the gray side down and the brown side up and with the holes positioned directly over the reaction wells.
6. Place the plate in the ABI 7000 and proceed as described in the Real-Time PCR Quantification of Human DNA procedure.

APPENDIX C

PCR Amplification using AmpF*l*STR Identifiler Kit

1. Sample Preparation (to be performed in the Biology lab)

   A. 30 minutes prior to using the PCR cabinet, sterilize the hood interior, equipment, and consumable supplies with the UV light. Turn off light just before you are ready to begin.

   B. List the samples to be amplified and reagent lot numbers on the amplification worksheet. Evidence and reference samples from the same case, or evidence samples from different cases can be amplified at the same time; however, references and evidence from each case should be set up at different times or in different locations. Open only one tube at a time when making up samples to be amplified. Amplify up to 96 samples, including a positive and negative control.

   C. Prepare the DNA samples in 0.5mL microcentrifuge tubes prior to starting on the PCR tubes. For Identifiler, use the template DNA concentrations listed on the Quantifiler results report to determine the volume that will give a maximum of 1.0ng of template DNA and a final volume of at least 11µL. Use TE^{-4} buffer to bring final volume to at least 11µL if the concentration of template DNA is less than 1ng/µL. Close and place the tubes in the refrigerator until ready to amplify.

   D. Dispose of gloves

2. Amplification Set-Up (to be performed under the PCR enclosure):

   A. Place the Reaction Mix, Primer Set, and AmpliTaq Gold DNA Polymerase under the PCR enclosure, mix, briefly spin in centrifuge, and place the reagents in a rack.

   B. Place one clean, autoclaved 0.2mL MicroAmp® reaction tube for each reaction, into a rack and label appropriately or use a 96-well optical reaction plate.

   C. Calculate the required amount of each component of the PCR master mix (listed below). Multiply the volume (µL) per sample by the total number of reactions plus two to obtain the final volume (µL).

PCR Master Mix Component	Volume per Sample (µL)
Reaction Mix	10.5
Primer Set	5.50
AmpliTaq Gold (at 5u/µL)	0.50 (2.50u)

   D. In the order listed in the above table, add the final volume of each reagent to a sterile tube labeled Master Mix, close the tube, mix, and briefly spin tube in centrifuge.

E. Return Reaction Mix, Primer Set, and AmpliTaq Gold DNA Polymerase to refrigerator and freezer. **NEVER OPEN THESE VIALS IN THE PRESENCE OF ANY TEMPLATE DNA.**

F. Add 15 µL of Master Mix to each tube or well. Pipette solutions carefully to prevent bubbles in the liquid and spatters on the walls. Close the tubes.

G. Remove prepared samples from the refrigerator.

H. Pipet 10.0 µL of the prepared sample into the respective tube or well containing the PCR Master Mix. Only keep one PCR tube and its corresponding DNA sample open at any time during preparation. Visually verify the presence of solutions in the pipette tip. The final volume is 25 µL. Close the tubes or seal the optical reaction plate with an adhesive cover.

3. PCR Amplification

A. Make sure the thermal cycler is on and allow the cover to reach the appropriate temperature before starting.

B. Assemble the tubes within the tray set in the thermal cycler. If using the optical reaction plate, place the insulating cover over the plate.

C. Cover the reaction tubes or plate with the thermal cycler lid.

D. Select and run the Identifiler amplification program on the GeneAmp® PCR System 9700. Use 25µL setting on thermal cycler.

E. Confirm the cycling parameters.

F. After completion of the thermal cycling protocol, store the samples at -20°C, protected from light.

APPENDIX D

Prior to sample preparation, remove POP-4 from refrigerator and allow it to equilibrate to room temperature.

1. Prepare Sample Sheet

A. Turn the CE 310 on.
B. Turn the computer on.
C. The 310 collection module software will launch automatically.
D. Obtain menu for ABD/PE 310 Collection Module.
E. Select "File".
F. Click on "New".
G. Click on GeneScan sample sheet 48 or 96 well format.
H. Select five dye sheet
I. Fill in the necessary information (**"sample info" field must be filled in!**).
J. Make sure that the allelic ladder has the word "ladder" in the "sample info" field on each of the dye colors (blue, green, yellow, red, and orange).
K. Save the file with an appropriate sample sheet name.

2. Preparation of Sample

A. Count the number of samples including the allelic ladders. At least one Identifiler allelic ladder is to be used per run.
B. Dispense 24.5 µL deionized formamide per sample (+2) into a 1.5 mL microcentrifuge tube; e.g. 48 total samples = 50 x 24.5 µL = 1225 µL formamide.
C. Add 0.5 µL of the GeneScan 500 LIZ Size Standard per sample (+2); e.g. 50 x 0.5 µL = 25 µL LIZ added to the formamide. Gently mix and briefly centrifuge.
D. Aliquot 25 µL of this formamide mix into appropriately labeled sterile 0.5 mL tubes or Microamp tubes. Use the 310 Sample location sheet to note the samples to be run and their positions in the tray.
E. Add 1.5 µL of amplified PCR product or ladder to each tube. For contact DNA samples, up to 3.0 µL of amplified PCR product can be used.
F. Place a septum on each sample tube.
G. Mix and centrifuge all the 0.5 mL tubes. The Microamp tubes do not have to mixed and centrifuged, but they must be free of bubbles.
H. Heat all samples at 95°C for 3 minutes (Optional: rapid chill for at least three minutes in freezer) and load onto the ABD/PE 310 CE genetic analyzer.

3. Preparation of ABD/PE 310 Genetic Analyzer (CE 310)

A. Prepare 15 mL of 1X capillary electrophoresis (CE) buffer from 10X stock CE buffer with EDTA (from supplier Applied Biosystems).
B. Assemble interior components of the CE 310 (pump block & anode buffer reservoir, etc.) according to the manufacturer's instructions.
C. Remove the capillary from its mailing tube and clean the capillary window with ethanol and a Kimwipe.
D. Install capillary by taking the capillary and threading it into the pump block ferrule. Place the end of the capillary at the edge of the tube descending from the syringe. Tighten the ferrule. Pull on the capillary to make sure it is tightly in place.
E. Align the capillary window with the laser window (**Do Not Touch Window Area**). Close the laser window door and tape down the capillary portion above the door to the heating block.
F. The other end of the capillary is passed through the electrode holder and lined up just past the end of the electrode holder. The capillary portion above the electrode is then taped to the heating block. Close the heating block door.
G. Calibrate autosampler (only when the ABD/PE 310 has been moved or the autosampler has been worked on) by going into the ABD/PE 310 collection module program and selecting "calibrate autosampler" and follow the instructions for the calibration.
H. Fill all the appropriate reservoir containers (buffer, water, syringe, waste, etc.) with the necessary fluid.
I. Under manual control adjust so that the syringe drive pump barely touches the top of the syringe plunger and it is now in the proper position.
J. Also under manual control set temperature at 60°C and execute.

4. Prepare Injection List

A. Select "File".
B. Click on "New".
C. Select GeneScan injection list.
D. Click on "sample menu" and select recently saved sample sheet (or any appropriate sample sheet containing the samples you want to run).
E. Modify injection list if necessary (if you plan on having multiple injections of the same sample or if you plan on shorter/longer injection times, etc.). Once started, the list can still be modified.
F. Make sure you inject allelic ladders at the beginning, middle, and end of every run. More ladder injections can be done between samples.
G. Make sure that the "GS STR POP-4 (1 mL) G5" module is selected for all the samples.
H. Make sure that you have selected the appropriate matrix file to fill the "matrix field" for all your samples in your injection list (you do not have to save the injection list since it automatically saves it when you run it).

5. Run Samples

A. Place samples into the sample tray in accordance with the sample sheet.
B. Open instrument door and under manual control select "present tray".
C. Load sample tray and select "return tray".
D. Close the instrument door.

E. If running a new column, reset counter for column.
F. Click on "Run" in the Injection List window to begin collecting data points on each of the samples.
G. Once a sample has been run through the capillary, a sample file is generated containing the raw data points which will be utilized during the analysis step in the GeneScan analysis module.
H. Close project and it will automatically save it to a run folder of the date the samples were run.

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